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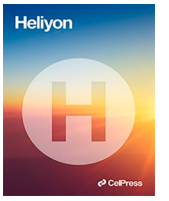

Insights into environmental caffeine contamination in ecotoxicological biomarkers and potential health effects of *Danio rerio*

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ABSTRACT

Caffeine (CAF) exposures have been shown to cause several pharmacological and biological effects in target and non-target organisms. Although there are already several ecotoxicological studies with CAF in non-target organisms, they are focused on marine organisms, with relevant concentrations in these ecosystems, therefore, less ecologically relevant to freshwater ecosystems (the main ecoreceptor of this type of anthropogenic contaminant). The present study aimed to assess the chronic effects (28 days) of sub-lethal and environmentally relevant concentrations of CAF (0.16, 0.42, 1.09, 2.84, 7.40, 19.23, and 50 μg/L) in *Danio rerio*. Biochemical endpoints as biomarkers of antioxidant defense, biotransformation, lipid peroxidation, energy sources, and neurotransmission were assessed. CAF exposure induced alterations in antioxidant defenses (superoxide dismutase and glutathione reductase activities, and glutathione content) preventing lipid peroxidation. Lactate dehydrogenase activity decreased in all the concentrations tested, while acetylcholinesterase activity was only affected by the highest concentrations tested (19.23 and 50 μg/L). We also utilized a multi-biomarker approach (Integrated Biomarker Response version 2, IBRv2) to investigate the effects of CAF in the dispersion scope of individual biochemical responses of *D. rerio*. IBRv2 showed that the concentration of 50 μg/L promotes the highest stress. However, the results showed that CAF induced disturbances in the metabolic pathways studied in *D. rerio*. These results demonstrated the toxic effects of CAF on freshwater fish, compromising their physiological functions and evidencing the need for monitoring the

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residues of CAF released into the inland aquatic environments. Furthermore, this research evidence that phylogenetically and physiologically different species may present different biological responses with concern for ecologically relevant environmental conditions. In this sense, the present study generated ecotoxicologically relevant data, that can be considered by environment regulators, since the here-endpoints evaluated showed sensitivity and consistency in the evaluation of caffeine risks in freshwater environments.

1. Introduction

Caffeine (CAF) is considered a suitable indicator of anthropogenic contamination of aquatic environments [1,2], and one of the most consumed substances in the world (e.g., 36.5–319.4 mg/day/adult in Europe) [3]. This natural substance appears in more than 60 species of plants (e.g., coffee, cacao, and tea plants) [4], and is used for different purposes as in beverages (e.g., coffee, tea, energy, and soft drinks), food products, and pharmaceuticals (cold medicines, analgesics, diuretic supplements, and stimulants) [5,6]. Currently, CAF can be produced synthetically and is considered a potential biologically active substance, and a contaminant of emerging concern [7,8]. This substance is relatively stable under different conditions (e.g., salinity; light, and temperature), with a high water-solubility

Table 1
Caffeine concentrations (μg/L) detected in water samples around the world.

Geographic area	Environmental Matrix	CAF concentrations (μg/L)	References
Europe	Rivers and lakes, Germany	0.53	[1]
	Rivers and lakes, Germany	1.27	
	Rivers and lakes, Netherlands	0.17	
	Rivers and lakes, Greece	0.16	
	Influent WWTP, Greece	2.88–4.97	[17]
	Influent WWTP, UK	150	[18]
	Effluent WWTP, Greece	0.02–0.12	[17]
	Effluent WWTP, UK	34.2	[18]
	Drinking water, Spain	0.50	[19]
	Groundwater, Italy	0.68	[19]
	Seawater, North Sea	0.016	[20]
	Seawater, Venice	0.08–0.37	[21]
	Baltic sea, Sweden	0.058	[17]
	Tagus river, Portugal	<0.004–0.05	[22]
	Arade river estuary, Portugal	0.60–0.80	[7]
	Freshwater, Belgium	39.8	[23]
	Schussen river, Germany	0.05–0.09	[24]
	Argen river, Germany	0.03	
America	Rivers and lakes, USA	0.01–0.10	[1]
	Rivers and lakes, USA	0.003–1.44	
	Rivers and lakes, USA	0.01–0.03	
	Rivers and lakes, USA	0.01–0.05	
	Rivers and lakes, USA	0.081	
	Rivers and lakes, USA	0.13–0.37	
	Influents, USA	64	[19]
	Effluents, USA	0.31	[19]
	Hospital effluent, New York	86,000	[25]
	Drinking water, Brasil	2.8	[5]
	Groundwater, USA	0.01–0.08	[1]
	Groundwater, USA	<0.04	
	Seawater and coastal areas, Canada	1.4	[7]
	Seawater and coastal areas, Costa rica	1.21	
	Seawater and coastal areas, Brasil	1.3	
	Sediments Java river, Costa Rica	1100	[23]
	Reservoirs, Brazil	27.7	[26]
Asia	Influent, Singapore	3594	[27]
	Drinking water, Turquia	35.0	[5]
	Seawater and coastal areas, China	3.06	[7]
	Seawater and coastal areas, Japan	8.23	
	Seawater and coastal areas, Malaysia	0.0003	
	Seawater and coastal areas, Singapore	1.39	
	Seawater and coastal areas, Taiwan	0.72	
	Seawater, Saudi Aradian	0.06–0.003	[16]
	Shijing river, China	0.87	[28]
Africa	Raw wastewater, South Africa	1214	[27]
	Msunduzi River, South Africa	33.2	
Oceania	Seawater, Australia	0.02–0.21	[7]
	Seawater, Australia	0.14–11.0	

(20 g/L), and a long half-life (variating from 100 to 240 days [7] to 10 years [9]) depending on the characteristics of the aquatic environment [7]. These characteristics and the input increase to the aquatic environment are greater than the degradation rate [10], as a result of human excretion and industrial wastewater, environmental detection has increased in freshwaters [11,12], estuarine and marine ecosystems (Table 1) [13,14], as well as in the biota (e.g., microalgae *Ulva lactuca* and fish *Chanos*) [15,16].

CAF is a psychoactive drug that belongs to the methylxanthines class and can cause several pharmacological (e.g., stimulant and diuretic) and biological (e.g., cell death and oxidative stress) effects in target and non-target species [8]. CAF is a non-selective antagonist of adenosine receptors [23], stimulating the central nervous system and affecting the activity of neurotransmitters [29,30]. Furthermore, several studies already showed acute toxicity of CAF to bacteria (*Pseudomonas putida,* EC_{50} (17 h) = 3490 mg/L), algae (*Scenedesmus subspicatus,* EC_{50} (72 h) > 100 mg/L), freshwater invertebrates (*Daphnia magna,* EC_{50} (48 h) = 182 mg/L), and fish (*Leuciscus idus,* LC_{50} (96 h) = 87 mg/L) [31], however, these studies report toxicity at concentrations well above those found in the environment (Table 1). Moreover, Zhou et al. [32], reported significant effects on locomotor activity in zebrafish larvae (5 days post-fertilization) after exposure to 1, 10, and 100 μg/L of CAF. Regarding, that the lowest concentration in the previous study (1 μg/L) is around 40 times lower than the maximum concentration (39.8 μg/L) detected in surface water in Europe (Table 1), which reinforces the potential environmental effects of CAF on non-target species in the aquatic ecosystem. Moreover, several studies have shown that CAF can cause alterations in psychomotor functions/behavioral profile, cell cycle regulation, and reactive oxygen species (ROS) generation in humans and a wide range of aquatic organisms [11,23]. Moreover, several ecotoxicological studies, with CAF, already evaluating neurotoxic, biotransformation, oxidative stress, and genotoxic effects in non-target aquatic organisms [7,26,33–37], mostly on marine organisms, with relevant concentrations in these ecosystems. According to Glazier [38] significant variations in the biological responses by the metabolic pathways and physiological functions are mainly described for different aquatic animals. Thus, phylogenetically and physiologically different species (freshwater *vs* marine) may present various biological responses, under ecologically relevant conditions, according to the environment where they live. Even if only results from phylogenetically close organisms (e.g. fish) are compared, results can vary abruptly, so conclusions are unpredictable and dissociated from generalizations, as mentioned in Rodrigues et al. [39,40], where the effects of an antibiotic in freshwater and marine fish, in environmentally relevant concentrations, were different.

Currently, the continuous input of CAF in the aquatic environment can classify this compound as a pseudo-persistent contaminant, as already shown by Mirasole et al. [41], and Zarrelli et al., [4]. Several entities, namely Organization for Economic Co-operation and Development (OECD), Environmental Protection Agency (EPA), and European Chemical Agency (ECHA), consider CAF as a substance with low priority to monitor in aquatic ecosystems due to the low bioaccumulation potential and the toxic effects to aquatic organisms [31,42,43]. Furthermore, the current databases do not provide chronic toxicity data for aquatic organisms, mainly freshwater, which contributes to a lack of ecotoxicologically relevant data about CAF in inland aquatic ecosystems. Effects of CAF exposure on freshwater species is a largely underexplored field, but toxicity levels for different aquatic species (mainly marine) have been identified by the scientific community [7,34,36,44–48], and therefore it is being increasingly explored and reported.

This study aims to assess the potential chronic effects of environmentally relevant concentrations of CAF on *D. rerio*. This evaluation was conducted on the cellular and biochemical levels, where different biomarkers involved in several metabolic pathways and physiological functions were measured, namely oxidative stress [Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed) activities, and glutathione (GSH) content], lipid peroxidation [Thiobarbituric acid reactive substances (TBARS) levels], neurotoxicity [Acetylcholinesterase (AChE) activity], and reserve energy content [Glycogen (GLY), lipids (LIP), protein total (PROT) contents], and energetic pathways [Lactate dehydrogenase (LDH) activity]. Biological responses (i.e. biochemical biomarkers) applied in this study represent sensitive and reliable responses when assessing the risk of environmental pollutants, such as CAF, in aquatic environments, since endpoints applied in current guidelines (e.g. OECD) may not be sufficient and adequate to evaluate the exposure risks [37]. Furthermore, the present study also aimed to evaluate the integrated biomarker response index (IBR) for an overview of how CAF can impact the fish and demonstrates the efficiency of the biomarker's response. The main reason to conduct this ecotoxicological research was to obtain reliable information that can be used by regulators, in order to protect the freshwater ecosystems from the CAF, where trends towards increasing environmental concentrations have been registered over the years. The present study also intends to be an important contribution to addressing knowledge gaps regarding the impacts of long-term exposures to CAF on freshwater fish.

2. Materials & methods

2.1. Chemicals and test solutions

Caffeine (1,3,7 trimethylxanthine; molecular weight 194.19 g/mol) was acquired from Sigma Aldrich (CAS: 58-08-2), with a degree of purity of 99%. A stock solution (1000 μg/L) was prepared by dilution of CAF in dechlorinated tap water. The concentrations for chronic exposure were chosen based on sub-lethal and environmental concentrations already detected in freshwater ecosystems (Table 1) [1,23]. To conduct this study, 8 nominal concentrations of CAF were defined: 0 (without caffeine - negative control), 0.16, 0.42, 1.09, 2.84, 7.40, 19.23, and 50 μg/L.

2.2. Test organisms: zebrafish

Zebrafish (*D. rerio,* wild type) is a small cyprinid widely used as a model organism in many fields of biological research [49]. Fish used in the experiment were born from a laboratory broodstock and reared under standard laboratory conditions in a zebrafish facility,

at CIIMAR - Interdisciplinary Centre of Marine and Environmental Research (Matosinhos, Portugal), until transferred to the experimental room for acclimation. The acclimation period (three weeks) was conducted in 60 L tanks with continuous aerated and dechlorinated tap water, with controlled conditions of photoperiod (16 h^L:8 h^D) and temperature (27.0 ± 2.0 °C). During this quarantine period, water quality parameters (temperature, pH, dissolved oxygen, ammonium, and nitrite levels) were monitored, and the organisms were fed *ad libitum* with commercial fish food (Tetra Goldfish), every 48 h. No disease signals or death were recorded during this period, and organisms were considered proper/healthy for chronic assay. The use of these organisms has been previously authorized by the Ethical Committee (ORBEA) of the host institution (CIIMAR).

2.3. Chronic ecotoxicity assay

The chronic assay was carried out according to OECD test guideline 215 [49]. This assay was performed under laboratory-controlled conditions similar to those adopted during the acclimation period. Juvenile individuals of *D. rerio* (2.00 ± 0.05 cm; 0.15 ± 0.01 g) were exposed for 28 days to the eight concentrations of CAF (0, 0.16, 0.42, 1.09, 2.84, 7.40, 19.23 and 50.00 μg/L). *D. rerio* was divided by twenty-four 4-L glass aquaria (randomly distributed in the exposure room), with three replicates per treatment (3 aquaria/replicate per treatment, each one with 5 organisms complying with the fish guideline assumptions - 0.8 g of fish mass/L). Every 48 h of exposure, fish were fed, and the medium was 80% renewed.

At the end of the exposure period, organisms were euthanized by immersion in a rapid ice-cold water bath (≤4 °C), according to Wilson et al. [50], and with few adaptations that are described in Rodrigues et al. [51]. When fish lose their opercular movements and swimming ability, were immediately sacrificed by decapitation. This practice agrees with the American Veterinary Medical Association (AVMA) guidelines for the Euthanasia of Animals and Portuguese animal welfare laws, taking into consideration the Portuguese animal welfare testing regulations (Decree-Law 113, 2013) [52], and was considered a procedure effective, rapid, and not stressful for the fish [50].

Physical and chemical water parameters (pH, temperature, and dissolved oxygen) were measured every two days using a multiparametric probe (Multi 3630 IDS SET F). For ammonium and nitrites determinations, water aliquots from the exposure aquarium were collected, before medium renewal, and a bench photometer (Spectroquant Multy Colimeter) was used to conduct the analysis.

2.4. Determination of CAF concentration

For the quantification of CAF analytical concentrations (Table 2), a volume of 100 mL of water was collected from all aquaria tests, at the beginning of the assay (0 h) and before the renewal of the medium (48 h). Samples were immediately frozen at −20 °C until further quantification of CAF. For this quantification, the samples were filtered using a regenerated cellulose syringe filter (0.22 mm pore). One milliliter of the filtered sample was spiked with 10 ng of internal standards of Caffeine-(trimethyl)-13C3 per aliquot of sample. The samples were analyzed by a DIONEX UltiMate 3000 ultra-high pressure liquid chromatography (UPLC) system (Thermo Scientific, Waltham, MA, USA) coupled to a triple quadrupole mass spectrometer (MS/MS) (TSQ QUANTIVA, Thermo SCIENTIFIC, Waltham, MA, USA). An Acquity UPLC BEH-C18 column (Waters, 100 mm × 2.1 i. d., 1.7 μm particle size from Waters Corporation, Manchester, UK) was used as an analytical column. The injection volume was 10 μL for all samples. A heated electrospray ionization (H-ESI) was used to ionize the target compound. The spray voltage was set to static: positive ion (V) 3500. Nitrogen (purity >99.999%) was used as a sheath gas (50 arbitrary units), auxiliary gas (15 arbitrary units), and sweep gas (2 arbitrary units). The vaporizer was heated to 400 °C and the capillary to 325 °C. The mobile phase consisted of MilliQ with 5 mM ammonium acetate and acetonitrile. The flow rate was 0.5 mL/min and the run time was 15 min. Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) was used to optimize the instrument methods and running of samples. The obtained data were evaluated using TraceFinderTM 3.3. Software (Thermo Fisher).

The linearity of the calibration curve was tested in the range from 0.01 ng/mL to 100 ng/mL. The limit of quantification (LOQ) was calculated as one-quarter of the lowest calibration point in the calibration curve where the relative standard deviation of the average response factor was <30%. LOQ was 0.31 μg/L. The precision of the method was evaluated by the repeatability of the study. For this

Table 2
Nominal and analytical concentrations (average ± standard deviation) of CAF in water samples, and physical and chemical parameters measured during chronic exposure. BLOQ – Below limit of quantification (< 0.31 μg/L).

Caffeine concentrations (μg/L)			pH	Temp (°C)	O_2 (mg/L)	Nitrites (mg/L)	Ammonium (mg/L)
Nominal	Analytical						
	0 h	48 h					
CTL	0	0	7.96 ± 0.04	27.5 ± 0.3	7.85 ± 0.03	0.19 ± 0.06	0.29 ± 0.17
0.16	BLOQ	BLOQ	8.04 ± 0.02	26.8 ± 0.3	7.91 ± 0.03	0.19 ± 0.08	0.27 ± 0.08
0.42	1.67 ± 0.18	0.78 ± 0.07	8.00 ± 0.01	26.9 ± 0.4	7.90 ± 0.06	0.32 ± 0.08	0.17 ± 0.07
1.09	2.03 ± 0.07	BLOQ	8.01 ± 0.04	27.0 ± 0.2	7.89 ± 0.03	0.28 ± 0.10	0.36 ± 0.23
2.84	3.47 ± 0.29	0.64 ± 0.24	7.99 ± 0.05	26.9 ± 0.2	7.89 ± 0.03	0.26 ± 0.03	0.39 ± 0.19
7.40	9.03 ± 0.50	2.47 ± 0.82	7.99 ± 0.05	27.5 ± 0.3	7.81 ± 0.06	0.26 ± 0.07	0.40 ± 0.30
19.23	22.67 ± 2.33	8.27 ± 2.03	7.95 ± 0.04	27.6 ± 0.1	7.77 ± 0.01	0.22 ± 0.10	0.52 ± 0.24
50.00	60.67 ± 2.73	38.67 ± 0.67	7.90 ± 0.06	27.1 ± 0.5	7.87 ± 0.07	0.27 ± 0.04	0.25 ± 0.15

purpose, all samples were prepared in triplicates. No target compound was detected in method blanks and control samples.

2.5. Biological samples collection and data analysis

Samples (bodies and heads) were stored at −80 °C until the biochemical determinations be conducted, according to the protocols described in Table 3. Body samples were used for evaluation of antioxidant defense [Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed) activities; and glutathione (GSH) content], biotransformation [Glutathione S-transferases activity (GSTs)], lipid peroxidation [Thiobarbituric acid reactive substances (TBARS) levels] biomarkers, and quantification of energetic reserves [glycogen (GLY), lipids (LIP), protein total (PROT) contents, and energy production via lactate dehydrogenase (LDH) activity] (Table 3). Head samples were used for acetylcholinesterase (AChE) activity determinations.

Data from biomarkers were checked for normality by the Shapiro-Wilk test and for homogeneity of variances by Levene's test. An analysis of variance (one-way ANOVA), followed by a Dunnett's test was carried out to determine significant differences between the treatments and the negative control (without caffeine). All statistical analyses were performed using SPSS Statistics v26 and a significance level of $\alpha = 0.05$.

2.6. Integrated biomarker responses version 2

The Integrated Biomarker Response version 2 (IBRv2) index was used to combine the responses of the biomarkers analyzed (organized in the following clockwise order: SOD, CAT, GPX, GRed, GSH, GSTs, TBARS, GLY, LIP, PROT, LDH, and AChE) to evaluate CAF effects. IBRv2 was calculated according to Beliaeff and Burgeot [65], with the update described by Sanchez et al., [66]. To estimate the IBRv2 index, the following calculations were performed:

mean X0 – stands for the mean results of each biomarker.

Yi = log (Xi/mean X0), where Xi is the data of each biomarker in each treatment (or replicate). After that, the general mean (μ) and standard deviation (s) of Yi for each biomarker were calculated.

Zi = (Yi - μ)/s.

A = mean Zi – mean Z0 (control)

Table 3
Summary details of the protocols for the here-biomarker determinations.

Biomarkers	Sample	Tissue preparation	Biomarker determinations			
		Homogenization Centrifugation	Dilution factor	Spectrophotometric readings (nm)	Result expression units	References
PROT	All	same to the corresponding biomarker	1:20	595	mg protein/body weight	[53]
SOD	Body (2 fish per replicate)	2.5 mL - phosphate buffer (50 mM, pH 7.0) with Triton X-100 (0.1%) 15,000 rpm; 10 min; 4 °C	1:15	500	units min/mg/ protein	[54]
CAT			1:100	240	μmol/min/mg protein	[55]
GPx			1:2	340	mmol/min /mg protein	[56]
GRed			1:2	340	μmol/min/mg protein	[57]
GSH			1:20	412	μg/mg protein	[58]
GSTs			1:20	340	mmol/min /mg protein	[59]
TBARS			1:10	535	mmol/mg protein	[60]
GLY	Body (1 fish per replicate)	2.5 mL - phosphate buffer (50 mM, pH 7.0) with Triton X-100 (0.1%) 4000 rpm; 10 min; 4 °C	1:1	490	μg/mg protein	[61]
LIP	Body (1 fish per replicate)	4 mL - chloroform: methanol (2:1) mixture 3000 rpm; 10 min	1:1	*	mmol/mg protein	[62]
LDH	Body (1 fish per replicate)	2.5 mL - TRIS buffer (0.1 M, pH = 7.2) 6000 rpm; 3 min; 4 °C	1:15	340	mmol/min /mg protein	[63]
AChE	Head (1 fish per replicate)	1.5 mL - phosphate buffer (0.1 M, pH 7.2) 6000 rpm; 5 min; 4 °C	1:20	412	nmol/min/mg protein	[64]

*Extraction procedure through the biphasic solvent system consisting of chloroform/methanol/water. The results (% of lipids) were obtained by the difference between the weight of tubes before and after the lipids extraction

$$IBRv2 = \sum |A|$$

The IBRv2 data were represented in a star plot, representing each biomarker's deviation in relation to the control (0 μg/L of CAF). Biomarker induction is represented by the area up to 0, and biomarker inhibition by the area down to 0. Star plots and IBRv2 values were performed using Microsoft Excel software.

3. Results

3.1. Water quality

During the chronic assay, water quality parameters measured (pH, temperature, dissolved oxygen, ammonium, and nitrites) were within the quality assurance criteria [49] (Table 2). Moreover, no mortality was recorded during the chronic exposure, complying with

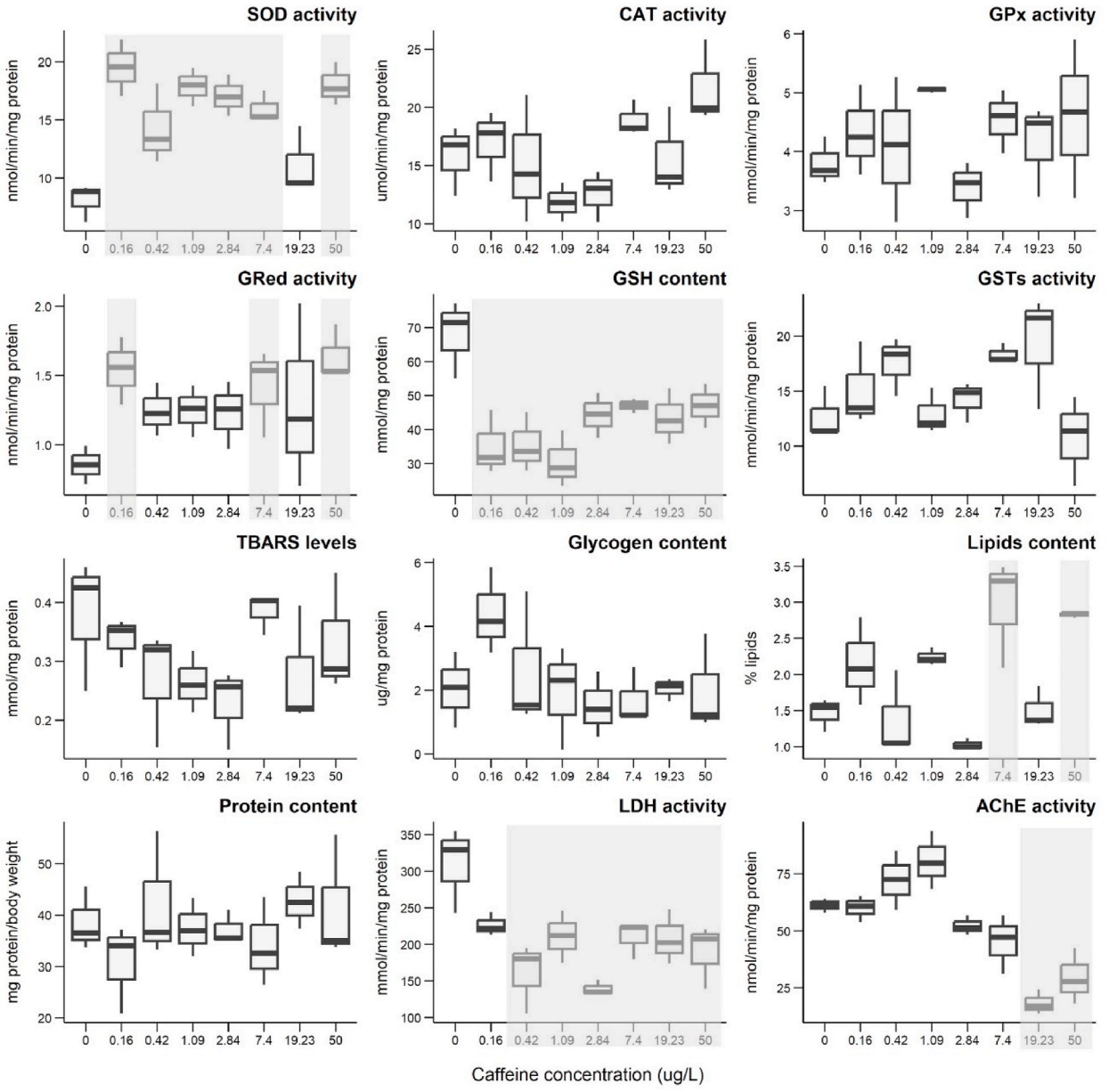

Fig. 1. Results of biochemical biomarkers on *D. rerio*, after chronic exposure to a range of caffeine concentrations. Data are shown as individual observations (white/open circles) and expressed as mean (n = 3) ± standard error (black circles and error bars). Grey shadows represent significant differences compared to the control treatment (Dunnett's test, $p < 0.05$).

the OECD 2000 requirements (mortality < 10% in the control group).

3.2. Biomarkers

The results of biochemical biomarkers are presented in Fig. 1. Relatively to antioxidant responses, the results showed that all caffeine concentrations tested, except for 19.23 μg/L, significantly increased the activity of SOD ($F_{[7,\ 23,]} = 9.146$; $p < 0.001$). A significant increase was also observed in GRed activity when the organisms were exposed to 0.16, 7.4, and 50 μg/L ($F_{[7,\ 23,]} = 4.523$; $p = 0.006$). The opposite was observed in GSH levels, which significantly decreased after exposure to all CAF concentrations tested ($F_{[7,\ 23,]} = 6.018$; $p = 0.001$). On the other hand, CAT ($F_{[7,\ 23]} = 2.885$, $p = 0.038$), GPx ($F_{[7,\ 23]} = 1.215$; $p = 0.350$), GSTs ($F_{[7,\ 23]} = 2.800$, $p = 0.042$) activities, as well as TBARS levels (lipid peroxidation) ($F_{[7,\ 23]} = 1.000$; $p = 0.466$) were not significantly altered after chronic exposure to different concentrations of CAF relatively to the unexposed organisms (0 μg/L of CAF; Fig. 1).

In terms of biomarkers of energetic reserves and the energetic pathway (Fig. 1), LIP content significantly increased after exposure to 7.4 and 50 μg/L ($F_{[7,\ 23]} = 8.401$; $p = 0.001$), while no significant effects were observed on GLY ($F_{[7,\ 23]} = 1.360$; $p = 0.287$) and PROT content ($F_{[7,\ 23]} = 0.730$, $p = 0.650$). LDH activity showed a significant decrease in almost concentrations tested ($F_{[7,\ 23]} = 5.389$; $p = 0.003$), except for the lowest (0.16 μg/L of CAF).

Regarding the results of the activity of AChE (Fig. 1), a significant decrease ($F_{[7,\ 23,]} = 14.326$; $p < 0.001$) was observed for the highest CAF concentrations tested 19.23 and 50 μg/L (Fig. 1).

IBRv2 results and the star plots for biomarker responses are shown in Fig. 2. The highest score obtained stands for the highest concentration tested (50 μg/L of CAF), and the effects on SOD, GRed, GSTs, and LIP, were the most relevant responses that explain the IBRv2 (Fig. 2). Overall, and regardless of the CAF concentrations tested, the most relevant responses arise in the increase of SOD and GRed activity (highest disturbances relatively to control) and the decrease of GSH and LDH (lowest values) (Fig. 2).

4. Discussion

4.1. Antioxidant responses and lipid peroxidation

In order to deal with oxidative damage (LPO), organisms developed multiple systems of antioxidant defense, which involve the activity of specific enzymes (e.g., SOD, CAT, GPx, and GRed, indirectly). Several studies showed that CAF activates the antioxidant enzymatic pathways in organisms exposed, to combat oxidative stress [6]. According to Liu et al. [67], CAF can be efficiently transported by the enzyme SOD, and this interaction can affect their conformation (change the secondary structure of SOD) but not the enzyme's activity. Nevertheless, several studies have shown that SOD activity tends to increase with CAF, once can superimpose/potentiate SOD antioxidant capacity [6,68], a fact also observed in our study after exposure to all CAF concentrations tested (except to 19.23 μg/L; Figs. 1 and 2). An increase in SOD activity was also reported in the fish *Carassius auratus*, after a short exposure (one week) to 3.2 μg/L to 10 mg/L of CAF [68], with greater activity of the highest concentrations (> 4.0 mg/L), which are well above the environmental levels reported for surface waters (Table 1). The same pattern was observed in marine species (e.g. *Diopatra neapolitana* and *Arenicola marina*) after exposure to the same range of CAF concentrations [48]. Moreover, Cruz et al. [46], showed that after 28 days of CAF exposure (0.5, 3, and 18 μg/L), a significant increase in SOD and CAT activity was observed in marine clam

Fig. 2. The IBRv2 index results and star plot, with all the biomarkers determined on *D. rerio*, after exposure to a range of caffeine concentrations.

Ruditapes philippinarum. CAT and GPx activities are enzymes responsible for removing H_2O_2 from the cell [40], however, our results showed an absence of effect after CAF exposure in these enzymatic activities (Fig. 1), probably because H_2O_2 levels (in part resulting from SOD activity) were not yet sufficient to significantly increase CAT and GPx activities. In this study, potentially the removal of H_2O_2 is still ensured by basal levels of these antioxidant enzymes. Pires et al. [34], also evaluate the effects of CAF (0.5, 3.0, and 18.0 μg/L) in the marine invertebrate *Hediste diversicolor*, after chronic exposure, however, no effects on SOD activity and a significant increase in CAT activity at 3 and 18 μg/L was recorded, contrary to the results obtained in the present study. Aguirre-Martínez et al. [35], observed an increase in GPx activity in marine crab *Carcinus maenas* exposed for 28 days to 5 and 15 μg/L of CAF. Several studies showed that antioxidant defenses (SOD, CAT, GPx) can be stimulated in several aquatic organisms (freshwater or marine), due to CAF exposure, demonstrating the activation of the antioxidant defense (with different biomarker responses depending on the organisms) for eliminating ROS and preventing oxidative damage [6,7,35].

In the present work, and regarding the previously reported set of antioxidant glutathione-dependent enzymes, only GRed activity increased, after exposure to 0.16, 7.4, and 50 μg/L of CAF (Fig. 1). Corroborating our results, Li et al. [6], reported that CAF exposure also induced GRed activity in marine invertebrates, such as the amphipod *Ampelisca brevicorni*. The concentration of 50 μg/L of CAF showed an increase in GRed activity also in the freshwater clam *Corbicula fluminea* after 21 days of exposure [44], and in the marine species *R. philippinarum* after 14 days [45]. A significant increase in this enzyme activity suggests that GRed possibly is involved in GSH restoration, for the glutathione cycle [69]. In addition to being a co-substrate of GSTs and a co-factor for GPx and GRed, GSH plays an important role in the non-enzymatic antioxidant defense [29], acting as a free radical scavenger, and preventing oxidative damage [48]. Our results showed a significant decrease in GSH content after exposure to all the CAF concentrations tested (Figs. 1 and 2), which demonstrated that GSH was used to conjugate with metabolites or ROS. As in our study, Pires et al. [48], report a decrease in GSH content in *D. neapolitana* and *A. marina* after 28 days of CAF exposure (0.5, 3, and 18 μg/L). However, this GSH decrease and the increase in antioxidant enzyme activities (SOD, CAT, and GSTs) were not enough to prevent LPO [48].

At phase II detoxification, the GSTs conjugate GSH with electrophilic substances [70], turning toxic compounds more easily excretable [71]. Several studies show that the activity of this enzyme increased in response to CAF exposure [29,45], however, in the present study GSTs activity was unaffected (Fig. 1). Indeed, Santos-Silva et al. [29], already demonstrated that the freshwater fish *Prochilodus lineatus* after exposure to 0.3, 3, and 30 μg/L of CAF, do not show differences in GSTs activity in the brain and liver, agreeing with our results. This finding suggests that GSTs are not involved in the phase II metabolism of CAF [29], and probably other enzymes, such as cytochrome monooxygenases P450 enzymes (CYP450) can be responsible for the detoxification/biotransformation of CAF, in these freshwater fish and under the conditions evaluated. In fact, different studies demonstrated that CAF is metabolized mainly by enzymes from phase I (CYP450) [29,72]. Contrary to what was observed here, studies with marine species such as *C. maenas* [35], and *H. diversicolor* [34], presented a GSTs activity induced, after exposure to relevant CAF concentrations in marine ecosystems.

TBARS levels appear as by-products after occur lipid peroxidation and reflect the cell/tissue toxicity induced by free radicals [71]. In the present study, no significant TBARS changes were recorded (Fig. 1), which showed that the detoxification and antioxidant defense pathways (enzymatic and non-enzymatic as SOD, GSH, and GRed) were efficient to fight against the excess of free radicals and to prevent lipid peroxidation. Contrary to what was observed here, most studies demonstrate that the antioxidant defense cannot neutralize the effect of CAF, causing lipid peroxidation, namely in estuarine and marine organisms [6,7,45,48]. A study with similar CAF concentrations (0.5, 3.0, and 18.0 μg/L) proved to be sufficient to cause a significant increase in the level of TBARS in the marine species *R. philippinarum*, after 28 days of exposure [46]. However, few studies also reported similar results in freshwater species. Santos-Silva et al. [29], reported that 0.3, 3, and 30 μg/L of CAF caused an increase in TBARS levels in the liver and brain of *P. lineatus* after 24 h and 7 days of exposure [29]. However, these exposure periods are short, given the environmental relevance that we demarcated in this study. In addition, organisms may have the ability to adapt and neutralize effects, which may be more noticeable in longer exposure periods. As we can observe in here obtained biomarker results, long exposure periods to ecologically relevant concentrations of CAF in freshwater ecosystems may be harmful to zebrafish, acting on specific pathways, which appear to have remarkable repercussions at the cellular level (Fig. 1). The different studies previously reported, allowed us to infer that the different biological responses (different biomarker responses) between different organisms (freshwater *vs* marine) indicate that CAF interferes with oxidative metabolism, in the different conditions of environmental relevance (that is, relevant environmental concentrations in freshwater and marine ecosystems evaluated in different studies, as well as exposure times).

4.2. Energy sources

The energy needs of fish are supplied by fats, carbohydrates, and proteins. In the presence of contaminants, such as CAF, and to activate detoxifying and antioxidant defense mechanisms, organisms can resort to energy reserves (e.g., GLY content) [46]. In the present study, no significant effects were recorded on GLY and protein content after exposure to CAF concentrations (Fig. 1). Regarding GLY content, Nunes et al. [33], reported that concentrations within the range of concentrations tested in our study (0.4, 2, 10, and 50 μg/L of CAF) did not affect the GLY content in *Daphnia magna*, corroborating our results. However, overall, the typical response to CAF exposure showed a GLY reduction in marine species *R. philippinarum*, *D. neapolitana,* and *A. marina* [46,48], which are phylogenetically and physiologically different from *D. rerio*. CAF can be responsible for the inhibition of phosphodiesterase activity and the consequent increase in cyclic adenosine 3′,5′-monophosphate levels (cAMP). This increase is responsible for activating an enzyme - 5′-AMP-activated protein kinase (AMPK) - which allows adjusting cellular energy homeostasis [33]. In the presence of CAF, AMPK promotes catabolism, leading degradation of fatty acids (lipid turnover and oxidation), and the consequent increase in glucose absorption. Thereby overall after CAF exposure organisms reduce their energy reserves resulting in lower GLY content in their tissue [33]. Regarding the Body PROT content was not also affected by CAF (Fig. 1) which can be related to the fact that proteins are the last

fraction of energy that is consumed [73]. However, Pires et al. [48], reported a significant decrease in PROT content after exposure to an increased CAF concentration range (0.5, 3, and 18 μg/L) in *D. neapolitana*. Pires et al. [48], argue that this decrease is due to energy expenditure associated with increased activity of antioxidant enzymes recorded (e.g., SOD increase). On the opposite, Cruz et al. [46], observed, for the same concentrations, a significant increase in PROT levels, in *R. philippinarum*. In this case, Cruz et al. [46], hypothesized that this increase can be the result of the stimulation of protein synthesis to guarantee the increase of metabolic defenses involved in detoxification pathways and consequently prevent oxidative stress.

There are no studies describing the relationship between the total lipids of aquatic organisms and CAF exposure. However, changes in lipid energy reserves can be a sensitive indicator of perturbations in zebrafish due to a source of stress (e.g., contaminants), given and allowing linking cellular effects to other ecotoxicological endpoints (e.g., individual responses) [74]. In our study, LIP content increased significantly after exposure to 7.4 and 50 μg/L of CAF (Fig. 1). However, it is known that CAF attenuates lipid accumulation, reduces triglyceride and cholesterol levels in concentration-dependent manners, and is mainly responsible for lipid turnover [75,76]. Notwithstanding, Aguirre-Martinez et al. [47], reported that CAF (0.1, 1, 10, and 50 μg/L) did not affect the total lipids in the freshwater bivalve *C. fluminea*, concluding that other energy sources, different from lipid reserves (e.g., protein), were used to supply the necessary energy. Differences in the reserve mobilization may be due to intrinsic factors, such as species (as observed here and in the cited studies), stage of the life cycle, degree of adaptation and resistance to the compound, as well as modulating environmental (e.g. temperature) factors, habitat, etc.

LDH activity is a biomarker that can be used to evaluate energetic metabolism disturbances since is a crucial enzyme of the anaerobic pathway for producing energy [40]. In the present study, a significant decrease in LDH activity > 0.42 μg/L of CAF was recorded, indicating that pyruvate conversion was perturbed (Fig. 1). Some studies in fish report that LDH inhibition may be directly related to alterations in mitochondrial membrane function or impaired carbohydrate metabolism [40,77]. However, in this case, this may occur due to the competition for the substrate pyruvate, since CAF binds at the substrate binding site and interacts allosterically with the coenzyme binding site (blocks the action of LDH in the transformation of pyruvate into lactate). Gardiner et al. [78], showed that CAF is a competitive inhibitor of the enzyme lactate dehydrogenase, preventing it from using the substrate pyruvate. Despite the lack of information, several authors have already reported the inhibition of this enzyme activity after exposure to different types of compounds, namely pharmaceuticals, metals, and hormones [40,79,80]. Thus, changes in the allocation of energy reserves, depending on their magnitude, may be closely related to changes in individual parameters of the life cycle of organisms (e.g., growth and reproduction), which may have negative consequences at higher levels of biological organization [74]. Generically, this work confirm that different organisms can use different ways of obtaining energy, and as such the biochemical responses can differ. However, a general finding is related to the fact that with the increase in exposure concentrations, organisms can alter energy reserve levels and pathways to obtain energy, to fight against oxidative stress caused by CAF.

4.3. Neurotransmission

Acetylcholinesterase (AChE) is an enzyme involved in neurotransmission, responsible for degrading the neurotransmitter acetylcholine [64], playing an important role in neuromuscular transmission, and neuronal differentiation [81]. CAF causes a blockage of adenosine receptors, preventing the binding of adenosine to its receptor [82]. This blockage can indirectly affect the release of neurotransmitters [e.g., dopamine, gamma-aminobutyric acid (GABA), and glutamate], cause inhibition of neurotransmission [83], and affect organisms' locomotor behavior [84]. Haskell-Ramsay et al. [85], identified CAF as a substance able to inhibit enzymes responsible for the breakdown of neurotransmitters, namely acetylcholinesterase. A structural feature of CAF, the N-methyl of the pyrrolidine ring is an essential binding receptor to AChE, causing its inhibition [86,87]. The results here-obtained (Fig. 1) are in agreement with other CAF studies where AChE activity decreases after exposure to a CAF concentration-dependent manner [88]. Likewise, Farias et al. [86], reported that AChE activity decreased in zebrafish embryos exposed to a range of CAF concentrations (0.0088, 0.16, 2.8, and 50 mg/L) for 7 days. Muñoz-Peñuela et al. [26], exposed *Astyanax altiparanae* (a freshwater fish species) to a 27.5 μg/L of CAF and observed inhibition of AChE activity only in muscle. Aguirre-Martínez et al. [45], evaluated the neurotoxicity of different CAF concentrations (5, 15, and 50 μg/L) in *R. philippinarum,* after 14 days of exposure, and concluded that the higher concentration tested (50 μg/L - the same concentration tested in our study) cause a significant decreased of AChE activity. Thus, and similar to the here-presented results, CAF was able to cause neurotoxic effects, due to the decrease in AChE activity. Therefore, the hazard of CAF for fish populations and aquatic organisms, in general, deserves further attention since unexpected effects on neuro-behavioral parameters occur at concentrations often detected in natural aquatic ecosystems. These results are disquieting and worrying since environmentally relevant concentrations of CAF are capable of inhibiting AChE activity in organisms of different aquatic ecosystems, which can cause prolonged over-stimulation of nerve and muscle fibers due to increased acetylcholine at synapses, affecting locomotor activities and compromising normal behavior, including feeding, escape response, and reproduction [26].

4.4. IBRv2 index - integrative stress response category

IBRv2 index has been widely used in the field and laboratory studies to analyze the combined effects of contaminants and their impacts on different organisms [89–91]. Biomarkers exhibit a response to different stress factors (in this study CAF concentrations; Fig. 2), and the representation of this response in a star plot provided a clearer visualization of which biomarkers were the most responsive/sensitive to the exposure compound [91]. The IBRv2 values obtained were similar independent of the CAF concentrations (Fig. 2). However, a strong induced response was observed for SOD, GRed, and GSTs activities, while a strong but inhibitory response was observed in LDH activities and GSH content, which could indicate that CAF caused several metabolic pathway disturbances in

zebrafish. This fact was more evidence regarding the results of 50 μg/L of CAF, where the IBRv2 value was the highest recorded (14.59), according to the known effects of this concentration in the activity of several antioxidant enzymes [44,45]. The IBR index showed clearly that biomarker responses were responsive and coherent to demonstrate the stress caused by CAF on fish.

5. Conclusions

Environmentally relevant concentrations of CAF after chronic exposure affected the different metabolic pathways in *D. rerio*, as demonstrated by the biochemical biomarker's individual and integrated responses. Using the integrative approach of biomarkers and IBRv2 values, the results reveal that exposure to CAF induces: i) significant disruptions in antioxidant defense pathways (SOD, GRed, and GSH); ii) cellular energy allocation mechanisms somewhat affected as LDH activity and lipids content; iii) the highest concentrations of CAF were responsible for neuro-oxidative disturbances. Regarding the here-presented results and the literature data (essentially with estuarine and marine organisms), CAF is capable of causing deleterious biological effects on aquatic biota at biochemical (e.g., metabolic pathways measured by biomarkers), cellular (e.g., physiological functions such as neurotransmission, respiration, hormone, and enzyme secretion), and individual levels (e.g., locomotor behavior, reproduction, growth), in environmentally relevant concentrations. Therefore, more research is crucial for revealing the potential repercussions of CAF in different aquatic ecosystems, with different levels of contamination. Furthermore, with the here-obtained and literature results we confirm that phylogenetically and physiologically different species (freshwater *vs* marine) may present different biological responses with greater or lesser environmental concern under ecologically relevant conditions. This study also intends to alert the scientific community and society in general to the problems of environmental contamination and ecosystem sustainability and security, associated with levels of CAF detected in surface waters.

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Author contribution statement

Bárbara S. Diogo, Sara C. Antunes, Ivo Pinto and Sara Rodrigues - Conceived and designed the experiments; Bárbara S. Diogo, Sara C. Antunes, Ivo Pinto, João Amorim, Cláudia Teixeira, Luís Oliva Teles, Oksana Golovko, Vladimír Žlábek, and Sara Rodrigues - Performed the experiments; Bárbara S. Diogo, Sara C. Antunes, Ivo Pinto, António Paulo Carvalho, and Sara Rodrigues - Analyzed and interpreted the data; Sara Rodrigues and Sara C. Antunes - Contributed reagents, materials, analysis tools or data; Bárbara S. Diogo, Sara C. Antunes, Ivo Pinto, João Amorim, Cláudia Teixeira, Luís Oliva Teles, Oksana Golovko, Vladimír Žlábek, António Paulo Carvalho, Sara Rodrigues - Wrote the paper.

Data availability statement

All data are included in the article.

Additional information

No additional information is available for this paper.

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.

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