RESEARCH ARTICLE

Dangerous connections: biochemical and behavioral traits in *Daphnia magna* and *Daphnia longispina* exposed to ecologically relevant amounts of paracetamol

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Abstract

Exposure of nontarget organisms to therapeutic agents can cause distinct toxic effects, even at low concentrations. Paracetamol is a painkiller drug, widely used in human and veterinary therapies, being frequently found in the aquatic compartment in considerable amounts. Its toxicity has already been established for some species, but its full ecotoxicological potential is still not sufficiently described. To characterize the ecotoxicity of paracetamol, the present study evaluated several parameters, such as acute immobilization (EC_{50} calculation), biochemical alterations, and behavioral effects, in two species of freshwater microcrustaceans of the genus *Daphnia* (*D. magna* and *D. longispina*). To increase the relevance of the data obtained, animals were exposed to levels of paracetamol similar to those already reported to occur in the wild. Data showed antioxidant responses in both species, namely an increase of catalase and GSTs activities in *D. magna*. On the contrary, effects of paracetamol on *D. longispina* included only an impairment of GSTs activity. Despite the absence of anticholinesterasic effects, behavioral modifications were also observed. This set of data indicates that realistic levels of paracetamol may trigger the activation of the antioxidant defense system of freshwater crustaceans, causing changes in behavioral traits (increase in swimming time, but with a reduction in swimming distance) of unknown etiology that are likely to affect normal life traits of wild populations.

Keywords Drugs · Crustaceans · Biomarkers · Population effects · Sublethal effect

Introduction

Pharmaceuticals have been considered important pollutants, due to their increasing detection frequency in environmental samples, namely those from the aquatic compartment (Ankley et al. 2007; Pugajeva et al. 2017). Being biologically active, pharmaceutical drugs have the potential to cause alterations and damages to organisms, contributing for adverse ecosystem changes (Elorriaga et al. 2013). Pharmaceutical residues can be found in different compartments, such as the aquatic environment (untreated raw sewage, sewage treatment plant effluents, surface water, groundwater, and drinking water), sediment, and soils subjected to sewage (sludge) application (Bila and Dezotti 2003; Fent et al. 2006; Elorriaga et al. 2013; Gaffney et al. 2014; Der Beek et al. 2016; Balakrishna et al. 2017).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used because of their analgesic, antipyretic, and anti-inflammatory properties. NSAIDs are, in general, weak acids and act as nonselective inhibitors of cyclooxygenase isoforms, COX-1 and COX-2 (Funk 2001; Hayashi et al. 2008; Santos et al. 2010). By acting this way, NSAIDs have the potential to cause also effects on many other organisms, environmentally exposed. The potential ecotoxicological effects of these pharmaceutical drugs are favored by the homology of routes in which these chemicals may act, which are in general evolutionary conserved; in fact, mammalian/human forms usually have homologous couterparts in many wild organisms. For instance, mammalian COX-2 equivalent enzymes have been detected in freshwater fish (e.g., *Carassius auratus*; Zou et al.

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1999). In invertebrates, prostaglandin biosynthesis has been reported to occur in many organisms, such as mussels (*Mytilus edulis*), sponges (*Reniera mucosa*), and cnidarians (*Gersemia fruticosa*), indicating that these organisms are susceptible to inhibition of their COX forms by the environmental presence of NSAIDs (Rowley 2005).

Not being fully classified as a NSAID, paracetamol (N-acetyl-p-aminophenol) is a drug widely used in human medicine (Nunes et al. 2014). Paracetamol shares with classic NSAIDS the same therapeutic uses, since it is also a weak inhibitor of cyclooxygenase. This property grants paracetamol analgesic and antipyretic effects, but without significant anti-inflammatory activity (Santos et al. 2010). Despite being largely eliminated by wastewater treatment plants (WWTPs), it is considered a pseudo-persistent pollutant due to its constant release into the ecosystem. Consequently, small amounts of paracetamol are detected in the aquatic environment (levels in general of the ng/l to the μg/l range) (Gómez et al. 2007). Paracetamol has already been found in distinct aquatic matrices, such as effluents from wastewater treatment plants, groundwater, and drinking water (Gros et al. 2012; Rabiet et al. 2006). In France, paracetamol was detected in waste water, in levels of 11.3 μg/l (Rabiet et al. 2006) and 200 μg/l (Togola and Budzinski 2008). In England, paracetamol was also reported, in surface waters (Tyne River), in concentrations of 69.5 μg/l (Roberts and Thomas 2006). In Portugal, paracetamol was found in the Arade river, in concentrations between 19 and 88 ng/l (Gonzalez-Rey et al. 2015); it was also found in seven rivers in the north of the country, in which not only the parental compound but also metabolites (p-aminophenol and paracetamol-glucuronide) were detected, with concentrations of 0.25 μg/l for paracetamol, 3.47 μg/l for the metabolite paracetamol-glucuronide, and 1.63 μg/l for p-aminophenol (Santos et al. 2013).

The toxicity of this compound in humans (and vertebrates in general) derives from its metabolism. In vertebrates, low dosages of paracetamol are excreted after conjugation with glucuronic acid and with sulphate; however, these cofactors are likely to be exhausted, especially if the ingested amount of paracetamol is high. In this case, both cofactors are depleted, and paracetamol is bioactivated by oxidative metabolism, via the cytochrome P450 (namely, the CYP2E1 isoenzyme) pathway. This route results in the formation of N-acetyl-p-benzoquinone imine, a highly reactive and electrophilic metabolite, which is in turn conjugated with glutathione (GSH) and excreted (Dahlin et al. 1984). However, if glutathione is also exhausted, this metabolite accumulates and exerts toxic effects (James et al. 2003), mediated by covalent modifications of thiol groups on cellular proteins, DNA and RNA damage, oxidation of membrane lipids, resulting in necrosis, and cell death (James et al. 2003; Brandão et al. 2011). Despite the absence of a large amount of data about the metabolic pathways involved in the metabolism of paracetamol

by invertebrates, the metabolism of this drug seems also to involve the cytochrome P450 route. In fact, the study conducted by Ding et al. (2020) showed that *D. magna*, after being exposed to paratecetamol, increased the expression of genes of the CYP450 system. Considering this involvement of the CYP450, and the putative production of a toxic intermediate, it is not surprising that the adverse effects of paracetamol, in most organisms, are mediated by oxidative effects. Paracetamol toxicity involving changes in the antioxidant defense system has been reported for other vertebrate animal models, such as fish (*Anguilla anguilla,* Nunes et al. 2015; *Cyprinus carpio,* Gutiérrez-Noya et al. 2021), but especially for invertebrates, such as *Ruditapes philippinarum* (Correia et al. 2016; Nunes et al. 2017), *Corbicula fluminea* (Brandão et al. 2011), and *Daphnia magna* (Oliveira et al. 2015; Daniel et al. 2019). Despite the absence of data on the metabolism of paracetamol by invertebrates, this similarity of trends in terms of its toxicological effects strongly suggests that a conserved pathway (which results in the activation of paracetamol into a toxic metabolite) exists among very distinct taxa.

The biological responses to the toxicity of paracetamol seems not to be exclusively limited to the antioxidant defense system or peroxidative damage. In fact, the data obtained by Nunes et al. (2015) and by Daniel et al. (2019) suggested that paracetamol is able to inhibit the cholinesterase activity by oxidative denaturation, in the fish *A. anguilla* and in the crustacean *D. magna*, respectively, thereby explaining its potential neuroactive and behavioral deleterious effects. In fact, this linkage between oxidative effects, cholinesterasic inhibition, and adverse behavioral effects was recently established by Matus et al. (2018) and Nogueira et al. (2018), in the fish *Phalloceros harpagos* and *Danio rerio*, respectively.

Toxicity protocols issued by international regulatory organizations (e.g., Organization of the Economic Cooperation and Development, OECD; American Society for Testing and Materials, ASTM; United States Environmental Protection Agency, US EPA) use *D. magna* as a model organism to evaluate parameters such as acute immobilization, reproduction, and behavioral changes (Persoone et al. 2009). *D. longispina* has been used as an indicator species to assess water quality (Neves et al. 2015), and recent studies have suggested that the behavior of these organisms may be a promising and effective tool to address subtle adeverse effects caused by a vast array of chemicals (Bownik 2017). Both species have been used as model organisms in drug trials (Dave and Herger 2012; Du et al. 2016) and for the toxicological evaluation of the effects of metals (Biesinger and Christensen 1972; Martins et al. 2017) and herbicides (Neves et al. 2015), among others. However, the performance of these two species when used in ecotoxicological tests is likely to evidence significant interspecific differences; *D. magna* is a long established standard microcrustacean species, while *D. longispina* is an autohctonous organism.

Consequently, their tolerance to environmental chemicals is not similar or even coincident, and such differences may be of high ecological interest (Gonçalves et al. 2007).

In addition, the existing literature about the acute toxic effects of paracetamol on microcrustaceans presents a considerable variation, not only of the type and duration of the tests but also in terms of results. The study by Nunes et al. (2014) summarized a few studies from the literature and showed that previously calculated EC_{50} values for *D. magna* varied considerably. The 24-h EC_{50} numbers varied from 13 to 293 mg/l; 48-h EC_{50} values were comprised bewtween 9.2 and 50 mg/l; and only a 96-h EC_{50} was found, of 26.6 mg/l. However, in this same study, Nunes et al. (2014) calculated another 96-h EC_{50} for this same chemical, of 4.7 mg/l. Given this extreme variation in the performance of *D. magna* in terms of acute toxicity tests (considering immobility as effect criteria), it is virtually impossible to ascertain the more realistic toxicity benchmark value for paracetamol. In addition, and despite the significant number of studies that have focused on the effects of paracetamol on aquatic species (viz., biochemical effects at high dosages of this drug; Daniel et al. 2019), no data exist on the effects on swimming behavior for the two species previously mentioned and also about the putative mechanistic trait linking behavioral alterations with biochemical changes. Thus, the present study aimed to evaluate the effects of paracetamol in both *D. magna* and *D. longispina*, by using a combination of standardized tests (acute immobilization), swimming behavior (horizontal distance and time of activity), and changes at the biochemical level, namely by determining the activities of catalase (CAT), glutathione-S-transferases (GSTs), and cholinesterases (ChEs).

Material and methods

Culture of test organisms

Animals genotypically well identified, from healthy cultures, kept under appropriate laboratory conditions (light, temperature, medium, food, number of animals per culture, and unit of volume, according to the procedures and recommendations of OECD guidelines; OECD 2012), were used in the present study. *D. magna* (clone K6) and *D. longispina* (clone E-89) were obtained from monoclonal cultures of the Applied Ecology and Ecotoxicology Laboratory, from the Center for Environmental and Marine Studies (CESAM), University of Aveiro, Portugal. For the beginning and renewal of the culture, only animals from the 3rd to 5th broods were used. The animals were cultured in synthetic hard water medium (ASTM 2007; USEPA 1993) and exposed to a photoperiod of 16-h light:8-h dark, with a temperature of 21 °C (± 2 °C), without aeration (OECD 2012). The medium was renewed three times a week, with the

addition of seaweed extract (*Ascophyllum nodosum*; Antunes et al. 2003; Loureiro et al. 2011) and a ration of the microalgae *Raphidocelis subcapitata*, grown under controlled conditions (Stein 1973). Cultures were fed with cells of this algal species, in an amount up to a 3.0×10^5 and 1.5×10^5 cells/ml, for *D. magna* and *D. longispina*, respectively (Antunes et al. 2003; Loureiro et al. 2011).

Chemical compounds and test concentrations

Paracetamol ($C_8H_9NO_2$, CAS: 103-90-2), with purity of 99%, was purchased from Sigma-Aldrich. Stock solutions of 200 mg/l were prepared by dilution in ultrapure water (MILLI-Q®) prior to each assay. The test solutions were prepared from the stock solution by dilution in ASTM medium.

Acute immobilization determinations

The data for acute immobilization of *D. magna* that were found in the literature showed to be extremely varied (see Introduction section); consequently, we determined the EC_{50} under our experimental conditions, not only for *D. magna* but also for *D. longispina*. Acute toxicity tests were performed with both species, according to Guideline 202 (OECD 2004). After the performance of a range finding test (data not shown), neonates of *D. magna* and of *D. longispina* up to 24 h of age were exposed to the following concentrations of paracetamol: 0 (control); 2.25; 3.37; 5.06; 7.59; 11.38; 17.07; 25.6; and 38.4 mg/l, with five replicates, containing a volume of 50 ml of each concentration, and with five neonates per replicate. The assays were maintained under the same conditions described for cultures, without the addition of food. Acute immobilization data were recorded at 48 h, considering the number of motionless neonates, after 15 s of mild agitation. The immobilized animals were counted, and these values were used for statistical analysis and for determination of EC_{50} values.

Exposure of organisms for the determination of biomarkers

For the evaluation of the effects of paracetamol on biochemical parameters, an acute exposure (48 h) was performed, following the recommendations of the previously described protocol (OECD 2004). However, animals were only exposed to sublethal concentrations of paracetamol. Neonates (less than 24 h old and born between the 3rd and 5th broods) of *D. magna* and *D. longispina* were exposed to concentrations of paracetamol of 0 (control); 5; 10; 20; 40; and 80 μg/l. These concentrations were selected considering their environmental relevance; this means that the here-selected levels of paracetamol were defined considering the already-reported environmental concentrations of this drug, such as 69.5 μg/l measured

at the Tyne River (Roberts and Thomas 2006) and 30.4 μg/l, detected at the Monjolinho River (Campanha et al. 2014). Different exposures were conducted for the determination of each biochemical marker. For *D. magna*, 50 neonates were exposed in vials containing 170 ml of the test solutions, with five replicates per concentration. Animals from the species *D. longispina* are considerably smaller than those of *D. magna*. So, exposure of individuals of *D. longispina* was carried out with 100 animals per replicate. At the end of each exposure, the neonates were manually collected, placed in Eppendorf microtubes, and stored at −80 °C for further processing.

Sample processing for determination of CAT and GST activities

For the determinations of CAT and GST activities, samples were thawed on ice, and a volume of 1 ml of 50-mM phosphate buffer, pH = 7, with 0.1% Triton X-100 was added to each sample. Samples were homogenized in an ultrasound homogenizer (Branson sonifier model 250) and centrifuged at 15,000 g for 10 min at 4 °C (Thermo Scientific Heraeus Megafuge 8R centrifuge). The supernatants after centrifugation were collected in properly identified Eppendorf microtubes and frozen at −80 °C.

For the determination of cholinesterase activity, the samples were homogenized with the same sonifier, in a volume of 1 ml of 0.1 M phosphate buffer, pH = 7.2, and centrifuged at 3330 g for 3 min (same centrifuge). The supernatants after centrifugation were collected in properly identified Eppendorf microtubes and frozen at −80 °C.

Quantification of catalase (CAT) activity

Catalase is an enzyme responsible for the decomposition of H_2O_2 in $H_2O + O_2$ and peroxidic activity, where H_2O_2 consumption occurs with the oxidation of hydrogen donors (methanol, formic acid, and phenols). The method consists in the monitoring of this decomposition, determined spectrophotometrically at 240 nm ($\varepsilon 240 = 0.00394 \pm 0.0002$ l mmol^{-1} mm^{-1}), in which the decrease in absorbance (Δ 240) (Aebi 1984) is observed. The enzymatic activity was expressed in nmoles of H_2O_2 consumed, per minute, per milligram of protein.

Quantification of the activity of the isoenzymes glutathione S-transferases (GSTs)

The activity of glutathione S-transferases (GSTs) was determined according to Habig et al. (1974). GSTs catalyze the conjugation of glutathione in its reduced form (GSH) with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), forming a thioether ($\varepsilon = 9.6$ mM^{-1} cm^{-1}) whose formation was observed by measuring the increase of the absorbance, detected spectrophotometrically at 340 nm. Enzyme activities were expressed in nmol of thioether produced, per minute, per milligram of protein (Habig et al. 1974).

Quantification of the activity of the enzymes cholinesterases (ChEs)

The quantification of the enzymatic activity of ChEs was performed by the Ellman method (Ellman et al. 1961) adapted to 96-well microplates (Guilhermino et al. 1996). The enzymes cholinesterases are responsible for the degradation of the synthetic substrate acetylthiocholine into acetate + thiocoline. In this method, the activity of the enzyme is measured by quantifying the increase in absorbance at 414 nm over time, which occurs with the increase in the yellow color produced when thiocoline is complexed with dithiobisnitrobenzoate (DTNB). The enzymatic activity was expressed as nmol of the complex formed, per minute, per milligrams of protein.

Determination of total soluble protein concentration

The protein concentration of samples was determined by the Bradford method (Bradford 1976; Qi et al. 2017) adapted to 96-well microplates, using a standard of γ-globulin 1 mg/ml. This method is based on the binding of a dye (Bradford's reagent) to the total soluble proteins, forming a stable complex, detectable at 595 nm. This quantification allows expressing the enzymatic activity as a function of the protein content of the sample.

Behavioral evaluation

To assess the effects of paracetamol on the behavior of *D. magna* and *D. longispina*, neonates (less than 24 h old) and adults (6 days old) were exposed to sublethal drug concentrations for 48 h (Loureiro et al. 2011; Nogueira et al. 2018). Animals were exposed to the following paracetamol concentrations: 0 (control); 5; 10; 20; 40; and 80 μg/l, selected considering their environmental relevance, as a function of the environmental concentrations already mentioned in the literature for paracetamol (Roberts and Thomas 2006; Campanha et al. 2014). Six well microplates were used for these exposures. Each well was filled with approximately 10 ml of test solution, for each concentration of test substance, and one organism was randomly added to each well. Each plate was composed of one control and one concentration to be tested, in a total of 20 organisms per concentration. Assays were maintained under conditions similar to those used for their culture but without food. After exposure, the organisms were transferred to 24-well plates, with 2 ml of the respective test solutions, and acclimatized for 10 min before being subjected to behavior assessment by the monitoring system. Each reading

plate was composed of four control organisms and 20 organisms of one of the concentrations tested. The evaluation of locomotor behavior was performed in an automated video recording system (Viewpoint Zebrabox®), with support for multi-well plates, equipped with internal LED lights (light recording; white light, max 15,000 lx; this light seems not to affect the behavior and orientation of animals from the *Daphnia* genus; Stearns 1975; Novales Flamarique and Browman 2000) and infrared (dark recording; the source of the previously referred white light is equipped with a filter that selects radiation at a wavelenght of 850 nm; Liu et al. 2015), with a camera mounted for motion detection. Alternating periods of light (5 min) and dark cycles (5 min) were programmed (species of the *Daphnia* genus may have their phototatic behavior compromised by environmental toxicants; Rivetti et al. 2016), totaling 20 min, and data of total horizontal swimming distance, TD (mm), and total swimming time, TT (s), were recorded and collected for statistical analysis. Behavioral determinations were performed simultaneously for animals of all treatments and started exactly at 9:00 am, to avoid the influence of nycthemeral cycles, which could work as an additional confounding factor biasing the interpretation of obtained data.

Statistical analysis

For EC_{50} calculation, the results were analyzed by Probit analysis, to calculate slope curves and EC_{50} with 95% confidence limit ($p = 0.95$) (OECD 2004). The analyses were performed using the IBM SPSS Statistics software (version 25).

The data from the biochemical and behavioral tests were previously analyzed to assure normality and uniformity of variance (Shapiro-Wilk and Levene tests, respectively). The biochemical and behavioral parameters (swimming and reproduction) were analyzed by analysis of variance (one-way ANOVA), followed, when necessary ($p < 0.05$), by a Dunnett test to discriminate significant differences among exposed treatments and the control condition. The level of significance was 0.05. The data are presented as mean and respective standard error. The analyses were performed using software Sigmaplot (version 12.5) and IBM SPSS Statistics (version 25).

Results

Acute immobilization after 48 h

Acute paracetamol toxicity (immobility after 48 h) was assessed by calculating EC_{50} values with their respective 95% confidence intervals (Table 1). For *D. magna* the calculated values were $EC_{50} = 3.180$ mg/l (2.94–3.46), and for

Table 1 EC_{50} values and respective confidence intervals (CI) of 95%

Paracetamol (mg/l)

Species	EC_{50} (48 h)	Lowest	Highest
D. magna	3.18	2.94	3.46
D. longispina	30.45	26.12	37.05

D. longispina, $EC_{50} = 30.45$ mg/l (26.12–37.05). The toxicity curves for this parameter are depicted in Fig. 1.

Biomarkers

Catalase activity

A significant increase in catalase activity (Fig. 2) was observed in individuals of *D. magna* exposed to the highest concentration of paracetamol (80 μg/l) ($F = 3.752$; $p = 0.012$; d.f. 5; 24). Catalase activity did not change significantly in *D. longispina* ($F = 1.910$, $p = 0.130$, d.f 5; 24).

Glutathione S-transferases (GSTs)

Exposure to paracetamol (Fig. 3) resulted in a significant inhibition of GSTs activity in *D. magna* individuals exposed to two intermediate concentrations (20 and 40 μg/l) ($F = 9.041$ and $p < 0.05$, d.f. 5;24). Paracetamol exposure was also capable of enhancing the enzyme activity in individuals of *D. longispina* exposed to the concentration of 40 μg/l ($F = 1866$, $p < 0.05$, d.f. = 5;24).

Cholinesterases (ChEs)

No significant differences were observed in cholinesterase activity (Fig. 4) of *D. magna* individuals after exposure to paracetamol ($F = 1.615$, $p = 0.194$, d.f. = 5; 24); a similar finding was obtained for individuals of *D. longispina* ($F = 1.24$, $p = 0.185$, d.f. = 5; 24).

Swimming behavior

Paracetamol exposure resulted in changes in swimming behavior of *D. magna* and *D. longispina*. Statistical analysis is summarized in Table 2, and comparative results between concentrations and control (Dunnett's test) are shown in Table 3.

Neonates of *D. magna* showed an increase in the total swimming time (TT) during light and dark cycles (Table 3), for the two lowest concentrations (5 and 10 μg/l) of paracetamol. Regarding the total horizontal swimming distance (TD), an increase was observed for animals exposed to concentrations of 10 and 80 μg/l, in the first light cycle. In adults, an

Fig. 1 Toxicity curves depicting data on the immobilization of *D. magna* (light bars) and *D. longispina* (dark bars)

Discussion

Acute immobilization
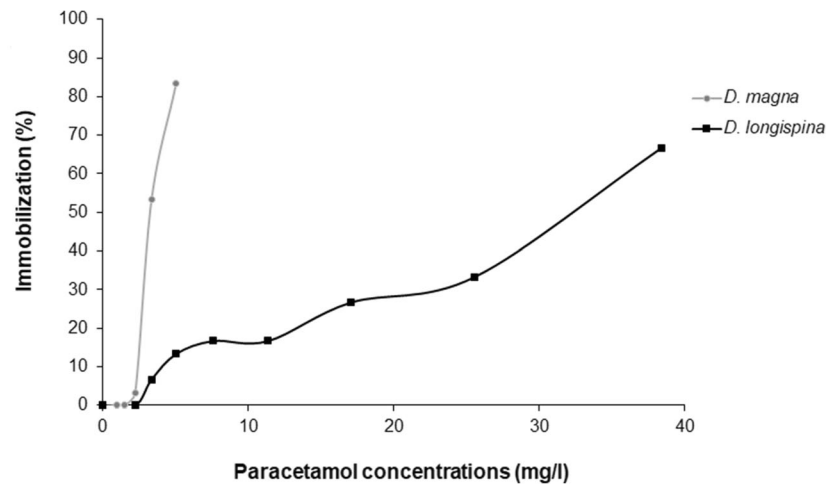
increase in total time was observed for those animals exposed to concentrations of 20 and 80 µg/l, during the first light cycle. During the dark cycle, a decrease in total time was observed in animals exposed to the highest concentration (80 µg/l). No changes were observed druing the light cycles in terms of the total distance traveled by animals. For dark cycles, it was possible to devise a decrease in total distance, but only for animals exposed to the two highest paracetamol concentrations (40 and 80 µg/l).

Neonates of *D. longispina* showed a decrease in total swimming time, when exposed to concentrations of 5 and 80 µg/l, for the second light cycle, with no changes in dark cycles. A decrease in the total distance traveled was observed for animals exposed to the concentrations of 5, 10, 40, and 80 µg/l, for the two cycles analyzed. For the adults of *D. longispina*, no significant differences were observed between exposed animals and those from the control treatment.

Several studies have reported the toxicity of paracetamol towards a large number of aquatic species. Nunes et al. (2014) evaluated the acute toxicity of different organisms exposed to paracetamol, by calculating the EC_{50} values for cladocerans (*D. magna* (4.7 mg/l), and *D. longispina* (65.9 mg/l)), bacteria (*Vibrio fischeri* (92.2 mg/l)), microalgae (*Pseudokirchneriella subcapitata* (317.4 mg/l)), cyanobacteria (*Cylindrospermopsis raciborskii* (192.9 mg/l)), and aquatic plants (*Lemna minor* (429.9 mg/l), and *Lemna gibba* (>1000 mg/l)) (Nunes et al. 2014). The here-calculated EC_{50} value for *D. magna* (EC_{50} = 3.180 mg/l) was in the same order of magnitude as the one previously calculated by Nunes et al. (2014). However, the now-calculated EC_{50} value for

Fig. 2 Catalase activity measured in *D. magna* (light bars) and *D. longispina* (dark bars) after acute exposure (48 h) to paracetamol, $n = 5$; error bars correspond to standard error, and * represents statistically significant differences (Dunnett's test, $p < 0.05$) between the different concentrations and the negative control
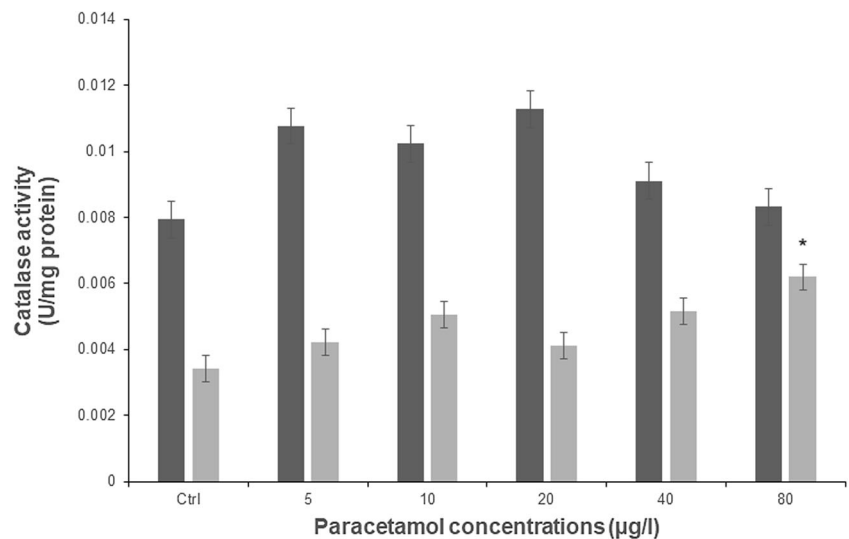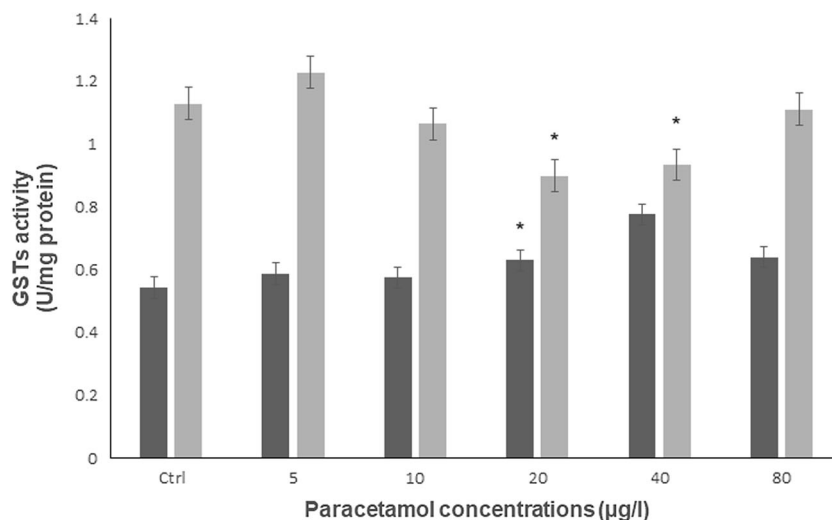
Fig. 3 Activity of glutathione S-transferases quantified in *D. magna* (light bars) and *D. longispina* (dark bars) after acute exposure (48 h) to paracetamol, *n* = 5; error bars correspond to standard error, and * represents statistically significant differences (Dunnett's test, *p* < 0.05) between the different concentrations and the negative control

D. longispina (EC$_{50}$ = 30.45 mg/l) is considerably lower than the initially calculated value of 65.9 mg/l (Nunes et al. 2014). These results suggest a higher sensitivity of the organisms used in this work, when compared to those used in the study by Nunes et al. (2014). The use of two or more species is important to establish comparative degrees of sensitivity (Nunes et al. 2014), and data from the literature already evidenced that these two species may have very distinct sensitivities to specific chemicals. Exposure of these species (*D. magna* and *D. longispina*) to different salinities, and to sediments from abandoned mine ponds, indicated a higher sensitivity of *D. longispina* (Antunes et al. 2007; Gonçalves et al. 2007). However, the observation of the format of the here-obtained toxicity curves allowed to conclude that *D. magna* was more sensitive than *D. longispina* towards paracetamol. A similar tendency, with higer sensitivity of *D. magna* when compared to *D. longispina*, was also reported by Sousa and Nunes (2020), when analyzing the toxicity of

the antidandruff and antifouling chemical zinc pyrithione. Thus, it is possible to conclude that sensitivity is a species-specific trend, and there are no species that are always more resistant or more sensitive to a wide variety of chemicals. In fact, differences in sensitivity of distinct species may be explained by their origin. *D. magna* is a well-established standard test organism, and its culture is a common procedure in a vast number of laboratories involved in ecotoxicological testing. To attain homogeneity of performance and constant biological responses, organisms of this species are cultured under optimal laboratory conditions. It is however important to mention that such maintenance conditions may have a decisive effect on the quality of results (Baird et al. 1989), and some, albeit low, level of fluctuation is possible among tests. On the other hand, *D. longispina* is a somewhat different case, since it corresponds to an autochtonous species that, despite being reared under strictly laboratory controlled conditions, may be strongly influenced by its origins. The here-used clone of

Fig. 4 Activity of cholinesterases measured in *D. magna* (light bars) and *D. longispina* (dark bars) after acute exposure (48 h) to paracetamol, *n* = 5; error bars correspond to standard error, and * represents statistically significant differences (Dunnett test, *p* < 0.05) between the different concentrations and the negative control
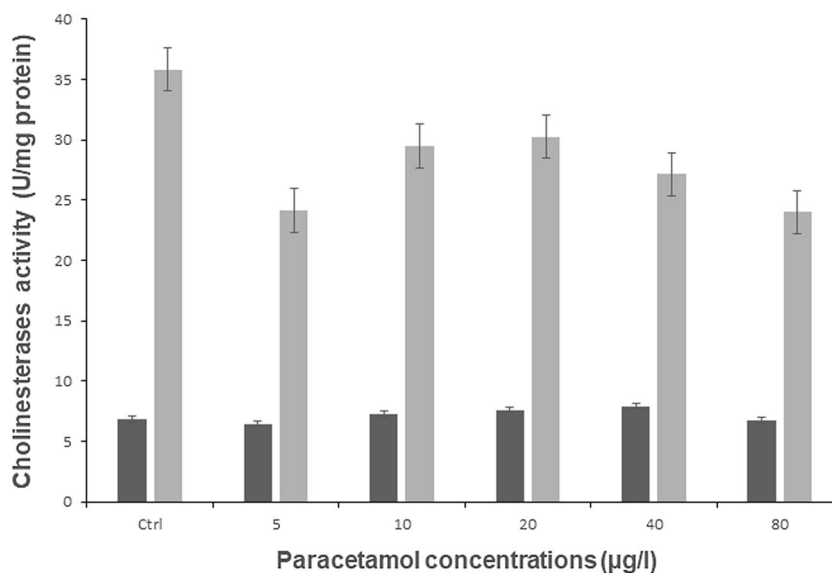
Table 2 Statistical analysis of the behavioral test after exposure to paracetamol

Cycles	D. magna (24 h)		D. magna (6 days)		D. longispina (24 h)		D. longispina (6 days)	
	F	p	F	p	F	p	F	p
TT-300	4.0	**0.002**	3.2	**0.01**	2.3	0.05	0.9	0.2
TT-600	4.5	**0.001**	7.2	**0.001**	1.3	0.3	0.5	0.2
TT-900	2.6	**0.03**	2.0	0.08	5.0	**0.05**	0.4	0.3
TT-1200	2.9	**0.02**	5.3	**0.001**	0.9	0.5	0.5	0.5
TD-300	1.3	**0.03**	3.0	0.08	4.1	**0.002**	1.6	0.1
TD-600	0.9	0.5	12.9	**0.001**	3.8	**0.003**	1.1	0.2
TD-900	1.1	0.4	3.0	**0.02**	10.1	**0.001**	0.8	0.2
TD-1200	1.3	0.3	11.0	**0.001**	4.8	**0.001**	0.7	0.4

TT is total swimming time (s), and TD is total distance traveled (mm), 300 and 900 (light cycle) and 600 and 1200 (dark cycle), with $n = 20$ and gl (5; 114). Values of F and p are inserted according to the cycles, age of the organisms, and compound analyzed

D. longispina was isolated from a field population (Lopes et al., 2009), being likely to have their physiological responses towards chemicals (or other abiotic/environmental stressors) somehow conditioned by its genetic background. This higher resistance to toxicants may be due to genetic erosion of wild *D. longispina* populations, which are known to be caused by

environmental exposure to chemical contaminations; genetic erosion of *D. longispina* populations has been involved in significant differences in the deployment of toxicological responses towards specific environmental chemical stressors, such as copper and salinity (Leitão et al. 2013). This may explain why *D. longispina* was more resistant to paracetamol

Table 3 Behavioral changes observed in *D. magna* and *D. longispina* after exposure to paracetamol

Concentration (µg/l)		*Daphnia magna*					*Daphnia longispina*				
		5	10	20	40	80	5	10	20	40	80
<24 h	TT-300	*(↑)	*(↑)								
	TT-900	*(↑)	*(↑)				*(↓)				*(↓)
	TT-600	*(↑)	*(↑)								
	TT-1200	*(↑)									
	TD-300		*(↑)			*(↑)	*(↓)	*(↓)			*(↓)
	TD-900						*(↓)	*(↓)		*(↓)	*(↓)
	TD-600						*(↓)	*(↓)		*(↓)	*(↓)
	TD-1200						*(↓)	*(↓)		*(↓)	*(↓)
6 days	TT-300			*(↑)		*(↑)					
	TT-900										
	TT-600					*(↓)					
	TT-1200					*(↓)					
	TD-300										
	TD-900										
	TD-600				*(↓)	*(↓)					
	TD-1200				*(↓)	*(↓)					

TT, total swimming time(s); *TD*, total distance traveled (mm), 300 and 900 (light cycle) and 600 and 1200 (dark cycle); (↑), increase in relation to the control; (↓), decrease in relation to the control; and *, statistically significant differences (Dunnett test, $p < 0.05$) between the different concentrations of paracetamol in relation to the negative control

when compared to *D. magna*. In addition, and similarly to what was mentioned for *D. magna*, its performance may also be modulated by biotic factors during its maintenance, namely by the food supply, as demonstrated by Antunes et al. (2003). The potential contribution of all these variables may, at least partly, contribute for the evident interspecific dfifferences that were observed in our assays.

EC$_{50}$ values found in the literature for paracetamol show great variability for the same species: *D. magna* 48-h EC$_{50}$ values vary from 4.7 to 50 mg/l; 24-h EC$_{50}$ differs from 13 to 293 mg/l (Calleja et al. 1994; Du et al. 2016; Henschel et al. 1997; Kim et al. 2012). These variations show that, even among experiments based on a single standard species, the results can be affected by other factors, requiring additional information to establish toxicity thresholds. In fact, acute immobilization is a blunt measurement of toxicity, since no mechanisms about the causes of death are provided in most cases. Furthermore, this type of determination is also subjected to critical influences by unavoidable sources of interference, which are inherent to culture and exposure conditions, such as crop handling, differences in the nutritional status of the test organisms, and also to genetic variation, since different clones were used by distinct authors. This was already the core of the discussion by Nunes et al. (2014), who proposed that several key factors may act as confounding factors in *D. magna*-based bioassays. In addition, the nutrients (source, amount, and quality) that are used to feed these animals may also play a decisive role in lethality-based bioassays, especially in the case of studies that adress the toxicity of paracetamol. Nutritional deficits may induce toxic effects of paracetamol in humans (Vale 2012), interfering with the metabolism of glutathione, thereby influencing the biotransformation and excretion of paracetamol (Geenen et al. 2013). This suggests that the nutritional status of organisms may alter the reserves of cofactors required for biotransformation of the drug, consequently modulating the toxicological response (Nunes et al. 2014). In addition, lethality values may also show an extreme variation when compared to other aquatic organisms, such as *Artemia salina* (Webb 2001), and *Dugesia japonica* (Li 2013); these two species showed much higher EC$_{50}$ values, namely of 577 mg/l and 858.6 mg/l, respectively. From this analysis, it is possible to sustain that EC$_{50}$ values may help identify patterns of toxicity, among distinct species, but are far from having ecological interest per se, and are not usefull to accurately predict the comparative sensitivity of multiple test species. In fact, it is not simple to understand how one species may be 100-fold more sensitive than another; comparison of EC$_{50}$ values may thus be useful to identify extreme species-specific differences that require further research about the underlying toxicological mechanistic traits that explain such differences. Consequently, these different mechanisms of toxic action may ultimately serve to understand interspecific differences, namely the exacerbated sensitivity of critical species

towards certain chemicals (or group of chemicals). However, and for the specific case of paracetamol (and of its toxicity to both species of *Daphnia*), it is not expectable that the actual amounts of paracetamol that occur in most locations may result in adverse effects—at least in terms of mortality. This occurs since the levels of paracetamol that are necessary to cause lethal acute effects in aquatic organisms are in the mg/l range and that its environmental concentrations are in the order of μg/l. However, characteristics such as high consumption, toxicity, occurrence, and pseudo-persistence make this drug a priority in the aquatic environment (Voogt et al. 2009), and its continuous release into the environment can cause subtle effects, such as changes at the cellular level to modifications at the population level and may be interpreted as evolutionary adaptations (Daughton and Ternes 2001). That is why a biomarker-based analytical approach is not only necessary but mandatory.

Biomarkers

Antioxidant defenses

In the present study, paracetamol induced the catalase activity in *D. magna*, suggesting the increase of hydrogen peroxide production, and concomitant activation of antioxidant defenses (Ozcan Oruc et al. 2004; Brandão et al. 2011). Our results are consistent with those found by most authors studying the ecotoxicity of paracetamol in aquatic species, namely invertebrates. The study conducted by Masteling et al. (2016) with *D. magna* showed that oxidative stress triggered the activation of the main antioxidant enzymes, including catalase, after subchronic exposure (8 days) to paracetamol in concentrations comprised in the interval between the EC$_{10}$ (3.7 mg/l) and the EC$_{40}$ (4.54 mg/l) values, previously determined in this same study. A similar trend was also reported by Daniel et al. (2019), after an acute exposure of *D. magna* to paracetamol, in a concentration of 2.56 mg/l. However, it is important to underline that in our study, all concentrations were sublethal (10 to 80 μg/l), well below those used by Masteling et al. (2016) and by Daniel et al. (2019), which were in the mg/l range. However, the here-adopted conditions also triggered the activation of antioxidant defenses (in support to an increased production of hydrogen peroxide), to cope with the presence of this pharmaceutical. This trend is however not surprising. Catalase enhancement was also observed after the exposure of individuals of the freshwater mussel species *Dreissena polymorpha* to environmentally realistic concentrations of paracetamol (0.154 to 1.51 μg/l), indicating a scenario of oxidative stress, confirmed by the increased activity of the enzyme superoxide dismutase (Parolini et al. 2010). Increased catalase activity was also evidenced in individuals of the bivalve species *R. philippinarum* exposed to paracetamol at concentrations of 0.05, 0.5, and 5 mg/l (Correia et al. 2016).

Studies of pulses of exposure of *R. philippinarum* to paracetamol, followed by recovery periods, indicated oxidative damage, with involvement of several biochemical pathways, namely of the antioxidant defense (Nunes et al. 2017). Vertebrates seem also to respond this way. Fish (*Oncorhynchus mykiss* and *Oreochromis mossambicus*) exposed to paracetamol had also increased levels of catalase activity (Ramos et al. 2014; and Kavitha et al. 2011, respectively). It is important to highlight that the concentrations used in this work, contrarily to what happened for most described studies, are similar to the concentrations already found in the wild, suggesting that toxic effects may occur at the environmental levels of paracetamol.

Different responses were obtained in *D. longispina*, and catalase activity was not significantly increased, suggesting that paracetamol did not trigger the formation of enough hydrogen peroxide to cause a significant increase in the activity of this enzyme (Brandão et al. 2011). This pattern of response, despite apparently paradoxical (and even opposite to pre-established scenarios), requires further discussion. Similar results were obtained by Oliveira et al. (2015) after acute exposure (48 h) of *D. magna* to paracetamol (0.01 to 1.0 mg/l), as no changes in this enzyme were reported. Paracetamol did not cause significant changes in catalase activity in the mollusk *C. fluminea* after acute exposure (96 h) to concentrations of 0.05, 0.48, 4.82, and 532.78 mg/l of this drug; similarly, no effects were found after a chronic (28 days) exposure of this mollusk to levels of 3.88, 7.74, 15.49, 30.98, and 61.95 µg/l (Brandão et al. 2011). Catalase activity was not altered after the acute (96 h) exposure of the fish *A. anguilla* to varying concentrations (5, 25,125, 625, and 3125 µg/l) of paracetamol (Nunes et al. 2015). These results may indicate that response patterns may be variable across species but also suggest nonspecific dose-related action. In such experiments (Brandão et al. 2011; Oliveira et al. 2015; Nunes et al. 2015), there was no pattern that evidenced dose (or concentration) dependent responses, for all mentioned species. Considering that the EC_{50} values here calculated for *D. magna* (4.7 mg/l) are almost ten times lower than the value for *D. longispina* (30.45 mg/l), it would be somewhat expected that stronger effects (in comparative terms) should be observed for *D. magna*.

Similarly to catalase, the glutathione-S-transferase isoenzyme group has anti-oxidant defense activity, and these enzymatic forms are important for the biotransformation and excretion of a wide range of compounds (including of the paracetamol metabolite, NAPQI; Leaver and George 1998). In our work, a significant inhibition of GSTs was observed for *D. magna*, after exposure to paracetamol. However, these results are not unique to this work. Antunes et al. (2013) observed a similar trend, in *Venerupis decussata*, after exposure to paracetamol (Antunes et al. 2013). Decreased GSTs activity was also observed in individuals of *C. fluminea* exposed to

paracetamol, in concentrations of 0.48, 4.82, and 532.78 mg/l for 96 h, and in concentrations of 30.98, and 61.95 µg/l for 28 days (Brandão et al. 2011). These results are not in agreement with those that would a priori be expected, since the occurrence of oxidative modifications and the metabolism of paracetamol into NAPQI would require the increase of the activity of this enzyme. In fact, the increase of the activity of GSTs after exposure to paracetamol was also reported and specifically for *D. magna*; however, this pattern of response was only attained at extremely high dosages, namely concentrations from 3.7 to 4.5 mg/l of paracetamol (Masteling et al. 2016), which well exceeded the magnitude of those that were analyzed in our study. A significant increase of GSTs activity was the major pattern that was reported for this same crustacean species, *D. magna*, following an acute exposure to concentrations of paracetamol between 0.08 and 2.56 mg/l (Daniel et al. 2019). However, oxidative stress may be the basis for deleterious effects, including enzymatic inactivation (Oliveira et al. 2015), so it is suggested that pro-oxidative effects indicated by increased catalase activity may have culminated in inactivation of enzymes such as GSTs. It seems that direct effect by reactive oxygen species (ROS), overproduced during the metabolism of paracetamol, may act directly on specific enzymatic forms (including GSTs), obscuring the enhancement of GST activity that could be expected given its role in the detoxification of the bioactive toxic metabolites of paracetamol, namely NAPQI. A decrease of GSTs activity was also observed in chronically exposed individuals of *D. magna*, which were subjected to levels of paracetamol between 5 and 20 µg/l (Daniel et al. 2019). It thus seems that such effects are only attained when individuals of this species are exposed to low, environmental realistic levels of this drug, regardless the duration of exposure. Consequently, it must be assumed that the metabolism of glutathione to cope with the presence of paracetamol, albeit critical, is dual (with opposite responses) and not totally straightforward to predict. It is possible to conclude that the most likely outcome of exposure of *D. magna* to paracetamol is oxidative stress (Nunes et al. 2014), with the potential trigerring of some responses, including of GSTs. In our study, results with *D. magna* evidenced this trend, with simultaneous alterations of catalase and GSTs, thus suggesting the establishment of a pattern of oxidative stress.

Despite the importance of the previous assumptions, the two evaluated species yielded divergent results in relation also to GSTs. Contrarily to the response reported for *D. magna*, increased GSTs activity was observed in individuals of *D. longispina* exposed to paracetamol. These findings are in agreement with other studies, such as those described by Parolini et al. (2010) in *D. polymorpha* mussels exposed to environmental relevant concentrations of paracetamol; Nunes et al. (2015) reported increased GST activity in European eels *A. anguilla* after exposure to 625-µg/l paracetamol

concentrations. Low concentrations of paracetamol (25 μg/l) caused pro-oxidative changes in the marine clam *R. philippinarum*, with involvement of several biochemical pathways, evidencing an increase in GSTs and superoxide dismutase activities (Nunes et al. 2017). Increased GSTs activity was also observed in *O. mykiss* after acute exposure (96 h) to sublethal (0.05, 0.50, and 5.00 mg/l) concentrations of paracetamol (Ramos et al. 2014). However, it can not be directly inferred that the increase in GSTs activity after exposure of *D. longispina* to paracetamol is a consequence of oxidative stress, since there was no change in catalase activity. On the contrary, it seems that GSTs activation corresponds to the activation of the conjugation route of paracetamol metabolites, rather than being a response to pro-oxidative conditions. As GSTs are phase II conjugating enzymes, also in invertebrates (Solé et al. 2010), their increase may be related to the metabolism of paracetamol, with the detoxification of NAPQI by conjugation with glutathione, making it more water-soluble to be promptly excreted (as summarized in the introduction section). In this sense, it is possible to conclude that *D. longispina* is much more capable of excreting paracetamol (and its metabolites) than *D. magna*; by doing so, it is able to detoxify more promptly this drug, not being affected by its toxicity. Extreme differences in paracetamol sensitivity even among phylogenetically close species are not new. Antunes et al. (2013) reported a similar differential response in GSTs activity when comparing two clam species, namely *R. decussata* and *R. philippinarum*, after being exposed to paracetamol (Antunes et al. 2013). The results of this work from the literature showed that GSTs were significantly inhibited in *R. decussata* (a native species), while being activated in *R. philipinarum* (an introduced/invasive organism). These findings are somewhat comparable to our data and may be associated with interspecific differences, wich may strongly contition underlying mechanisms of detoxification (Antunes et al. 2013). These distinct patterns of response have granted the invasive species (*R. philipinarum*) a considerable competitive advantage over the native organism (*R. decussata*). This is extremely important in ecological terms, since a competive advantage stemming from a higher detoxication capacity may result in an enhanced capacity to withstand adverse environmental conditions, such as exposure to toxic chemicals. In our particular case, *D. longispina* may have a more effective detoxification capacity, when compared to *D. magna*. This is not entirely surprising, given the origin of both species. While the here-used specimens of *D. magna* have been obtained from a decades-long established laboratory clonal culture, the individuals of *D. longispina* that were used in our bioassays were obtained from a wild population. Despite being reared as a stock clonal culture, under strictly controlled laboratory conditons, this clone of *D. longispina* was obtained from a wild population that naturally occurred at a heavily contaminated site (Lopes et al. 2004). This

ancestry may have contributed for a naturally increased capacity of conjugating toxicants with GSH, via GSTs, to favor their detoxification/excretion that was now triggered in response to paracetamol.

Neurotoxicity

Neurotoxicity, with the occurrence of anticholinesterasic effects, may have two independent causative factors, namely effects of anticholinesterasic compounds (Nunes et al. 2005; Nunes 2010) or oxidative denaturation of the enzyme itself, after exposure to oxidant chemicals (Delwing-de Lima et al. 2010). In the present study, exposure to paracetamol did not trigger neurotoxic anticholinesterasic effects in any of the studied species. This finding was not consistent with the work conducted by Oliveira et al. (2015), which demonstrated the occurrence of cholinesterase inhibition in *D. magna* individuals exposed to concentrations of 0.1 and 1 mg/l paracetamol for 48 h; in addition, the data obtained by Daniel et al. (2019) showed a similar trend: daphnids exposed to high levels of paracetamol (0.32 to 2.56 mg/l) evidenced significant cholinesterase inhibition as well. However, it is paramount to stress that the concentrations used by these authors exceeded in one order of magnitude those used in our study. It is interesting to note that chronic exposure of individuals of *D. magna* to concentrations of paracetamol that are close to those here tested also resulted in negligible effects in terms of ChE activity (Daniel et al. 2019). *D. magna* cholinesterasic activity seems thus to be sensitive to paracetamol exposure, but only in dosages that far exceed the ones here-tested. This comparison between our data, and those from the literature, strongly suggests that the adverse effects caused by paracetamol on the cholinesterase activity in this species are much more dependent on the levels of paracetamol, rather than on the duration of the exposure. Other studies point to the neurotoxic effects caused by paracetamol, evidenced by the inhibition of this enzyme, in species as diverse as the fish *A. anguilla* (Nunes et al. 2015), the bivalves *Ruditapes philippinarum* (Nunes et al. 2017), and *Mytilus galloprovincialis* (Solé et al. 2010). This cholinesterasic inhibition is likely to result from the relationship between oxidative stress and the inhibition of the enzyme, by direct denaturation resulting from oxidative damage. In fact, this relationship has already been evidenced in previous studies, as demonstrated by Delwing-de Lima et al. (2010), by inducing oxidative stress in rats, following the administration of guanidine derivatives, which resulted in the inhibition of cholinesterases (acetylcholinesterase and butylcholinesterase); these effects were later reverted by antioxidant compounds, demonstrating the oxidative nature of this inhibitory effect. Weiner et al. (1994) also demonstrated that reactive oxygen species inactivated the acetylcholinesterase of the fish *Torpedo californica* by altering its conformational state (Weiner et al. 1994). However, in our work,

oxidative stress after exposure to paracetamol was not evident, as discussed in the previous section. This assumption allows us to suggest that cholinesterase activity was not altered by the lack of evident oxidative stress, indicating that activated antioxidant defenses were effective in preventing proteic/enzymatic damage.

Behavioral changes

Behavioral responses are sensitive indicators of stress because they integrate biochemical and physiological processes (Chevalier et al. 2015). Individual swimming activities in *Daphnia* species result in important defensive population behaviors, such as vertical and horizontal migrations (Burks et al. 2002; Dodson and Ramcharan 1991; Lass and Spaak 2003), which may end up in large-scale modifications, at the ecosystem level, as suggested by Uttieri et al. (2014). The analysis of this movement on an individual scale contributes to the understanding of the adaptations to the different environmental conditions (food, light intensity, type and size of the container; Dodson et al. 1997; Nikitin 2019), as well as the population dynamics (Ziarek et al. 2011). Swimming can be affected by several factors, such as light, water temperature, food presence, and predators (Hamza and Ruggiu 2000; Ziarek et al. 2011). Changes in mobility have been described after exposure to pesticides (e.g., carbaril; (Dodson et al. 1995), drugs (hormones; Goto and Hiromi 2003), cyanobacterial toxins; (Ferrão-Filho et al. 2014; Restani and Fonseca 2014), metals (cadmium; Restani and Fonseca 2014), organophosphate insecticides (Duquesne and Küster 2010), among others). Consequently, the distance traveled by individuals of species of the *Daphnia* genus seems to be an excellent parameter to assess potential neurotoxic effects caused by a vast array of substances, as summarized by Bownik (2017). In our study, neonates (less than 24 h) and adults (6 days), of both *D. magna* and *D. longispina* reacted to cycles of light and darkness after being exposed to paracetamol, with behavioral changes in horizontal swimming. These changes were not totally clear, and diffuse changes in relation to the exposed parameters and cycles, with some moments of excitation (increase in horizontal distance and swimming time) or inhibition (decrease of the same parameters). Some studies were developed to analyze locomotor activity after paracetamol exposure in zebrafish embryos (*D. rerio*) without altering any other biochemical or physiological traits (Selderslaghs et al. 2013; Xia et al. 2017). Nevertheless, behavioral changes must be connected to underlying biochemical and physiological mechanisms, and cholinesterasic inhibiton was a likely hypothesis. As suggested by previous data, several authors have reported that oxidative stress induced by paracetamol could trigger adaptive responses in protective systems, modify macromolecules, or cause cell damage or even death (Bajt et al. 2006; Solé et al. 2010). However, and from the here-

obtained data, already discussed in the previous section, no solid indication of cholinesterasic inhibition by oxidative effects resulted from our experimental design. In this work, it was made clear that the activation of antioxidant defenses (viz., catalase) was observed only in *D. magna*, whereas in *D. longispina*, it remained unchanged. For both species, a common response was made evident by GSTs alterations, which indicated the toxic potential of this drug. However, it was not possible to establish a comprehensive link between oxidative stress, enhancement of antioxidant defense efficacy, oxidative damage of macromolecules (e.g. cholinesterases, with loss of enzymatic activity), and changes in behavioral traits.

In addition to the differences between species, age also influenced behavioral changes, as observed in this study. The fact that neonates and adults of *D. magna* exhibited significant behavioral differences reflects the toxic profile of this drug during distinct life stages of this species. From the obtained results, it is possible to support that *D. longispina* seems to present a higher capacity for the detofixication of paracetamol in adult individuals. However, adults were not used to determine biochemical biomarkers, requiring further studies to understand underlying mechanisms that may justify behavior alterations.

In addition, it is also important to interpret the here-obtained behavioral data, considering that the methodology for behavioral assessment (ZebraBox) is always a simplification of conditions that occur in the wild. Our option was to calculate swimming distance and swimming time, which are somewhat limited, albeit important, behavioral parameters that may be measured by using this automated system. This was our initial choice, considering that the concept underlying this study was to integrate levels of responses (biochemical alterations vs. behavior). So, the here-adopted behavioral measurements were not aimed at a thourough evaluation of all forms of *Daphnia* behavior (comprehensive behavioral assessments have been adopted before by several authors in an ecotoxicological context; Lovern et al. 2007; Rivetti et al. 2016; Simão et al. 2019), since only two parameters were measured (horizontal distance, and time). However, our approach intended to add an additional insight into the toxicological profile of paracetamol, by measuring an endpoint of undisputable environmental significance. Despite being limited, the occurrence of behavioral changes in animals exposed to paracetamol demonstrated the usefulness of our approach. This trade-off between a limited number of behavioral parameters, and the simultaneous determination of biochemical traits, occurs due to the limitations of laboratory-based bioassays. In fact, the use of behavior as an ecotoxicological endpoint requires reproducibility, and all potential confounding factors are excluded from the experimental setup. The experimental setup itself may compromise the realism of the observed responses; experimental apparatuses are conditioned

by a series of factors, such as light spectra, shape, height, width, or diameter of test vessels, which are likely to affect normal behavior of analyzed individuals, as summarized by Bownik (2017). This author observed that physical parameters of water, viscosity of test substances and cosolvents/carriers, may also affect the behavioral measurements, in a way that has no biological meaning if the animals were environmentally exposed to the same chemicals. Interindividual differences in behavioral responses, and the interactions among distinct animals, are also strong factors that affect behavioral endpoints measured under strictly controlled conditions (Heuschele et al. 2017). In addition, in the wild, animals may also face other variables that are not included in laboratory-based experimental designs, such as metereological conditions (temperature, sunlight, and wind), water turbidty, and microbial fauna. The combination of all these factors may somehow modulate their behavior, and these variables are not included in common laboratory tests. This laboratory-based, automated approach is however much more suited to observe and interpret mechanistic traits, than to replicate the natural behavior of animals when in the natural environment. Despite being scientifically sound, and therefore valid, we cannot assume that all measured alterations that were observed in our experimental organisms are likely to occur if animals were in the wild. However, the toxicological mechanisms that underlie the observed behavioral changes are unequivocal, and such deleterious changes may indeed occur if animals were environmentally exposed to paracetamol. So, even if we assume the likelihood of behavioral alterations as a consequence of exposure to toxic agents, this assumption is somewhat limited due to the simplistic (albeit necessary) nature of the behavioral monitoring systems and experimental setups that are commonly used in ecotoxicological assessments.

Conclusions

Pharmaceutical compounds may exert subtle chronic effects that are likely to occur at the biochemical level, which may extend to populations and communities, not triggering visible acute lethal effects but activating imperceptible toxicity mechanisms. This study attempted to address the different susceptible levels of paracetamol-triggered effects by assessing acute immobilization, swimming behavior, and biochemical parameters (oxidative stress and neurotoxicity) in two cladoceran species, *D. magna* and *D. longispina*. By doing so, it also intended to establish a connection betweeen oxidative stress, antioxidant defense responses, oxidative damage to macromolecules, impairment of enzymatic activity, and consequent behavioral modifications. In this study, *D. magna* was shown to be more sensitive than *D. longispina* to paracetamol exposure. Antioxidant defenses were activated in *D. magna* after exposure to paracetamol, namely with the activation of

catalase and impairment of GSTs; however, in *D. longispina*, the results were not conclusive, as there were no changes in catalase activity, indicating that the change in GSTs activity may be due to the augmented detoxification metabolism of paracetamol. Neurotoxicity was not observed, since no alterations in cholinesterase activity were reported for the two analyzed species. Nevertheless, behavioral changes were observed after paracetamol exposure, confirming that even sublethal levels of paracetamol can affect organisms by altering their functions, in this case, by unknown mechanisms. Our results demonstrate that paracetamol is a potential threat to aquatic organisms, namely to zooplanktonic species, such as cladocerans. The results of this nature are of concern, as exposures were short-termed but performed at environmentally relevant concentrations. However, these conditions were undisputably capable of triggering biochemical and functional changes, indicating that significant toxic effects may be induced by this pharmaceutical drug at the environmental level.

Authors' contributions Ana Paula Sousa was involved in formal analysis; investigation; methodology; and writing of the original draft.

Bruno Nunes was involved in conceptualization; data curation; formal analysis; funding acquisition; project administration; resources; supervision; validation; and in writing, namely, reviewing and editing the manuscript.

All authors read and approved the final manuscript.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval   Not applicable.

Consent to participate   Animals used in this assay are from an invertebrate species, which do not require previous ethics approval.

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