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Suborganismal responses of the aquatic midge *Chironomus riparius* to polyethylene microplastics

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Abstract

Freshwater riverbeds are a major repository of microplastics (MPs) from inland activities. Benthic macroinvertebrates that live in close contact with sediments seem to ingest a considerable amount of such plastic particles. The effects of MPs on life-history traits are relatively well-known, but the suborganismal mechanisms underlying such effects remain unclear. This study addressed the potential effects of low-density polyethylene (LDPE) MPs on *Chironomus riparius* larvae at cellular and molecular levels. Fourth instar *C. riparius* larvae were exposed to 0.025 and 2.5 g LDPE/kg of dry sediment (size, <32 and 32–45 µm; shape, irregular) under laboratory conditions for 48 h. These short-term exposures to environmental concentrations of LDPE MPs induced changes in the energy reserves (mostly by decreasing carbohydrates and increasing lipids),

increased antioxidant and detoxification responses (tGSH, CAT, and GST), and induced increases in the activity of AChE (related to neurotransmission). In addition, at the gene level, exposure to MPs modified mRNA levels of *InR*, *Dis*, *EcR*, *Dronc*, *Met* (endocrine system), *Def* (immune system), *PARP*, *ATM*, *NLK*, and *Decay* (DNA repair), generating important alterations in the *C. riparius* development and response to unfavorable situations. This study provides new evidence of the effects of LDPE MPs at the suborganismal level, filling the gap in knowledge regarding the mechanisms underlying the toxicity of MPs and spotlighting gene expression analyses as early indicators of MP toxicity in *C. riparius* which were confirmed by Integrated biomarker response analyses highlighting the gene expression as sensible parameters for LPDE pollution in freshwaters. These results, coupled with previous investigations on responses at the organismal level, highlight the potential adverse effects of LDPE MPs on *C. riparius*, which may compromise freshwater benthic communities, considering its ecological role within these habitats.

Keywords: plastic pollution, aquatic toxicology, ecotoxicity, molecular response, aquatic insects

1. Introduction

Microplastics (MPs) encompass all particles with sizes of less than 5 mm that are composed of one or more polymers and functional additives, and they even contain residual impurities from manufacturing (da Costa et al., 2016). MPs can reach the environment from diverse sources, such as industrial effluents, airborne pollution, or sewages from agriculture (Eerkes-Medrano et al., 2015;

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Herrera et al., 2018; Wang et al., 2017). Freshwater systems (rivers and lakes) are major repositories of MPs from inland activities and are the main sources of these particles in seas and oceans. Rivers and lakes around the world display MP concentrations of up to 1 g kg^{-1} or higher (Hurley et al., 2018; Klein et al. 2015; Scherer et al. 2020), with hotspots detected in highly urbanized areas, as observed in Asia, such as those established in Haihe, Suzhou, and Huangpu Rivers (Liu et al., 2020; Luo et al., 2019). Thus, there is growing awareness of the impact of MPs on the environment, biota, and human health since such particles are capable of drifting far away from the original release source, so MPs have become an emerging pollutant worldwide (Royal Society, 2019). Polyethylene (PE) is considered one of the most manufactured plastic polymers (Conkle et al., 2018), and it is one of the main polymers found in wastewater treatment plants (Barcelo and Pico, 2020) and freshwater ecosystems (Rodrigues et al., 2018; Sruthy and Ramasamy, 2017).

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Low-density polyethylene (LDPE) is a polymer with multiple applications as manufacturing containers, dispensing bottles, wash bottles, tubing, plastic bags for computer components, and laboratory equipment. LDPE particles are relevant since they are the most detected MPs in for example Indian lakes (Sruthy and Ramasamy, 2017). LDPE MPs accumulate in sediments through aggregation or biofouling processes (Hurley et al., 2018), posing a potential threat to benthic organisms such as *Chironomus riparius* larvae. *C. riparius* is a dipteran species with great ecological relevance, acting as recyclers of organic matter and indicators of the quality of aquatic ecosystems, and they are the

basis of many freshwater food webs (Pèry and Garric, 2006; Sahandi, 2011; Stoian et al., 2009).

The exposure to and consequent ingestion of MPs by chironomids has been shown to alter larval growth and development/emergence rates (Silva et al., 2019; Stanković et al. 2020; Ziajahromi et al., 2019), causing mouthparts deformities during the fourth instar larval stage and altered wing shape in adults (Stankovic et al., 2020). In addition, acute exposure to PE MPs causes oxidative damage and decreased aerobic energy production in *C. riparius* larvae (Silva et al., 2021). However, it remains unclear which mechanisms underly the effects of PE MPs at the molecular level, with few studies focusing on specific genes in aquatic organisms, such as fishes (Granby et al., 2018; LeMoine et al., 2018) and mussels (Avio et al., 2015), which show altered gene expression. Therefore, it is expected that PE microplastics can induce changes in gene expression of *C. riparius* larvae, and those alterations can be detected after short exposures (48 h), which could be early alarm signals of damage anticipating individual and population level effects.

In this sense, this study addressed the potential cellular and molecular level effects of LDPE MPs in *C. riparius* larvae by combining different biochemical endpoints with a specific array for gene expression analysis, filling the knowledge gap regarding the mechanisms of LDPE toxicity. The final aim was to integrate the data and to relate this data to the observed effects at higher levels of biological organization (protein/enzyme, energy reserves for growth and development), searching for suitable early indicators of MP toxicity.

2. Materials and methods

2.1 Chironomus culture

The *Chironomus riparius* culture was maintained for several generations in the Centro de estudos do ambiente e do mar (CESAM) laboratory at the University of Aveiro using glass aquaria with inorganic fine sediment (Quartz sand, previously, washed with deionized water, sieved (<1 mm,) and burnt at 500ºC for 4h) and American Society of Test Materials (ASTM, 1980) hard water medium (1:4 ratio). The aeration, temperature (20±1°C), and photoperiod (16:8 light:dark) were controlled according to Organization for Economic Co-operation and Development (OCDE) guidelines. The cultures were fed three times a week with macerated fish food (TetraMin®, Tetrawerke, Melle, Germany) dissolved in Milli-Q water.

2.2 Polyethylene particle preparation

LDPE MPs with an average size of 40–48 μm were purchased from Sigma-Aldrich UK (ultra-high molecular weight powder, CAS No. 9002-88-4). The desired particle size was obtained by vibratory sieve shaking (mesh pore sizes of 32 and 45 μm), giving final particle sizes of <32 and 32–45 μm. These two different sizes were selected since they are within the optimal feeding range for particles in 3rd and 4th stage *C. riparius* larvae (Silva et al., 2019). The concentrations were selected considering concentrations reported in environment (Hurley et al., 2018) and considering the previously effects observed at the organismal level in *C. riparius* (Silva et al., 2019).

2.3 Experimental design

Experimental work was performed according to Silva et al., 2021. Briefly, the bioassay consisted of exposing fourth instar larvae of *C. riparius* (12 days post hatching) for 48h to two different sizes (<32 and 32–45 µm) and two concentrations of LDPE microplastics (0.025 and 2.5 g/kg) Each replicate (10 in total) consisted of a glass vial (250 mL) containing 15 larvae, 50 g of inorganic fine sediment (< 1mm; previously burnt at 500 ºC, for 4h) mixed with the respective concentration and size of LPDE and 150 mL of ASTM hard water medium. In the case of control treatments, no LDPE were added to sediment. From the ten replicates of each treatment, seven replicates were used to assess the biochemical responses employing pools of fifteen larvae, whereas three replicates were used for PE quantification and gene expression analysis. From these three replicates, a pool of five larvae was used for PE quantification, and three (random) individual larvae were selected for gene expression. The remaining larvae were stored (-80ºC, see below) as backup samples. The exposure was run at 20±1°C with a 16:8 h light:dark photoperiod. No food was added during the exposure.

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2.4 Quantification and size of LDPE particles in the *Chironomus riparius* larval gut

The method for the extraction and quantification of MPs in *C. riparius* larvae was performed as described elsewhere (Silva et al., 2019). In summary, larvae were rinsed in Milli-Q water, gently dried, and freeze-dried. Samples were then digested with 3 mL of nitric acid (<65%, HNO_3) for 3 h at 60°C. Once cooled (room temperature), complementary digestion to eliminate organic remains was done with 2.6 mL of hydrogen peroxide (35%, H_2O_2). Samples were diluted in Milli-Q water (10×) before vacuum filtration onto grid-lined cellulose nitrate filters

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(Ø 47 mm, 1.5 µm pore size). The biological material was absorbed onto the membrane, placed in a glass Petri dish, and dried at 25°C in the oven for 2–3 days. For the determination of the particles ingested by larvae, a stereoscopic zoom microscope (SMZ 1500, Nikon Corporation) associated with NIS-Elements D 3.2 microscope imaging software was used.

2.5 Biochemical response

After 48 h of exposure, the larvae selected for the biochemical studies were collected, quickly rinsed with Milli-Q water, dried on filter paper, weighed, and frozen in liquid nitrogen and then stored at –80°C until use. The larvae were homogenized in 1600 µL of Milli-Q water by the TissueLyser method, which is based on high-speed shaking with beads to beat and grind the samples, using a Qiagen TissueLyser II. The total homogenate was divided in aliquots. A volume of 200 µL was used for lipid peroxidation (LPO), adding 4 µL of 4% BHT (2,6-diter-butyl-4-methyl phenol), 300 µL for proteins and carbohydrates, 300 µL for lipids, and 300 µL for electron transfer system analysis (ETS). The remaining volume (~500 µL) was mixed with K-phosphate buffer (pH 7.4, 0.2 M) (v1:1) and then centrifugated at 10,000 × g for 20 min at 4°C. Then, the supernatant was collected in aliquots for determination of glutathione-S-transferase (GST), catalase (CAT), and acetylcholinesterase (AChE) activities and for total glutathione (tGSH) and protein quantification.

The optimized protocol for biochemical analysis of this species has been described by Silva et al. (2021) and Campos et al. (2017). The carbohydrate, lipid, and protein contents were measured following the methodology of De Coen and Janssen (1997) and modified by Rodrigues et al. (2015). The

energetic values were calculated based on the energy of the combustion of the different fractions (De Coen and Janssen, 1997). The lipid, carbohydrate, and protein contents were expressed as millijoules per mg of tissue. Protein quantification to calculate the enzymatic activities was done based on the Bradford method (Bradford, 1976) using Bio-Rad Bradford reagent and α-globulin as a standard, while following the manufacturer's instructions.

2.6 RNA extraction, complementary DNA (cDNA) synthesis, and Real-time polymerase chain reaction (RT-PCR)

The frozen larva was homogenized in Trizol reagent (Life Technologies, USA), and total RNA extraction was performed following the manufacturer's protocol. Total RNA was treated with RNase-free DNase (Roche, Germany) to remove any remaining DNA. A final purification with phenol:chloroform:isoamyl alcohol (Fluka, Germany) organic extraction and Phase Lock Light tubes (Quantabio, USA) was done. RNA was precipitated with isopropanol and washed with ethanol. Finally, the RNA was resuspended in diethylpyrocarbonate (DEPC) water and stored at –80°C.

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Complementary DNA (cDNA) was synthesized by reverse transcription using 1 µg of RNA and 100 units of the Murine leukemia virus (M-MLV) enzyme (Invitrogen, Germany) according to Martínez-Guitarte (2018). The cDNA was stored at −20°C.

Real-time PCR was used to evaluate the levels of messenger RNA (mRNA) of the selected genes. The protocol for amplification and analysis was previously described (Muñiz-González and Martínez-Guitarte, 2020). The ribosomal

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protein (*rpL13*), phosphofructokinase (*Phfk*), RNA polymerase (*RNA pol*), and Tata Binding protein (*TBP*) genes were employed as endogenous references. Efficiency was determined as previously described (Ozáez et al., 2016). The primers employed and their efficiencies are listed in Table S1.

2.7 Integrative biomarker response (IBR)

To integrate the results from the different biochemical biomarkers and gene expression analysis aiming to understand the global responses, the integrated biomarker response (IBR) was calculated. In this case the biomarkers with significative differences were selected for the IBR analysis according to a previous work with aquatic another invertebrate (Bertrand et al., 2016). The IBR values were calculated according to Beliaeff and Burgeot (2002) and following the detailed explanation from supplementary data (Ferreira et al., 2015). The calculates and figures were done employing R.4.0.4, RStudio and Microsoft 365 excel software's.

2.8 Statistical analyses

For the statistical evaluation, SPSS 25 software was used. Firstly, normality and variance homogeneity were checked using Shapiro-Wilk and Levene tests, respectively. For normally distributed data, the analysis was done with the Analysis of Variance (ANOVA) unifactorial test and post hoc analysis using the two-sided Dunnett's test to assess which treatment groups showed differences relative to the control and differences between themselves. Non-normal data

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were analyzed by non-parametric tests (Kruskal-Wallis) and the Bonferroni correction was applied for post-hoc analyses. Significant differences were considered when $p \leq 0.05$.

3. Results

Bioassays fulfilled the validity criteria with pH (pH = 7.610 ± 0.080), the dissolved oxygen above 80% and with no organisms showing signs of injury in any of the treatments.

3.1 LDPE MPs ingestion by *C. riparius* larvae

The number of LDPE MPs present in the *C. riparius* larval gut is shown in Fig. 1A. The ingestion of MPs was related to the tested sediment concentrations rather than the size. At 0.025 g/kg, each organism presented an average of 284.2 and 209.6 particles in the gut for <32 µm and 32–45 µm size classes, respectively. At 2.5 g/kg, each group presented 5687 and 5487 particles in the gut for <32 µm and 32–45 µm size class, respectively. Concerning the size of particles found in larvae guts, it is observed that, as expected, the size of the particles used is reflected in the size range (Fig. 1B).

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3.2 Biochemical responses

The energy reserves, energy consumption, and lipid peroxidation results, as well as the tGSH, CAT, GST, and AChE activities are depicted in Table 1. In comparison with the control treatment, a significant increase in the lipid content of *C. riparius* larvae exposed to 0.025 g/kg for both LDPE size classes was observed [$F_{(df)}$=11.576; p=0.000 for <32 µm size; p=0.002 for 32–45 µm size]. A significant reduction in carbohydrate content was only observed for larvae

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exposed to the highest concentration of 32–45 μm LDPE particles in comparison with the same concentration of 32 μm particles [$F_{(df)}$=3.247; p=0.019]. No significant effects in terms of protein content ($F_{(df)}$ = 0.973; p=0.437) and ETS activity [$H_{(KW)}$ =5.646; p=0.227] were observed for any of the particle sizes/concentrations tested. Larvae exposed to the highest concentration of 32–45 μm LDPE MPs also showed significantly higher levels of tGSH [$F_{(df)}$=28.386; p<0.05] and significantly higher activities of CAT [$F_{(df)}$=28.256; p<0.05], GST [$F(_{df})$=21.095; p<0.05], and AChE [$F(_{df})$=18.222; p<0.05] in comparison with the control. Besides the tGSH and the activities of CAT, GST, and AChE were also significantly higher in larvae exposed to 2.5 g/kg of 32–45 μm LDPE particles in comparison with the same treatment of the lower particle size. In the case of GST, increased activity was also observed with the 32–45 μm particles at a concentration of 0.025 g/kg respect to the control [$F(_{df})$=21.095; p=0.000]. Finally, no evidence of lipid peroxidation (LPO) was observed for any of the particle sizes/concentrations tested in comparison with the control treatment [$F_{(df)}$=1.241; p=0.315].

3.3 Gene expression

Forty-one genes associated with relevant pathways in invertebrates were selected to evaluate the effects of LDPE particles at the transcriptional level in *C. riparius*. The statistical significance values of the altered genes can be consulted in Table S2. From the genes analyzed, 10 were related to the endocrine system, 10 were involved in detoxification, 14 coded heat shock proteins (HSPs). In addition, 2 proteins were related to immunity, and 5 have a role in DNA-repairing mechanisms. From endocrine system five genes showed

altered expression; Insulin receptor (*InR*), Disembodied (*Dis*), Death regulator Neddd2-like caspase (*Dronc*), and Methoprene tolerant protein (*Met*) with upregulation in all the cases while Ecdysone receptor (*EcR*) had decreased mRNA levels with respect to the control (Fig. 2). The rest of the genes related to the hormonal system (membrane-associated progesterone receptor [*MAPR*], ecdysone-induced protein [*E93*], cytochrome 18a1 [*Cyp18a1*], juvenile hormone acid O-methyltransferase [*JHAMT*] and Krüppel homolog 1 [*kr-h1*]) were unaltered (Fig. S1). The detoxification response was not altered in any of the genes analyzed from phase I (cytochromes P450 (Cyps) *Cyp4d2*, *Cyp6b7*, *Cyp9f2*, *Cyp12a2*), phase II (Glutathione-S-transferases *GSTd3*, *GSTe1*, *GSTo1*, *GSTt1*), or phase III (ABC transporters) with *ABCB6* and the multidrug-resistance associated protein 1 (*MRP1*) as representatives (Figs. S1, S2, S3). Concerning the stress response, all of the genes showed stable mRNA levels (*hsp70*, hypoxia up-regulation factor 1 [*HYOU1*], *hsc70*, *hsp90*, glycoprotein 93 [*Gp93*], *hsp40*, *hsp60*, *hsp10*; and the small HSPs [sHSPs] *hsp17*, *hsp21*, *hsp22*, *hsp23*, *hsp24*, and *hsp27*), as observed in Figs. S3–5. Two genes related to the humoral immune response were studied, with increased expression for defensin (*Def*) (Fig. 3) and no effects on Prophenoloxidase (*Proph*) (Fig. S5). Finally, the DNA repair response showed altered expression in four genes with increased expression respect to the control in all the cases: (poly [ADP-ribose] polymerase [*PARP*], ataxia telangiectasia mutated gene [ATM], Nemo-like kinase [*NLK*], and death executioner caspase [*Decay*]) (Fig. 3), but there were no effects on X-ray repair cross-complementing protein 1 (*XRCC1*) (Fig. S5). Moreover, *ATM* exposed to 32–45 µm particles showed significant differences with respect to the 32 µm particles at 0.025 (Fig. 3). In

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summary, 10 out of 41 genes were modulated by LDPE MPs exposure, increasing their expression respect to the control, except for *EcR*, with downregulated expression.

3.4 IBR

Due to the different nature of the analyzed biomarkers and in order to know the integrated response in each case, the IBR calculation was first performed separately for the biochemical markers (Carbohydrates, Lipids, GST. tGSH, CAT, and AChE), for the gene expression, genes from the endocrine system (*InR, Dis, EcR, Dronc* and *Met*) and from the immune system / DNA repair (*Def, PARP, ATM, NLK,* and *Decay*). Finally, the total IBR from these biomarkers was analyzed to observe the global response and the relationship of the IBR with each of the sizes of MPs (32, 32-45 µm) as well as the concentrations (0.025 and 2.5 g/kg) evaluated. The results are presented in the table 2 and figures 4, and 5.

Biochemical markers

In the case of biochemical markers, a good visual concordance was observed between the GST, tGSH, CAT and AChE respect to the treatment (2.5 g/kg 32-45 µm; figure 4A). However, the other treatments followed a similar patten without remarkable correlations, altough the lipids present higher scores at 0.025 g/kg (32µm). The s score values (Table 2) were higher for GST, tGSH, CAT and AChE being the main contributors for the IBR. This tendency was confirmed in the IBR start plots (figure 5A) and IBR value (table 2). The IBR analyses for biochemical markers showed worse scores (higher IBR values) for

the both concentration of the bigger MPs particles (32-45 µm) being notable in the case of the 2.5 g/kg than the other treatments, in line to the results observed in the 3.2 section.

Gene expression:

Contrary to the biochemical markers as in line to previously observe in figure 2, the endocrine system genes showed similar s scores for all the treatments (figure 4B: table 2) with all genes being affected to a lesser or greater extent by all treatments. No clear gene contribution can be stablished between each gene analyzed and the endocrine system IBR. Altough focusing on the IBR values the worse score was observed for 0.025 g/kg (32 µm; table 2) followed by the same concentration at 32-45 µm LDPE, showing IBR= 7.70, and 4.37 respectively. This pattern was visually confirmed in the figure 5B. For the other genes analyzed, those belonging to the immune system and DNA repairing, a strong visual concordance was detected for *NLK* at 0.025 g/kg 32 µm and for *Decay* at 2.5 g/kg 32-45 µm (Figure 4C). Analyzing the s scores for each gene (table 2), the main contributors to the IBR are *PARP*, and *ATM* for the first concentration and *Decay* and *PARP* for the last concentration analyzed. This response was confirmed trough the IBR values with high values for 0.025 g/kg 32 µm (7.22) and being the IBR= 5.21 for 2.5 g/kg 32-45 µm. The pattern was similarly observed in the figure 5C.

Finally, the total IBR for all the biomarkers was obtained showing bigger IBR values for 0.025 g/kg (32 µm) and 2.5 g/kg (32-45 µm) with 5.20 and 4.47 values (table 2), respectively. For the two other conditions the values for IBR were similar.

4. Discussion

The toxicity of MPs to aquatic fauna has been evidence on organismal endpoints including growth, reproduction, or immune responses, among others (Green et al., 2019; Lanctôt et al., 2020; Mak et al., 2019). The MPs particles used in this study have been previously shown to be ingested by *C. riparius* larvae at a concentration of around 1 g/kg (Silva et al., 2019, 2021), reflecting the non-selective feeding of chironomids with no discrimination between sediment itself and MPs (Nel et al., 2018). In line with previous studies, we have shown that the number of plastic particles found within the larvae gut is in accordance with their concentrations in the sediment.

The blocking and clogging of the midgut of *C. riparius* larvae could impair feeding and digestion, leading to reduced energy acquisition and assimilation and, thus, to changes in energy reserves. However, only a significant decrease in carbohydrate content was observed for larvae exposed to the 32–45 µm LDPE MPs; although, a decreased tendency was detected for the exposure to 32–45 µm particles. This reduction, which was in line with what was observed for *Lumbriculus variegatus* that was exposed to the same LDPE particles (32-63 µm) for the same 48 hours of exposure (Silva et al., 2021b), is usually an indication of either reduced feeding and energetic constrains related to the digestion and egestion of these inert and non-nutritive particles or it is linked to the energetic costs related to the activation of detoxification and antioxidant mechanisms and/or immune responses (Silva et al., 2021; Trestrail et al., 2020). Given that our exposures were done without food, the differences are most likely related to the energetic costs of antioxidant responses, which, in our study, were corroborated by the increased activity of CAT and GST and by the glutathione content. In fact, carbohydrates are another example of classical cell

energy storage; therefore, the reduction in content can be explained by the use of energy in the process of metabolism and detoxification. Previous studies have shown a similar effect with the same MPs on earthworms (250 μm, GST) for 28 days, *L. variegatus* (48h), and mussels and even by polystyrene (PS) MPs (1 mg/L, 20 μm) in clams for 14 days (Green et al., 2019; Ribeiro et al., 2017; Rodríguez-Seijo et al., 2018; Silva et al., 2021b). The activation of detoxification mechanisms is usually associated with some kind of chemical compound, but increased reactive oxygen species (ROS) due to the activation of the immune response related to LDPE MPs ingestion has also been suggested (Silva et al., 2020). The obtained results demand further research to elucidate the possibility that some kind of chemical transfer could be in progress, causing activation of the detoxification response. Nevertheless, it is clear that activation of the antioxidant system avoided oxidative damage in *C. riparius* larvae since the analysis of LPO showed no changes. This is somewhat surprising given that previous studies with slightly larger LDPE particles (32–63 μm) have shown oxidative damage with altered LPO in the same species and no increases in the antioxidant defenses, such as CAT and GST activity (Silva et al., 2021a), the longer particle size seems to explain the greater toxicity in the work by Silva et al., 2021, generating the response in LPO. However, in our study the CAT and GST increased activity alleviating the global effect at the level of oxidative damage could explain the absence of alteration in LPO. Oysters showed similar response by PE MPs with no effects on LPO by 10 days exposure (Revel et al., 2020) besides in earthworms exposed to LDPE MPs < 400μm stimulated the CAT at 1 mg/L for 28 days (Chen et al., 2020).

Despite the above-mentioned responses, ETS activity was not altered, suggesting that aerobic energy production was not affected by exposure to LDPE MPs. Previous studies have shown that aerobic energy production can be reduced due to exposure to PE MPs in freshwater invertebrates (Silva et al., 2020; Silva et al., 2021), and these differences might be related to the lower size of the particles and the lower concentrations used in the present study.

Also, an increase in the lipid content in larvae exposed to the lowest concentration of both particle sizes tested was observed. A similar response has been observed in algae after PS nano-plastic (0.05 µg/mL) exposure for 96h (González-Fernández et al., 2020). In agreement with these results, PS MPs (50 µm, 100 µg/L) for 7 days of exposure increased the expression of genes related to lipogenesis, such as fatty acid synthetase and acetyl-CoA carboxylase, in larvae zebrafish (Wan et al., 2019). This increase could be derived from the stress induced by the MP exposure. Moreover, lipid accumulation is a typical way to keep reserves for future actions in the cells. The obtained results could reflect the effect of MPs at low concentrations, affecting, in some way, lipid synthesis. The carbohydrate content can also be biased to lipid synthesis (Hudgins et al., 2000) , and this would explain why the carbohydrate content was reduced in longer particles tested. The question that arises is why the increase in lipid content is not also observed at the higher concentrations. A putative explanation would be a reduced ability to feed off the larvae due to the block of the midgut by the greater presence of particles difficulty the digestion and nutrients assimilation as previously observed in crustacea exposed to PP MPs for 10 days (Au et al., 2015). Another explanation is the increased energy demand as a consequence of the

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detoxification response, which tries to compensate for the toxicity of the LDPE (De Coen and Janssen, 2003), as is suggested with the GST activity increase at the concentration of 2.5 g/kg.

MPs have been associated with mechanical damage, which could be translated into nervous alteration. The analysis of the typical biomarker AChE showed that its activity increased for the 32–45 µm particles at the highest concentration tested. This increased AChE activity is in agreement with the response of *C. riparius* exposed to 20 g LDPE/kg sediment, *Eisenia fetida* exposed to 1 and 1.5 g/kg, and *Lumbriculus variegatus* exposed to LDPE MPs (Chen et al., 2020; Silva et al., 2021a, b). However, AChE is usually inhibited by exposure to xenobiotics and mainly by pesticides (Pérez et al., 2013; Pham et al., 2017). The observed increase could be due to the irregular shape of these LDPE particles and could be related to the efforts in the peristaltic movements to eliminate the plastic particles, then altering nervous transmission. Additional research analyzing the motility in the midgut could raise some light on this possibility. A potential explanation might be related to extra effort to egest MPs as observed in rotifers exposed to PS MPs (0.1 µm) for 48h (related to peristaltic movements; De Oliveira et al., 2012) and/or gut inflammation during egestion (Gambardella et al., 2017). In summary, it seems that the 32–45 µm particles showed greater toxicity at the level of biochemical response. The analysis of transcriptional activity showed toxicity for both particle sizes, but it is hard to define which concentration elicited stronger effects. These results are in contrast to those observed with biochemical markers, where particle sizes of 32–45 µm showed the strongest effects. Focusing on the endocrine system, a

mixed response was observed in comparison with the control treatment, with some genes upregulated (*InR*, *Dis*, *Dronc*, and *Met*) and one gene downregulated (*EcR*). It can be proposed that 20-E synthesis increased since *InR*, which is involved in the synthesis that controls the insulin action, and *Dis*, which is a member of the Halloween set of genes (Gilbert, 2004; Keshan et al., 2017), were disturbed. It could be an attempt to compensate for the decrease suggested by *EcR* downregulation. The reduced transcription of *EcR* would reflect a decrease in the 20-E levels. Then, synthesis of the hormone could be activated. It is essential to consider that larvae were in the fourth instar, which is the last larval stage before pupation, with high endocrine activity. Furthermore, the overexpression of *Dronc*, an effector gene belonging to the signaling pathway of this hormone, could reflect the previous activation of the *EcR*, suggesting that endocrine activity was in progress. Although additional research is needed, it is clear that the MPs used seem to alter ecdysone metabolism.

On the other hand, these LDPE MPs also affect the juvenile hormone (JH) response pathway. The upregulation of *Met* suggests the induction of the response. However, it is not accompanied by any alteration in JH synthesis since *JHAMT*, the gene coding the key enzyme of biosynthesis, was not altered. 20-E and JH work coordinately to regulate growth, development, and metamorphosis, with coordinated temporal pulses (Belles, 2020; Truman, 2019). Both responses share some factors, such as *Kr-h1* and *E93*, genes which were not altered in exposed larvae. Kr-h1 acts by repressing E93, avoiding the precocious metamorphosis (Kayukawa et al., 2014). Moreover, a preliminary study on *C. riparius* employing LDPE showed decreased growth and delayed emergence, mainly by 32–63 μm particles (Silva et al., 2019). It is

evident that endocrine regulation is affected by the MPs, suggesting a complex interaction that impacts the development of the larvae. Similar results were observed in another chironomid, *C. tepperi*, by PE MPs (10-27 µm (500 particles/kg sediment); Ziajahromi et al., 2018). Moreover, a general decreased expression of developmental genes on zebrafish was detected, inducing alterations in the embryo, and organ development after exposure to 5-20 mg/L (10-45 µm) PE MPs for 48 h (LeMoine et al., 2018). These results suggest that endocrine disruption at the molecular level can be directly linked to the developmental effects and potential population level alterations already observed for *C. riparius* exposed to these LDPE MPs (Silva et al., 2019), interfering with the viability of the population. In addition, biochemical markers suggested a deviation of energy to detoxification; therefore, less energy could be available for development. Additional research is required to elucidate the mechanisms involved in the endocrine disruption induced by MPs.

The set of genes analyzed covering the detoxification mechanisms, surprisingly, showed unaltered response to LDPE MPs. These genes are involved in the response to xenobiotics; therefore, it is possible that the lack of induction is a consequence of the fact that no substance was released when the particles were used. Also, it is worthy to consider that the exposure time, 48 h, could be too short to detect any response. In contrast to our results, it has been shown that diverse MPs modulate GST expression, with upregulation in fishes (PVC, 45.55 to 136.65 µg/L for 30 days) and by PS (50 nm to 10 µm, for 48h) in crustacea (Choi et al., 2019; Xia et al., 2020). The increase in GST activity that we observed was not accompanied by an increase in mRNA levels of the genes

studied, but it could be due to the fact that we did not analyze the transcriptional activity of the specific GST class involved. In any case, it seems that we did not detect any transcriptional activity of the genes involved in the three detoxification phases. This is in contrast to the data obtained for fishes, which showed altered Cyps by MPs (Xia et al., 2020). A putative explanation is the different time of exposure, which was 30–40 days, in contrast to the 48h used in this work. In short, exposure to LDPE MPs causes stimulation of GST activity, but, although it is usually related to detoxification mechanisms, it would also be activated in response to oxidative stress. The exposure time, 48 h, supports the possibility that the activation of GST was related to oxidative stress more than the detoxification mechanisms. Longer exposures times could help to solve this question, which could be a two-step process with an initial activation by oxidative stress and a second activation due to the detoxification of xenobiotics.

Linked to stress, 14 genes coding for HSPs were analyzed

with lack of response in all of the HSPs (*hsp70*, *HYOU1*, *hsc70*, *hsp90*, *Gp93*, *hsp40*, *hsp60, hsp10*, *shsp17*, *shp21*, *shsp22*, *shsp23*, *shsp24*, *and shps27*) suggests that the cells did not suffer direct damage by the MPs used, at least for these exposure periods. The results are in contrast to other studies that observed a decrease in *hsp70* transcriptional activity by PE MPs in ternary mixtures in *D. magna* (Imhof et al., 2017), and in single exposure (77.5 µm, 100, 500 mg/kg) for 21 days (Espinosa et al., 2019). However, *hsp90* showed no change in different generations on *D. pulex* after exposure to PS nano-plastics (1 µg/L, 75 nm) for 21 days (Liu et al., 2020). *hsp60* and *hsp10* are located in the mitochondria working coordinately, which is an organelle sensitive to oxidative stress. A previous study on crustacea observed upregulation of *hsp60*

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after MPs mixture exposure including PE MPs (40 µm for 48h), although *hsp10* was not evaluated (Imhof et al., 2017). The absence of response seems to indicate a low oxidative damage due in part to the palliative effect of increased GST and CAT activity. For the rest of the proteins, there have been no previous studies with MPs that can provide information on other organisms or with different MPs. In any case, there is a limited impact of the MPs on the stress response, at least in the short-term. It is possible that the particle size used in this work could reduce the impact since they are not able to enter the cell. Combining different MP sizes and longer times could cause a stronger effect.

To evaluate immunity, we analyzed two genes belonging to the humoral response: *Proph* as an inactive form of phenoloxidase (PO) and *Def* as a representative of the antimicrobial peptides (AMPs). While *Proph* did not change, *Def* was upregulated. Previous studies have demonstrated the ability of MPs to modulate the immune response in aquatic invertebrates. Concretely, higher concentrations of larger LDPE MPs (32–63 µm) induced the activity of PO in *C. riparius* (Silva et al., 2021) at 48 h. The larger size of the particles and the higher concentration (5 g/kg and 20 g/kg) can explain the differences. Regarding other MPs, high-density PE (HDPE) MPs (25 µg/L, 102.6 µm) for 52 days of exposure altered the immunity of blue mussels, with overexpression of immune proteins such as cytokines or complement system components (Green et al., 2019). Another exhaustive study on crabs observed altered immune response, with increased activity of acid phosphatase and PO, among others, and modified mRNA levels for hemocyanin and lysozyme after PS (0.04 to 40 mg/L, 5 µm) exposure for 21 days (Liu et al., 2019). The increased activity of PO in crabs' contrasts with our results, but it could be due to the longer

exposure time employed (7 days) and the different nature of the MPs used. Moreover, in fish, changes in the immune response were detected after PE and PS MPs exposure, confirming the strong effects on this system in aquatic vertebrates (Espinosa et al., 2019; Wan et al., 2019). The activation of the immune system emphasized the possible oxidative stress damage derived from exposure to LDPE MPs, as suggested by the activation of CAT, GST, and tGSH. The putative oxidative damage can be explained by the relationship between ROS and activation of the immune response (Mittal et al., 2014). Moreover, in copepods, the production of ROS was detected in response to MP exposure (Choi et al., 2019). Also, it is important to consider that *Def* usually acts against pathogens such as bacteria or viruses. The upregulation of Def gene suggests that the immune response is activated because *C. riparius* identified the MPs as foreign particles similar to bacteria, stimulating the production of AMPs against them. Then, the MPs could have a double effect by activating a response that could produce free radicals that modulate the immune system and activate the response against pathogens by AMPs. Immune system activation is likely linked to mechanical/proteolytic damage of the gut epithelium of *C. riparius* larvae since the size of the LDPE MPs used was not small enough to cross biological barriers. As a consequence, immune response activation can be, at least in part, responsible for the production of ROS and the resulting oxidative stress observed (suggested by the observed activity of CAT and GST and by the glutathione content) and, thus, with the previously assessed life history responses (Silva et al., 2019).

The last set of genes allowed for evaluation of the DNA-repairing mechanisms. Three of them showed an increased expression (*ATM*, *PARP*, and *NLK*), even

the *Decay* involved in apoptosis, but *ATM* was downregulated by the large particles. However, the X*RCC1* participating in the single strand break (SSB) was unaltered. The pattern of the response is complex, and since the genes analyzed are involved in different DNA-repairing mechanisms, it could reflect a variety of damages. However, considering the data with other genes and the enzyme activities, it can be suggested that DNA was indirectly damaged by oxidative stress events. Then, MPs would affect the DNA by activating the release of free radicals that can alter the DNA and produce DNA breaks. The damage would not be uniform and would involve single and double strand breaks (SSB, DSB), activating different repair mechanisms. However, the particular behavior of *ATM* still has to be explained, but it is similar to that observed for the exposure of C. riparius to vinclozolin (Aquilino et al., 2019). There have been no previous reports on the effects of MPs on genes related to DNA-repairing mechanisms, but an alteration was detected by PS nanoplastic (0.5 to 50 mg/L,110 nm) for 96h exposure in mussels (Brandts et al., 2018). Nevertheless, our results are consistent with previous effects described for MPs on DNA, showing damage on DNA strands as confirmed by comet assay results. Only one study detected damage from PE MP exposure in mussels; although, this type of MP did not cause alterations in oysters (Avio et al., 2015; Revel et al., 2020). Furthermore, several works evaluating PS MPs showed strong alteration on DNA, showing strands breaks in earthworms (100-1300 nm,14days) and clams (Jiang et al., 2020; Ribeiro et al., 2017) and on fish larvae after MPs mixture exposure including PE for 30 days (Pannetier et al., 2020). In conclusion, LDPE MPs seem to follow the same effects on DNA as the previous studies in other MPs. According to previous observations, damage

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to DNA can be driven by a process of oxidative stress by ROS liberation, as previously detected by other particles after nanoparticles exposure (Dayem et al., 2017).

In recent years, interest in the evaluation of different biomarkers has increased in order to provide a more global analysis of the effects at suborganismal level. With this objective, the IBR was implemented for each group of biomarkers as well as the global IBR value. From the IBR analysis was confirmed that the main contributors for the biochemical markers IBR response were lipids and GST for the 0.025 g/kg (32 µm) and GST, tGSH, CAT, and AChE for the 2.5 g/kg (32-45 µm). However, no clear contribution was established between endocrine genes analyzed and the IBR, altough their similar response at each exposure condition indicates greater sensitivity of these molecular markers respect to the other parameters studied. For the genes involved in immune system and /DNA repairing the main contributors for the IBR were *PARP*, and *ATM* for the first concentration and *Decay,* and *PARP* for the last concentration analyzed. In general, seems that the 0.025 g/kg (<32 µm) was the most toxic for all the biomarkers analyzed (IBRt = 5.19). Closely, followed by the 2.5 g/kg (32-45 µm) with a IBRt= 4.47. Being the main contributors for the total IBR values, the gene expression from endocrine and immune/DNA repair, reaching to a 7.70 and 7.22 IBR for the first concentration and with 5.61 and 5.21 for the last condition, respectively. These results for IBR seem to be indicate that *C. riparius* could be a good bioindicator for LDPE pollution in freshwater sediments, highlighting the great sensitivity of gene expression changes. Besides the total IBR showed a globally response integrating the all biomarkers

analyzed reducing the need to employ bigger data sets, similarly to previously observed in shrimps after pesticide exposure (Bertrand et al., 2016).

5. Conclusions

Concern about the possible damages from MPs on aquatic organisms has been increasing over the years due to their exacerbated presence in surface waters. The present study represents the first evaluation of the effects of MPs at the cellular level by employing multiple molecular biomarkers on the insect *C. riparius*. The biochemical markers and gene expression results suggest that ingestion of LDPE MPs induced negative effects ranging from endocrine disruption to an immune response and damage to DNA, mainly associated with oxidative stress events. Being notable the effects by the 0.025 g/kg (32 µm) and 2.5 g/kg (32-45 µm) LDPE exposure as confirmed by the IBR analysis.

This study supports that ROS production due to an immune response and consequent oxidative stress (increased CAT and GST activities and tGSH levels) is one of the principal mechanisms of action behind the deleterious effects of MPs in aquatic biota. The energetic costs of the induced responses coupled with the likely feeding and digestive impairments caused by clogging of larval mid-gut and also endocrine disruption most likely contributed to the previously observed effects on growth and developmental rates of *C. riparius* exposed to these plastic particles (Silva et al., 2019; Silva et al., 2021).

In summary, the exposure and ingestion of LDPE MPs disrupt the metabolic and cellular processes essential for the life of *C. riparius*, despite the short exposure time employed. The molecular response shows the importance of these parameters as early signs of MP toxicity for aquatic organisms such as *C. riparius*. Comparing both particle sizes, the 32–45 µm particles showed slightly

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stronger effects. Assessment of the effects of longer exposures are essential to confirm these results and to better evaluate some of these endpoints in light of the much-desired adverse outcome pathways (AOPs) approach for MPs.

Figure 1. A) Number of LDPE particles ingested by fourth instar *C. riparius* larvae exposed to two concentrations (0.025 and 2.5 g/kg) and two size classes (<32 and 45–63 μm) of LDPE particles for 48h, employing 5 larvae per condition. B) Sizes of LDPE particles ingested by *C. riparius.*

Figure 2. Expression of *InR*, *Dis*, *EcR*, *Dronc*, and *Met* in fourth instar *C. riparius* larvae after exposure to LDPE MP concentrations of 0.025 and 2.5 g/kg of two size classes (<32 and 32–45 μm) for 48 h. mRNA levels were normalized using *rpL13*, *RNApol*, and *TBP* as reference genes. Whisker boxes are shown. (The horizontal line indicates the median. The boundaries indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box). The differences with respect to the control *were defined according to $p<0.05$.

Figure 3. Expression of *Def*, *PARP*, *ATM*, *NLK*, and *Decay* in fourth instar *C. riparius* larvae after exposure to LDPE MP concentrations of 0.025 and 2.5 g/kg of two size classes (<32 μm; 32–45 μm) for <32 and 32–45 μm) for 48 h. mRNA levels were normalized using *rpL13*, *RNApol*, and *TBP* as reference genes. Whisker boxes are shown. (The horizontal line indicates the median. The

boundaries indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box). The differences with respect to the control and the differences between treatments are defined in the legend. All significant differences were defined according to $p<0.05$.

Figure 4. Star plots indicating the s scores for each treatment for A) Biochemicals markers B) Endocrine system genes, and C) Immune/DNA repair genes, D) Total biomarkers of *C. riparius* exposed for 48h. The legend indicates the color line for each treatment.

Figure 5. Integrated biomarker response (IBR) represented by star plot of *C. riparius* in the control and exposed to LDPE MPs (0.025 and 2.5 g/kg at <32 and 32-45 μm) for 48h. The IBR values correspond to A) Biochemicals biomarkers B) Endocrine system genes, and C) Immune/DNA repair genes, D) Total IBR.

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Table 1. Biochemical responses of fourth instar *Chironomus riparius* larvae after low-density polyethylene (LDPE) microplastic (MP) exposure for 48 h. The values are presented as the mean ± standard error of the mean (SEM). The significant differences with respect to the control are marked with **c**, and with respect to <32 µm particles sizes at 2.5 g/kg. In all the cases the differences are according on ($p < 0.05$).

		LDPE treatments			
		<32 µm		**32-45 µm**	
Biomarkers	**Control**	**0.025 g/kg**	**2.5 g/kg**	**0.025 g/kg**	**2.5 g/kg**
Carbohydrates (mJ /mg organism)	181.84 ± 16.59	165.34 ± 18.78	189.33 ± 24.61	130.94 ± 15.23	144.67 ± 19.22
Lipids (mJ /mg organism)	508.02 ± 36.91	937.64 ± 80.43 c	611.56 ± 45.28	870.37 ± 63.05 c	507.01 ± 51.50
Proteins (mJ/mg organism)	265.07 ± 8.21	256.08 ± 12.51	253.61 ± 12.42	238.26 ± 11.63	267.12 ± 5.79
ETS (mJ/h/mg organism)	33.81 ± 0.6[illegible]	34.83 ± 1.01	32.50 ± 0.43	32.90 ± 1.69	35.45 ± 0.96
LPO (nmol TBARS/mg organism)	114.73 ± 4.89	111.41 ± 2.22	120.71 ± 4.21	133.21 ± 14.00	129.81 ± 7.98
TGSH (µM/mg organism)	13.58 ± 0.50	13.17 ± 0.98	13.02 ± 0.54	14.90 ± 1.90	26.20 ± 2.70 c
CAT (µmol/min/mg protein)	33.37 ± 3.89	38.34 ± 2.61	39.31 ± 2.50	64.02 ± 8.50 c	80.84 ± 4.07 c

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GST (nmol/min/mg protein)	32.70 ± 0.66	32.58 ± 1.20	36.26 ± 1.46	60.02 ± 8.91[c]	77.85 ± 3.35 [c]
AChE (nmol/min/mg protein)	6.81 ± 0.42	7.37 ± 0.87	7.58 ± 0.36	11.68 ± 1.48	17.21 ± 1.37 [c]

Table 2. Standardized biomarker responses (s scores) and integrated biomarker response (IBR) values for biochemical markers, endocrine genes and immune/DNA repair genes, the colors indicated the IBR values for each group of biomarkers. In bold the highest contributing biomarker scores (S) for the IBRt value. (C= control; 1= 0.025 g/kg <32 µm; 2= 2.5g/kg <32 µm; 3= 0.025 g/kg 32-45 µm; 4= 2.5g/kg 32-45 µm).

	Score of biomarkers (S value)																			
	Carbohydrates	**Lipids**	**GST**	**TGSH**	**CAT**	**AChE**	***Dis***	***Inr***	***FcR***	***Dronc***	***Met***	***Def***	***PARP***	***ATM***	***NLK***	***Decay***	**IBR**	**IBR**	**IBR**	**IBRt**
C	0,34	1,07	1,20	0,67	1,42	1,22	0,65	0,84	0,00	0,66	0,59	0,4	0,23	0,02	0,17	0,16	0,03	0,00	0,00	**0,01**
1	0,06	**4,26**	**1,21**	0,74	1,13	1,02	0,32	**0,93**	0,00	0,15	0,12	0,82	**0,97**	0,53	**1,18**	0,77	0,66	7,70	7,22	**5,19**
2	**3,69**	0,63	1,04	0,77	1,13	1,11	**1,20**	0,38	-0,34	0,25	**0,69**	0,58	0,56	0,55	0,34	**1,02**	0,52	3,42	3,94	**2,62**
3	0,60	**1,09**	1,01	0,44	**1,16**	0,83	0,49	0,43	-0,54	0,70	0,55	0,42	0,37	0,14	0,43	0,53	2,43	4,37	4,14	**3,65**
4	0,63	1,07	**1,94**	**1,96**	**2,00**	**2,00**	0,53	0,29	-0,1	0,22	0,33	0,54	**0,82**	0,13	0,76	**1,24**	5,61	2,58	5,21	**4,47**

									4											

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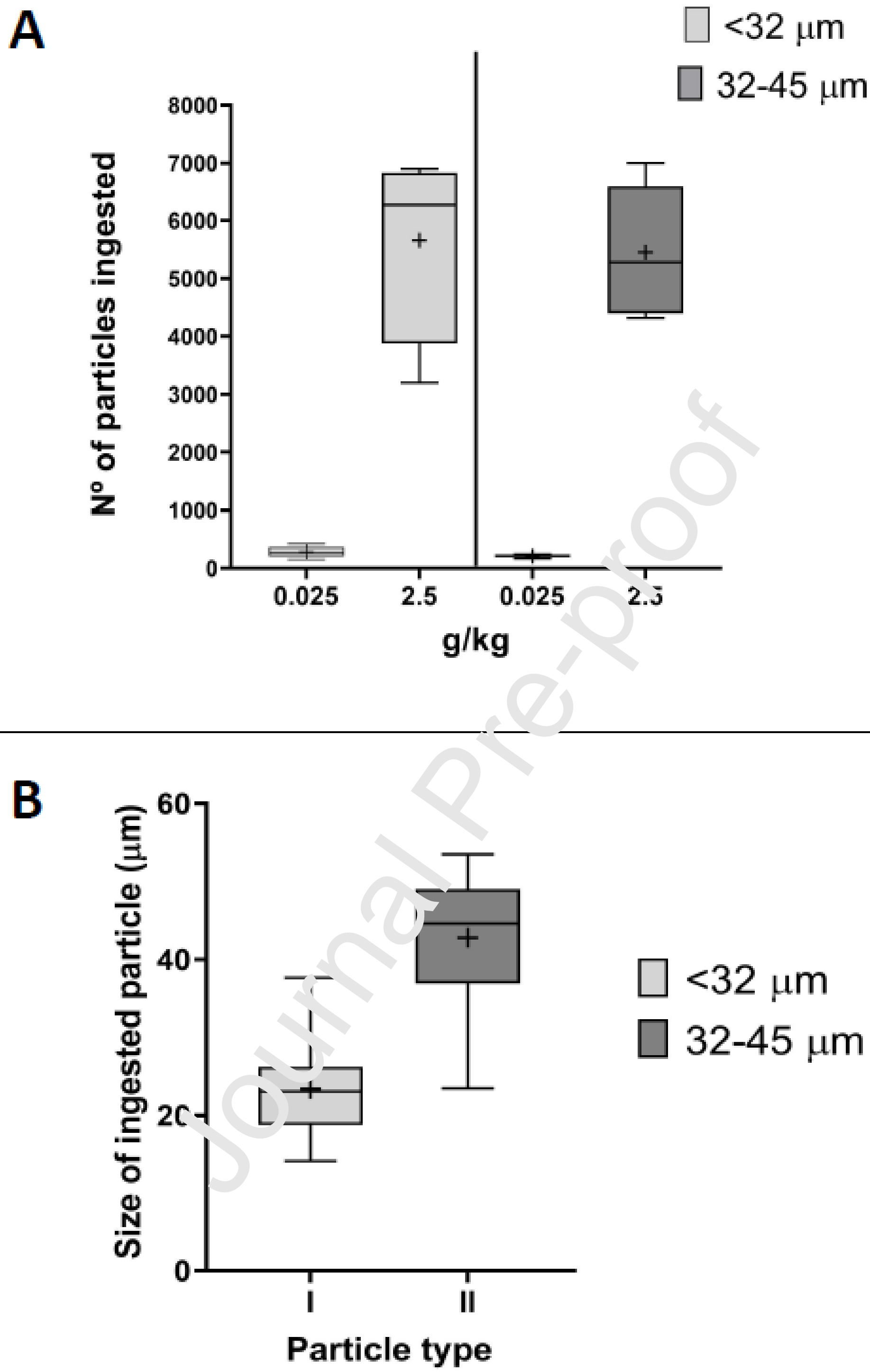

Fig. 1

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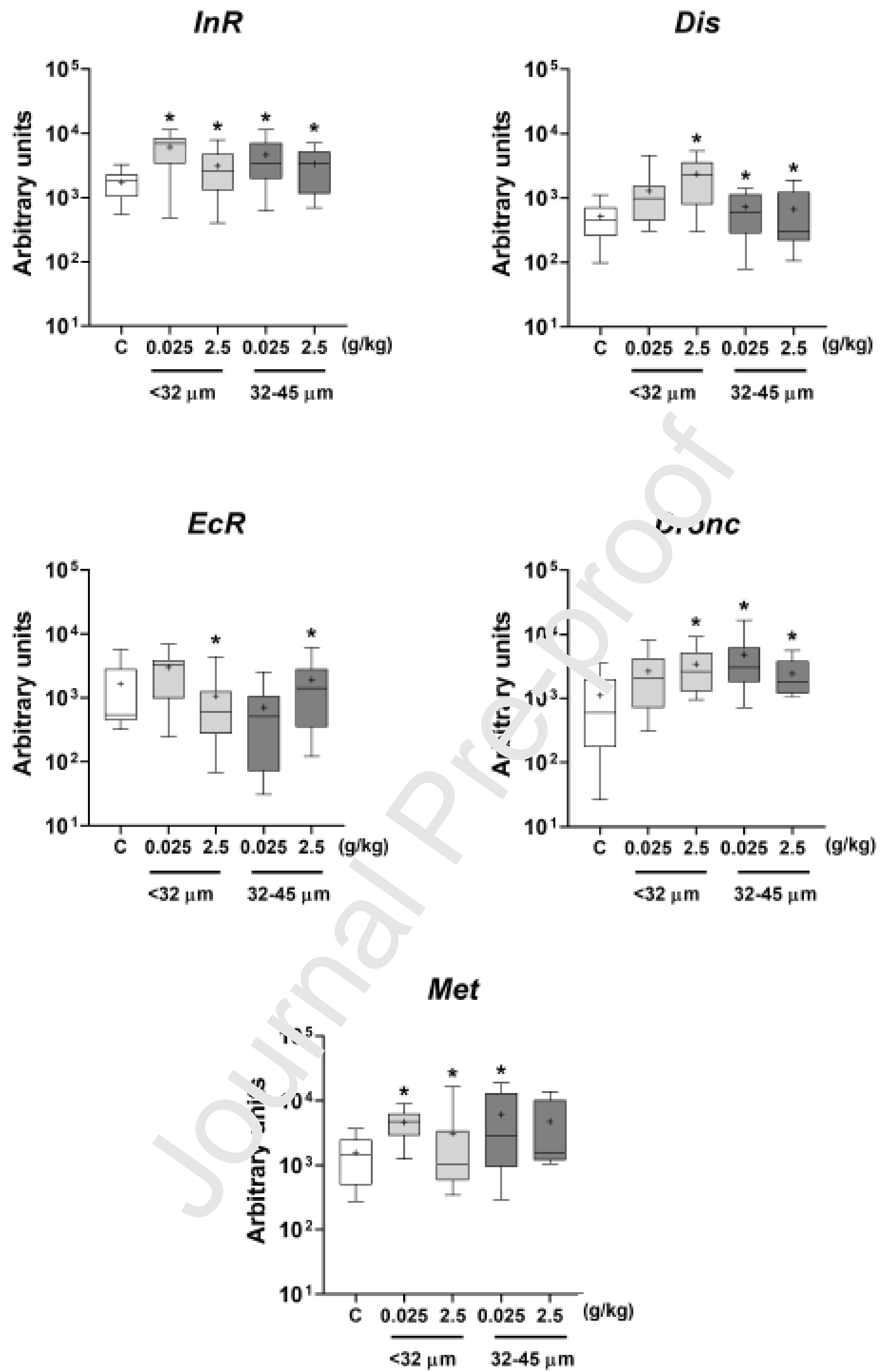

Fig. 2

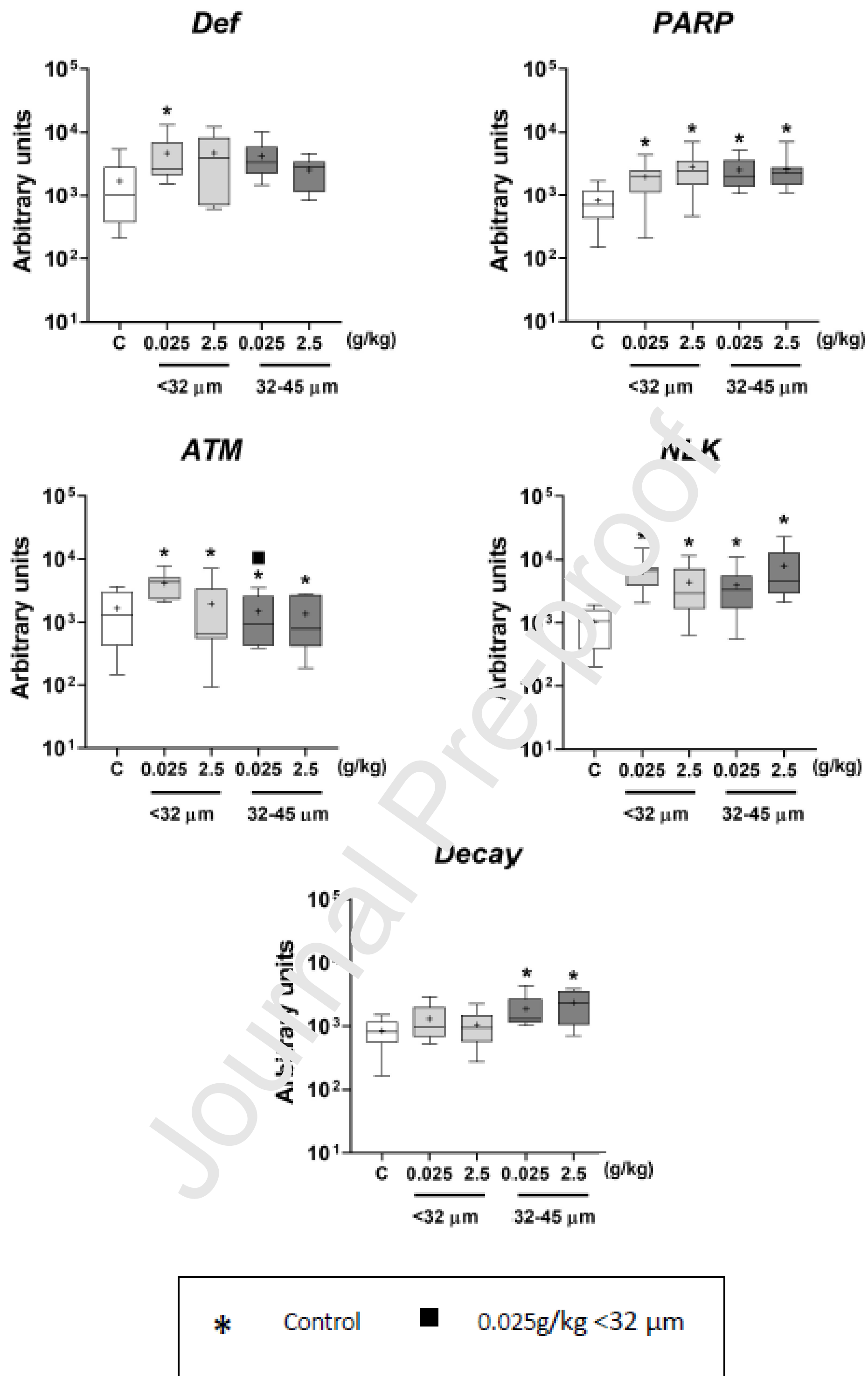

Fig. 3

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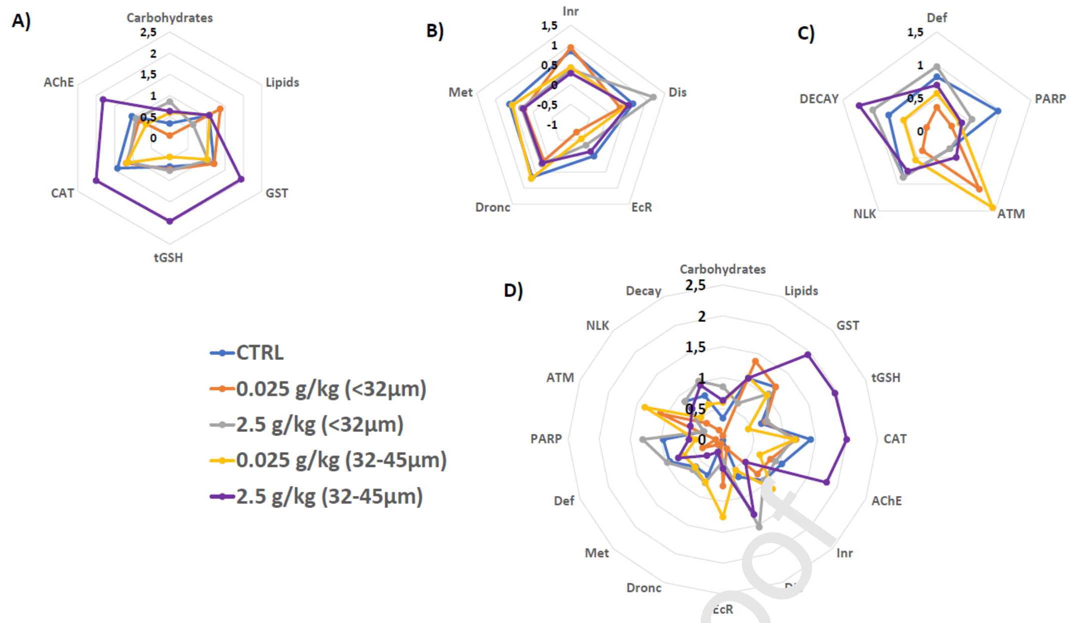

Fig. 4

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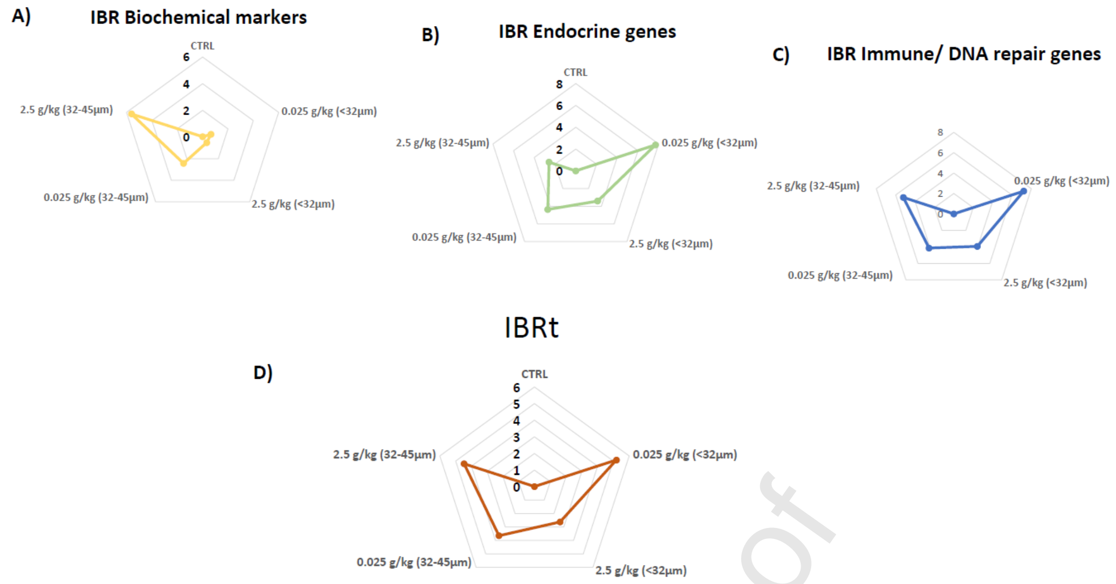

Fig. 5

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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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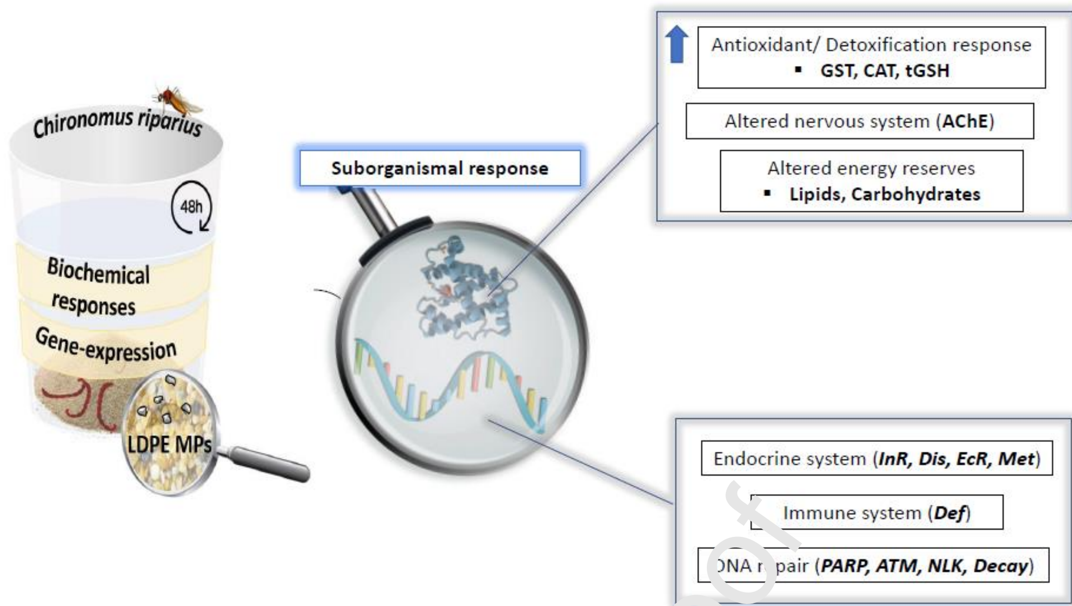

Graphical abstract

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Highlights

- 32–45 µm LDPE particles showed higher toxicity at the cellular level in *C. riparius*.
- Increased GST and CAT activities and *Def* expression support oxidative stress damage.
- The endocrine disruption observed confirmed LDPE MPs as EDCs.
- LDPE MPs altered DNA-repairing gene expression, acting as genotoxic compounds.
- Gene expression is an appropriate early alarm signal of MP toxicity in Chironomids.

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