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DNA damage and molecular level effects induced by polystyrene (PS) nanoplastics (NPs) after *Chironomus riparius* (Diptera) larvae

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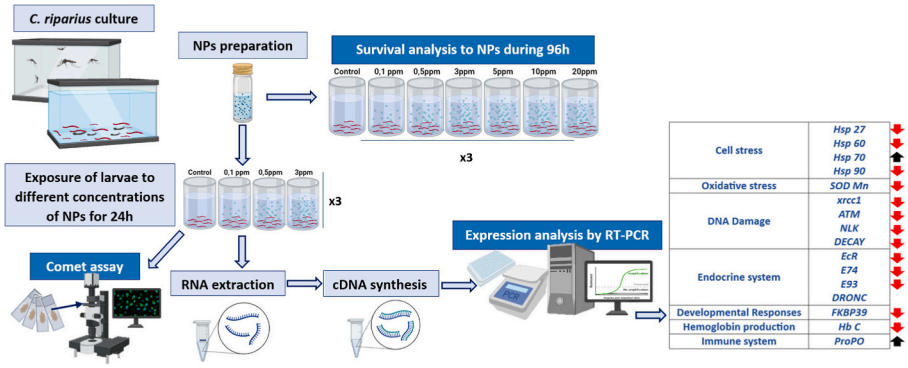
HIGHLIGHTS

- NPs inhibited the expression of genes involved in endocrine system and development.
- NPs down-regulated genes involved in cellular and oxidative stress and DNA repair.
- NPs exposure inhibited oxygen transport gene in *C. riparius* at 24 h.
- NPs induced expression of a gene involved in humoral immune response in *C. riparius*.
- NPs induced DNA breakage after 24 h of exposure at all concentrations tested.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, we analyzed the early molecular effects of polystyrene (PS) nanoplastics (NPs) on an aquatic primary consumer (larvae of *Chironomus riparius*, Diptera) to evaluate their potential DNA damage and the transcriptional response of different genes related to cellular and oxidative stress, endocrine response, developmental, oxygen transport, and immune response. After 24-h exposures of larvae to doses of PS NPs close to those currently found in the environment, the results revealed a large genotoxic effect. This end was evidenced after significant increases in DNA strand breaks of *C. riparius* larvae quantified by the comet assay, together with results obtained when analyzing the expression of four genes involved in DNA repair (*xrrc1*, *ATM*, *DECAY* and *NLK*) and which were reduced in the presence of these nanomaterials. Consequently, this reduction trend is likely to prevent the repair of DNA damage caused by PS NPs. In addition, the same tendency to reduce the expression of genes involved in cellular stress, oxidative stress, ecdysone pathway, development, and oxygen transport was observed. Taken together, these results suggest that PS NPs reduce the expression of hormonal target genes and a developmental gene. We show, for the first time, effects of PS NPs on the endocrine system of *C. riparius* and suggest a possible mechanism of blocking ecdysteroid hormones in insects. Moreover, the NPs were able to

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inhibit the expression of hemoglobin (*Hb C*), a protein involved in oxygen transport, and activate a gene of the humoral immune system. These data reveal for the first time the genomic effects of PS NPs in the aquatic invertebrate *C. riparius*, at the base of the food chain.

1. Introduction

The presence of nanoplastics (NPs) and microplastics (MPs) in consumer products, together with the fragmentation of the large amount of plastic reaching the environment, increases their occurrence in aquatic ecosystems and causes enormous concern about their potential toxic effects in aquatic environments (Jambeck et al., 2015; Hernandez et al., 2017; Mattsson et al., 2018; Tamayo-Belda et al., 2023). Detection of NPs in ecosystems remains a problem due to the absence of analytical methods to identify and quantify them (Cai et al., 2021; Wang et al., 2021a,b), and only few studies show data on the presence of NPs in environment. Particles between 1 and 1000 nm have been found in the North Atlantic Subtropical Gyre, but could not be quantified (Ter Halle et al., 2017). Nevertheless, Materić et al., 2022a,b,c were able to quantify MPs and NPs at ng/mL concentrations in Alpine Snow by thermal desorption mass spectrometry (TDS) and NPs were also detected in the Dutch Wadden Sea, in polar ice, and in rural and remote surface waters (Sweden and Siberian Arctic tundra) (Materić et al. 2022a, 2022b, 2022c).

Previous toxicological studies on the effect of NPs on producer and consumer organisms have described negative effects at all levels of the food chain. In primary producers (plants, microalgae, phytoplankton, and cyanobacteria), alterations in photosynthesis, increased reactive oxygen species (ROS), oxidative stress, membrane damage, and changes in gene and protein expression have been shown (Nolte et al., 2017; Rist et al., 2017; Frehland et al., 2020; Cao et al., 2022; Verdú et al., 2022). In addition, in primary or secondary consumers (i.e. zooplankton, fish) NPs produce mortality, body internalization and dissemination, bioaccumulation and effects on growth, reproduction, immune response and ROS production (Cui et al., 2017; Chae and An, 2017; Liu et al., 2020; Tamayo-Belda et al., 2023; Ramasamy and Palanisamy, 2021; Agathokleous et al., 2021; Martín-Folgar et al., 2023; Torres-Ruiz et al., 2021; Tamayo-Belda et al., 2023). However, information on the impact of NPs on freshwater organisms, especially benthic organisms, is limited and more studies are needed (Han et al., 2022). To further explore this topic, we set out to investigate on the effects of nanoparticles on fourth instar larvae of *C. riparius*. This organism is a model used in ecotoxicology (Armitage et al., 1995), and several assays have been developed to study chemical deleterious effects (OECD, 2011, 2010, 2004). The feeding behavior of C. *riparius* is mainly non-selective, so they could ingest NPs along with organic and inorganic matter and other contaminants (Armitage et al., 1995). The life cycle of *C. riparius* comprises different stages: embryo, larva, pupa, and adult (Armitage et al., 1995). Polystyrene (PS) is a plastic polymer used worldwide (Mathalon and Hill, 2014) and its commercial availability in carefully controlled shapes and sizes, make them a suitable and feasible option to study NPs toxicity. The target of this study was to provide information and evaluate on the early molecular effects (24 h of exposure) of PS NPs in *C. riparius*, focusing on the effect on DNA integrity and on the expression of different genes related to cell stress, oxidative stress, DNA damage, endocrine system, development, oxygen transport, and immune system. This is one of the first studies to evaluate a group of key regulatory genes directly related to the ecdysone pathway cascade. The results of this study will allow us to understand the mechanisms underlying NPs toxicity in an aquatic invertebrate consumer specie at the base of the food chain and provide scientific data for the development of biomarkers of early response to NPs.

2. Material and methods

2.1. Polysterene (PS) nanoplastic (NPs) preparation

Pristine polystyrene particles (30 nm) were acquired from Thermo Scientific™· NPs were provided as a 10% solution in water with <2% surfactant (SDS). NPs solutions were characterized with a Tecnai 12 FEC transmission electron microscope (TEM). The samples were sonicated for 5 min, then incubated on copper grids for 10 min, washed with MQH_2O and stained with uranyl acetate (2%) prior to examination. NPs characterization was performed and presented by our group in a previous paper (Martín- Folgar et al., 2023).

2.2. Animals and Polysterene (PS) nanoplastic (NPs) treatments

The model organism used was the fourth instar *C. riparius* larvae. The organisms were cultured following the guidelines indicated for conducting toxicity tests (OECD, 2010, 2011). Larvae from the midge *C. riparius* were grown in culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4) supplemented with nettle leaves, commercial fish food, and cellulose tissue (Martínez-Paz et al., 2012). Fourth instar larvae of *C. riparius* were treated with PS NPs for 24 h. The treatments lasted 24 h, and the culture medium was not changed. The PS NP stock solution was shaken and sonicated for 10 min before use and diluted in 50 mL of in *C. ripairus* culture medium to obtain the concentrations selected for exposures (0.1, 0.5 and 3 ppm. Cultures were maintained under constant aeration at 20 °C and under standard 16-h light–8-h dark cycle. Larvae were exposed in glass recipients (200 mL) and covered with aluminium foil to avoid photodecomposition. Each treatment consisted of three replicates, and three independent experiments were performed in each analysis using samples from different egg masses.

2.3. Toxicity test

Survival analysis of *C. riparius* larvae was performed at 24, 48, 72 and 96 h and PS NPs concentrations of 0.1, 0.5, 3, 5, 10 and 20 ppm were used to assess effects on mortality. When larvae did not move, they were considered dead.

2.4. RNA isolation

RNA extraction was carried out from *C. riparius* treated with 0.1, 0.5 and 3 ppm NPs and untreated control cells using a commercial kit (Trizol, Invitrogen) following the manufacturer's instructions. RNA treatment with RNase free DNase (Roche) following the method of Martín-Folgar et al. (2023) (Supplementary Material and methods). The quantity and quality of total RNA was controlled by absorbance (Biophotomer Eppendorf) and agarose electrophoresis. RNA was stored at −80 °C until use.

2.5. Complementary DNA synthesis

The cDNA was synthesized from 500 ng total RNA, 500 ng Oligo dT20 (Invitrogen), and 100 u/μL MMLV enzyme (Invitrogen, Germany) (Martín-Folgar et al., 2023) (Supplementary Material and methods).

2.6. Real-time polymerase chain reaction (RT-PCR)

From cDNA, real-time polymerase chain reaction (PCR) was

performed to analyze mRNA expression. For this purpose, SsoFast EvaGreen Supermix (BioRad, Hercules, CA, USA) was used in a CFX96 thermal cycler (BioRad, CA, USA) (Morales et al., 2020) (Supplementary Material and methods). The reference genes used in this study were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13 (rpL13). The oligonucleotides used are shown in (Supplementary Table S1).

2.7. The comet assay

The comet assay was carried out with 5 fourth instar larvae control and PS NPs treated following the method of Singh et al. (1988) modified according to Morales et al. (2013). Cells were stained with *SYBR*® Gold Staining Solution (Thermofisher) for 15 min and slides were observed under a Zeiss Axiophot photomicroscope equipped with an epifluorescence system and a Photometrics Cool Snap CCD camera. A total of 150 cells (50 cells from each of the three repeated slides) were scored. The images were analyzed with Image J software. The percentage of DNA in the tail and olive moment were used to assess DNA damage.

2.8. Statistical analysis

The mRNA expression levels of the different genes evaluated were normalized against the mRNA expression of the two reference genes (GAPDH and ribosomal protein L13). For the analysis of the percentage of DNA in the tail and olive moment, the values were normalized against the control. Statistical analyses were performed as in previous studies (Martín-Folgar et al., 2023; Morales et al., 2013). Three levels of significance are indicated: $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Statistical tests were performed using SPSS® 27.0.01 (SPSS Incorporated, Chicago IL, USA).

3. Results and discussion

3.1. Larval survival

Larval mortality was not found at PS NPs exposures of 0.1, 0.5, 3, 5, 10 and 20 ppm, with practically 90% survival rate in all treatments (data not shown). PS NPs exposures at environmental concentrations and short duration (24 h) were performed to analyze possible alterations in gene expression profiles in order to know the early response of genes related to different systems.

3.2. Stress response

Heat shock proteins (HSPs) work as chaperones in protein folding and unfolding. These proteins respond to cellular stress, they are also involved in other cellular processes (cell division, apoptosis, development, and differentiation) (Bakthisaran et al., 2015; Morrow et al., 2015). Some of these proteins are also antiapoptotic: HSP90, HSP70, and HSP27 (Joly et al., 2010; Wang et al., 2014) and can block the cell death process at different points (Bruey et al., 2000). In insects, HSPs are also implicated in some principal biological processes such as diapause, membrane fluidity, cell growth, apoptosis, differentiation, and starvation resistance (Arrigo, 1998). Since the expression of HSPs enzymes is essential in the maintenance of cellular homeostasis, these proteins have been suggested as biomarkers for environmental biomonitoring (Gupta et al., 2010). In this work, the response of stress genes (*hsp90*, *hsp70*, *hsp60*, and *hsp27*) after exposure to different concentrations of PS NPs for 24 h was analyzed. Our results showed an increase in PS NPs-induced *hsp70* mRNA expression that increased in a dose-dependent manner at 0.1 and 0.5 ppm and started to decrease expression at 3 ppm. In contrast, a trend for a decrease in the expression of *hsp90*, *hsp60,* and *hsp27* genes was observed, which was significant and dose-dependent in the case of *hsp90* (Fig. 1B). These results show that PS NPs alter the cellular stress response of NPs-treated *C. riparius*. HSP60 is a protein that localizes to the mitochondria (Zininga et al., 2018; Cappello et al., 2008; Lund et al., 2003). In addition, the antioxidant activity of HSP60 has been described, participating in the defense of cells against oxidative stress (Kaźmierczuk and Kiliańska, 2010). In this study, it has been observed that PS NPs tend to reduce the expression of this mitochondrial gene, this inhibition being statistically significant only at the concentration of 0.1 ppm. These results contrast with the up-regulation following exposure to MPs after 36 h exposure (Carrasco-Navarro et al., 2021) and agree with a recently published study showing that *hsp60* is inhibited in *C. riparius* larvae exposed to PS MPs for 24 h, but no changes of this gene are observed after exposures of 48 h and 72 h (Kalman et al., 2023). Clearly, the plastic particle size (micro vs nanoplastic) as well as the exposure regime are affecting this response.

On the other hand, the observed up-regulation of *hsp70* agrees with previous results in *Daphnia pulex* exposed to PS NPs (Liu et al., 2019). However, the Liu et al. (2019) study observed an upregulated *hsp90* expression in *Daphnia*, contrary to the inhibition observed in *C. riparius* in the present study. Our present results are also different from those found in zebrafish embryos (Zfe) exposed to PS NPs in which an inhibition of *hsp70* expression was observed, and the expression of *hsp27* and *hsp90* did not change (Martín-Folgar et al., 2023). It is thus clear that the response of these genes to NP exposure is different depending on the trophic level and the species studied. The activation of *hsp70* observed in *C. riparius* may be due to stress, induced by PS NPs and, in particular, to hider the stress-induced cell death process (Fig. 1A). Several studies established that overexpression of the human HSPB1 protein (equivalent to invertebrate's HSP70) can inhibit the intrinsic apoptotic pathway at various levels (Kennedy et al., 2014; Mosser and Martin, 1992; Mosser and Morimoto, 2004). Fig. 1A shows the possible interactions of HSP70 protein at various points in the apoptosis pathway, thereby altering induced cell death. Overexpression of this antiapoptotic protein suggests that exposure to PS NPs in the dipteran *C. riparius* induces an activation of apoptosis. It is certain that PS NPs modify the gene expression of these *hsps* in *C. riparius* and observed differences depending on species or trophic level indicate that further research is needed.

3.3. Oxidative stress response

Studies in vertebrates (Lu et al., 2016), invertebrates (Yu et al., 2018; Liu et al., 2020; Nogueira et al., 2022; Tamayo-Belda et al., 2023), algae (Bhattacharya et al., 2010), cells (Im et al., 2022; Poma et al., 2019), and bacteria (Sun et al., 2018) have shown that PS NPs stimulate the production of ROS. Elevated levels of ROS can cause lipid peroxidation, oxidative stress, membrane damage, mitochondrial damage, increased inflammatory cytokines, and induce pro-apoptotic factors that could induce apoptosis in mammals, thus affecting the organism (Lushchak, 2011; Banerjee and Shelver, 2021). ROS-mediated pathways are related to the mechanisms of toxicity produced by many xenobiotics or stress. Manganese superoxide dismutase (SODMn) is a protein that protects the cell from oxidative stress and helps maintain cellular balance (Wang et al., 2018; Livingstone, 2001). In this research, we analyzed the change in mRNA expression of *SODMn,* in *C. riparius* larvae exposed to PS NPs. The results obtained showed that *SODMn* gene expression is reduced at all three concentrations used, although only statistically significantly with the 0.5 ppm and 3 ppm exposures. The trend observed in all three cases suggests a dose-dependent inhibition, when compared to the control group (Fig. 2). This decrease in *SODMn* expression suggests a gradual depletion of this enzyme and, as a consequence, indicates the inability of *SODMn* to eliminate excess of superoxide radicals. This agrees with previous studies in Zfe treated with NPs in which *SODMn* expression levels decreased (Lu et al., 2018a,b; Zhang et al., 2020; Hu et al., 2020). Other studies suggest that excess ROS induced by PS NPs is a likely mechanism to cause cellular toxicity (Liu et al., 2020; Cao et al., 2022), and that a decrease in *SODMn* expression may reflect toxicity and depletion of the antioxidant response (Mu et al., 2016). Our results agree with those obtained in the insect

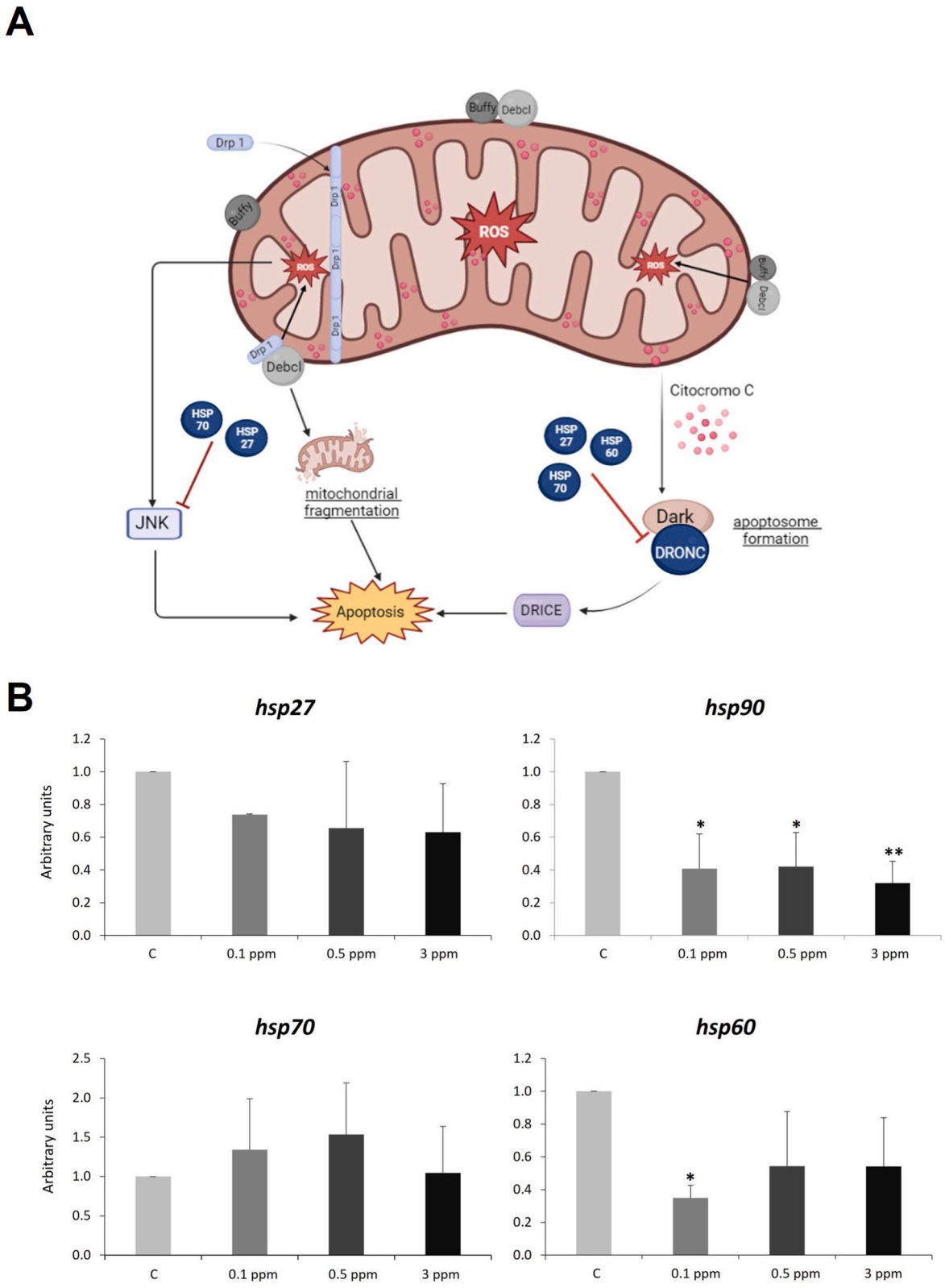

Fig. 1. A. Diagram of the possible actions of the antiapoptotic proteins (HSP70, HSP27, and HSP60) whose genes have been studied in the programmed cell death pathway. HSP27, HSP70, and HSP60 inhibit cell apoptosis at different points. *DRONC* gene is a caspase that regulates the activation of apoptosis (Khan et al., 2017). Genes evaluated are represented in blue. Biorender generated image. **B.** Expression of genes from stress response (*hsp27*, *hsp90*, *hsp70* and *hsp60*) in larvae after exposure to 0.1, 0.5, and 3 ppm PS NPs for 24 h mRNA levels were normalized using *GAPDH* and *rpL13* as reference genes. Significant differences: *$p < 0.05$ and **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

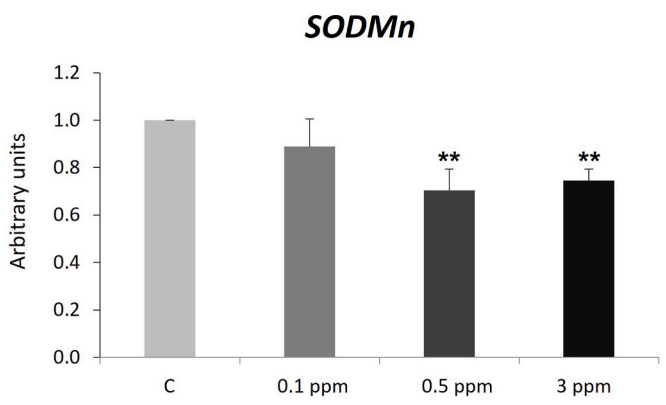

Fig. 2. Relative mRNA levels of *SODMn* larvae exposed to PS NPs (0.1, 0.5 and 3 ppm). Values were normalized against two reference genes (*GAPDH* and *rpL13*). Differences between PS NPs-treated and untreated samples were significant at $*p \leq 0.05$ and $**p \leq 0.01$.

Bombyx mori in which exposure with PS-NPs inhibited SOD expression (Muhammad et al., 2021). In contrast, other studies using vertebrates (Zfe) show up-regulation of other stress-protective proteins such as *SOD 1* and *SOD 2* gene expression after PS NPs exposure (Martín-Folgar et al., 2023). Further studies are needed to know the antioxidant effect of PS NPs on aquatic organisms and causes of these differences but it is clear that responses could vary with species.

3.4. DNA damage response

NPs can cause DNA damage, as has been observed in fish, human cells and this is thought to be related to the increased production of ROS demonstrated in fish and invertebrates (Gonçalves et al., 2022; Estrela et al., 2021; Guimarães et al., 2021; Brandts et al., 2018, 2021; Ballesteros et al., 2020; Gopinath et al., 2019). DNA damage can result from single nucleotide or single or double stranded modification (SSBs or DSBs). Cells respond to this by activating specific mechanisms of DNA damage response (DDR) (Fig. 3A). In this study, we have examined some of these mechanisms by analyzing the expression of four genes (*xrcc1*, A*TM*, *DECAY*, and *NLK*) involved in DNA repair. XRCC1 is a protein implicated in single-strand break (SSB) repair processes and cleaved sequence repair (Brem, 2005; Hanssen-Bauer et al., 2012). The *xrcc1* gene encodes for a base pair cleavage enzyme (BER). XRCC1 has an intimate relationship with poly (ADP-ribose) polymerase 1 (PARP1) activity and its critical role in the prevention of inherited neurodegenerative diseases (Caldecott, 2019). Ataxia telangiectasia mutated protein (ATM) belongs to the phosphatidylinositol 3-kinase family and is related in double-strand break (DSB) repair and it is also involved in chromatin remodeling, cell cycle, and metabolic regulation (Stracker et al., 2013). NLK protein belongs to the family of NEMO-like kinases (NLKs) (Ishitani and Ishitani, 2013) that is also implicated in DNA repair systems. This protein is required for p53 protein activation in response to genotoxic damage. NLK inhibition would lead to inactivation of p53, preventing the triggering of cell apoptosis, observed after exposure to NPs (Wang et al., 2014). DECAY protein is an effector caspase (class II) homologous to mammalian caspases 3 and 7. In *Drosophila*, this protein is implied in programmed cell death processes during development (Dorstyn et al., 1999). Our results revealed a significant inhibition of gene changes in *NLK* mRNA levels (Fig. 3B). In addition, there was a significant downward trend in *DECAY* expression at 0.3 ppm, while at 0.5 ppm there was no change in expression and at 3 ppm there was a downward trend, but not significant (Fig. 3B). This response with a wave-like trend may be the response of an inhibition at the lower concentration and a subsequent return to normal levels and so on, possibly due to compensatory mechanisms. In addition, a downward trend in *xrcc1* and *ATM* gene expression is observed, although it was not statistically significant (Fig. 3B). The pooled analysis of the results suggests that these DNA repair genes have reduced expression and therefore the cells do not respond to the damage caused by PS NPs under these conditions and time. These results are consistent with the decreased *ATM* expression observed in response to toxicants such as caffeine, and this inhibition is generally associated with genomic instability, immunodeficiency, and cancer susceptibility, among others (Weber and Ryan, 2015). Other studies have also shown inhibition of *xrcc1* in the presence of metals such as cadmium (Cd) or lead (Pb), thus impeding its function in BER repair mechanisms (Antoniali et al., 2015; Liu et al., 2018). Moreover, similar results were obtained in previous studies after exposure of *C. riparius* to carbon nanotubes (Martínez-Paz et al., 2019). On the other hand, even though inhibition of *NLK* and *DECAY* could suggest that apoptosis would not be able to be activated, the observed overexpression of the anti-apoptotic protein gene (*hsp70*) would indicate that this cell death process could be activated through other pathways.

3.5. Endocrine system

To evaluate the impact of PS NPs on the hormonal system of *C. riparius*, four genes associated to the endocrine system were chosen (Fig. 4A): ecdysone receptor (*EcR*), early ecdysone-inducible genes (*E74*, *E93*), death regulator Neddd2 like caspase (*DRONC*), and hormones that control insect metamorphosis, ecdysone (20E), which promotes molting, and juvenile hormone (JH), inhibiting morphogenesis. 20E is an ecdysteroid that controls ecdysis and metamorphosis in arthropods. The hormone binds to its heterodimer receptor, formed by the ecdysone receptor (EcR) and the ultraspiracle (USP) (Yamanaka et al., 2013) initiating the cascade of changes in gene expression responsible for insect development (Ashburner et al., 1974). The binding of 20E to its receptor (EcR/USP) activates the transcription of genes *E74, E75, E93,* and *broad* (Ashburner et al., 1974; Ashburner and Richards, 1976; Koelle et al., 1991; Karim and Thummel, 1992; Yao et al., 1993), which encode proteins that act as early response transcription factors to ecdysone, activating transcription of the late and subsequent response effector gene (*DRONC*) (Fig. 4A). *DRONC* gene is a caspase that regulates the activation of apoptosis in damaged cells in *Drosophila* (Khan et al., 2017). Changes in expression patterns triggered by ecdysone are responsible for most of the transformations that insects undergo during development (Yao et al., 1992, 1993).

In this work, a decrease in *EcR* gene expression is observed after 24 h of exposure with PS NPs in *C. riparius* (Fig. 4B). This affects the 20E signaling pathway, as shown by the results obtained leading to an inhibition of the expression of the early response genes to ecdysone (*E74* and *E93)* (Fig. 4B) and the late response gene (*DRONC*), which shows an impact on ecdysone synthesis. These genes were selected because of the particular relevance of the ecotoxicological level effects of endocrine disruptors (EDCs) and the lack of information on the mechanisms of action of NPs in invertebrate endocrine pathways. The expression of nuclear receptor genes are potential biomarkers, as they represent a key element in the development and physiology of these insects. The inhibition of the expression of *EcR* mRNA level was also reduced after 48 h exposure in *C. riparius* exposed to known endocrine disruptors such as benzyl butyl phthalate (BBP) (Herrero et al., 2015) and Bisphenol S (BPS) (Herrero et al., 2018), and in crustaceans exposed to BPS (In et al., 2019). Holometabolous (complete) metamorphosis includes the pupal stage, an intermediate stage between the fourth larval stage and the adult. In holometabolous species, Broad-Complex (BR-C) transcription factors determine the pupal stage and E93 stimulates BR-C expression in the prepupa (Belles, 2020). Alterations in the levels of these hormones, which are influenced by food intake, and contaminants, can produce effects on insect development and metamorphosis. As the larvae reaches the late juvenile stages, there is a drop in juvenile hormone levels and an increase in *E93*, at which point metamorphosis begins (Belles, 2020). The absence of *E93* gene expression shown in this study may be related to the continuation of juvenility. The statistically significant inhibition

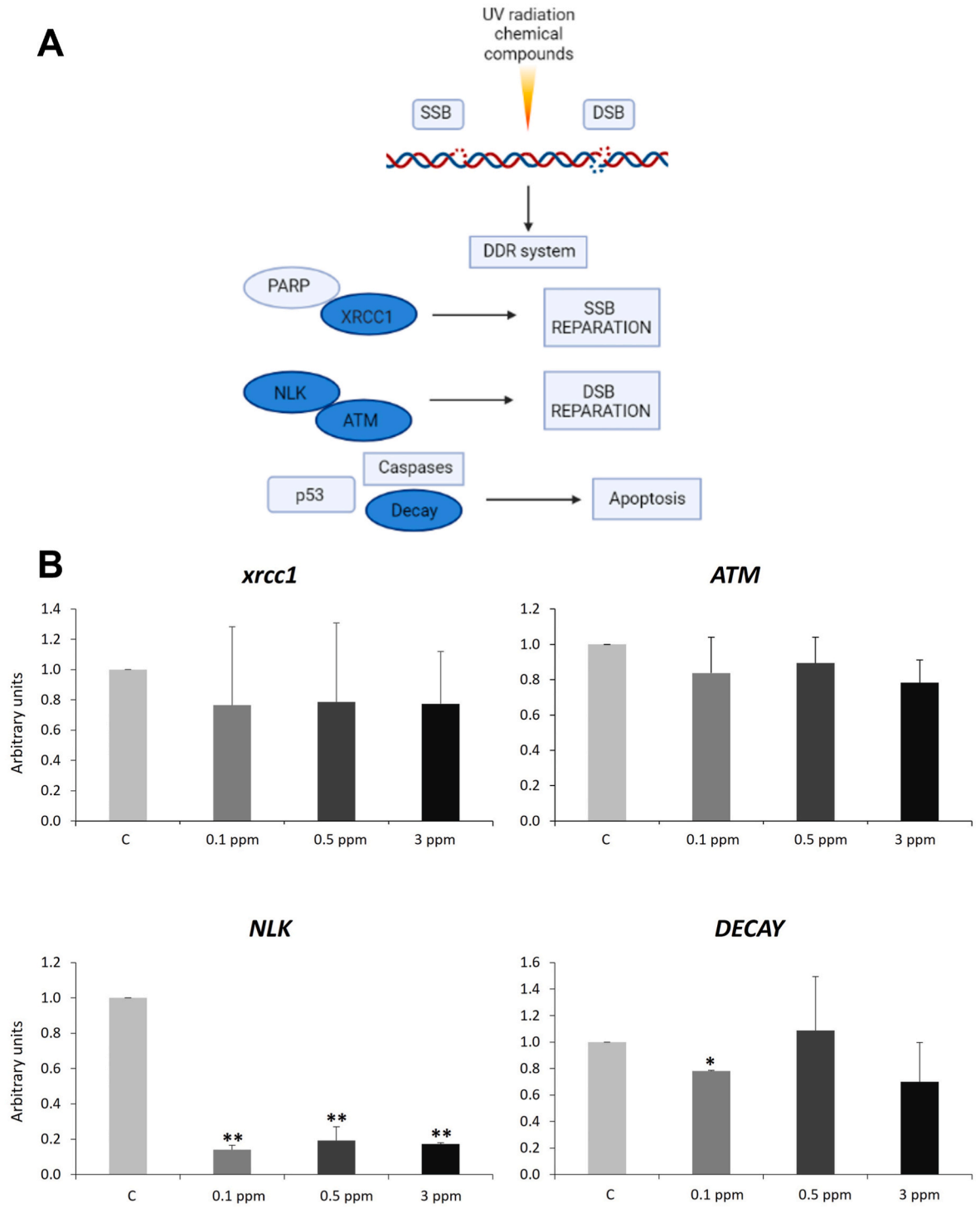

Fig. 3. A. Schematic representation of DNA repair response with genes evaluated on *C. riparius*. Biorender generated image. **B.** DNA repair expression genes (*xrcc1*, *ATM*, *NLK* and *DECAY*) on larvae after exposure to 0.1, 0.5, and 3 ppm PS NPs for 24 h. Differences between PS NPs control and treated samples were considered significant at * $p \leq 0.05$ and ** $p \leq 0.01$.

of *EcR* gene expression and the tendency to a decreased *E74* expression, with only the group of larvae treated at 0.5 ppm PS NPs being significant, could be preventing the activation of the ecdysone response cascade and thus delaying metamorphosis. On the other hand, a slight tendency to reduce *DRONC* expression at 3 ppm was observed. This is the first time that NPs are shown to reduce the *EcR*, *E74*, *E93,* and *DRONC* gene expression in an aquatic invertebrate. We believe that further studies are needed to understand the effect of NPs on the molting process.

3.6. Developmental responses and oxygen binding protein (hemoglobin)

FKBP39 is a protein implicated in growth and development (Li et al., 2007). Recent studies have found that two proteins Chd64 and FKBP39 have a key role in the interaction between juvenile hormone (JH) and 20E, although the molecular basis of this interaction is still unknown. These two proteins have been shown to bind to the JH response element (JHRE) and the nuclear EcR/USP receptor, suggesting that these two proteins are required for JH action and thus are involved in development

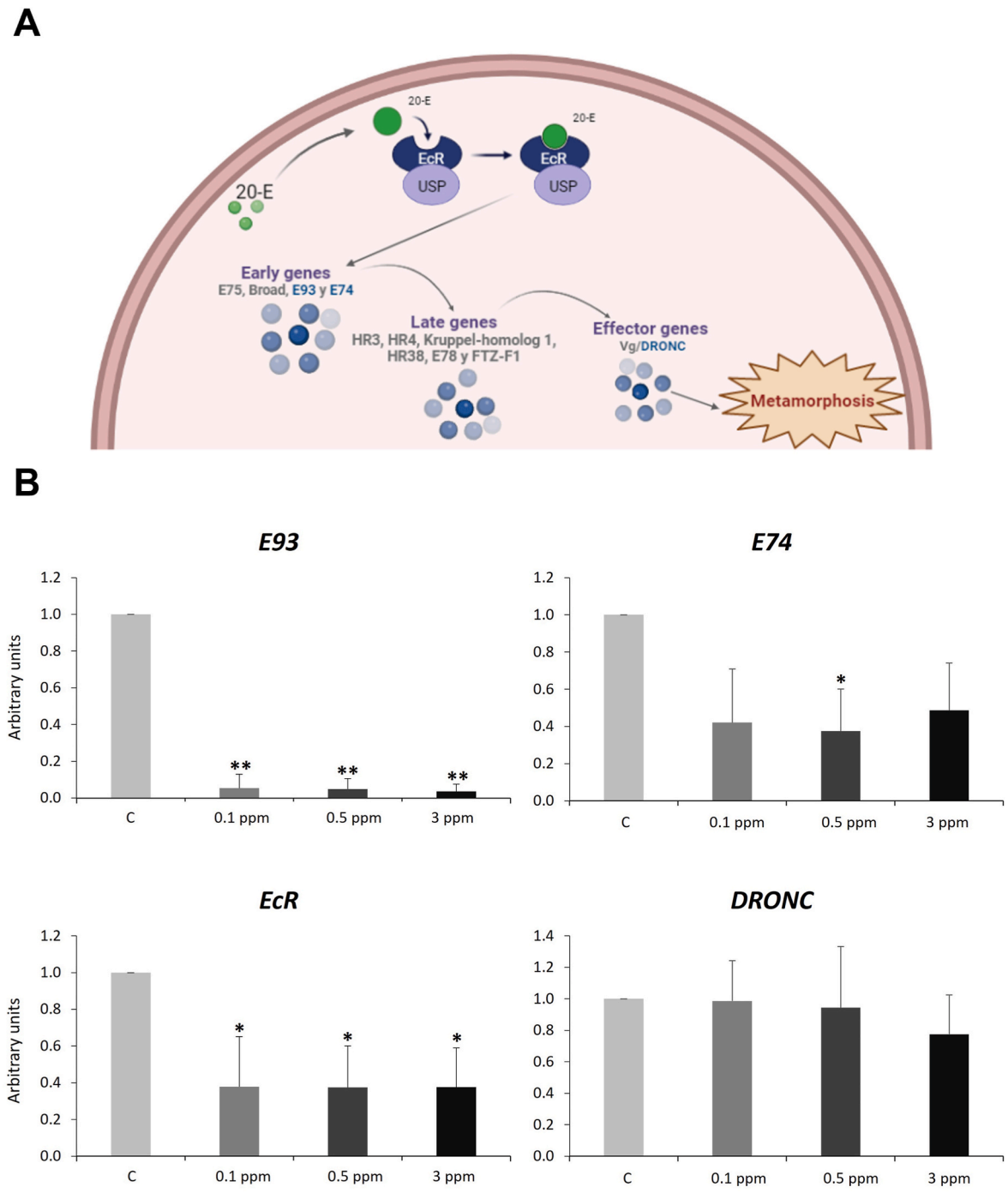

Fig. 4. A. Schematic representation of the ecdysone hormone pathway with the genes evaluated on *C. riparius*. Biorender generated image. **B.** Expression of endocrine system genes (*EcR, E74, E93 and DRONC*) in larvae exposed to 0.1, 0.5 and 3 ppm of PS NPs at 24 h. Differences between PS NPs samples were considered significant at * $p \leq 0.05$ and ** $p \leq 0.01$.

(Li et al., 2007). In addition, FKBP39 is involved in protein folding, receptor signaling, transcription regulation, and histone modification (Harikishore and Yoon, 2015) and shows an essential role in inhibiting autophagy by regulating the FOXO transcription factor pathway (insulin receptor pathway) (Juhász et al., 2007). Thus, for metamorphosis to take place, induction of autophagy must occur. Inhibition of *FKBP39* expression can prevent autophagy from occurring and delay metamorphosis.

In our study, we analyzed the expression changes of the *FKBP39* gene after exposure of *C. riparius* larvae to different concentrations of PS NPs. The results show an inhibition of the expression of this gene at all NPs concentrations studied (Fig. 5A). These results agree with previous work with PS MPs (Kalman et al., 2023) that also down-regulated *FKBP39* expression in *C. riparius* larvae, but contrast with another previous study with PS MPs that up-regulate *FKBP39* expression that could be due to particle size differences (Carrasco-Navarro et al., 2021). With this study we can conclude that NPs are altering the expression of a gene involved in the development and growth of *C. riparius*. Previous studies have shown that ingestion and persistence of polyethylene (PE) MPs cause alterations in the life cycle of *C. riparius*. It is possible that the PE particles in the gut of larvae can have adverse effects (Silva et al., 2019). There are also studies showing the effects of PE MPs on the development

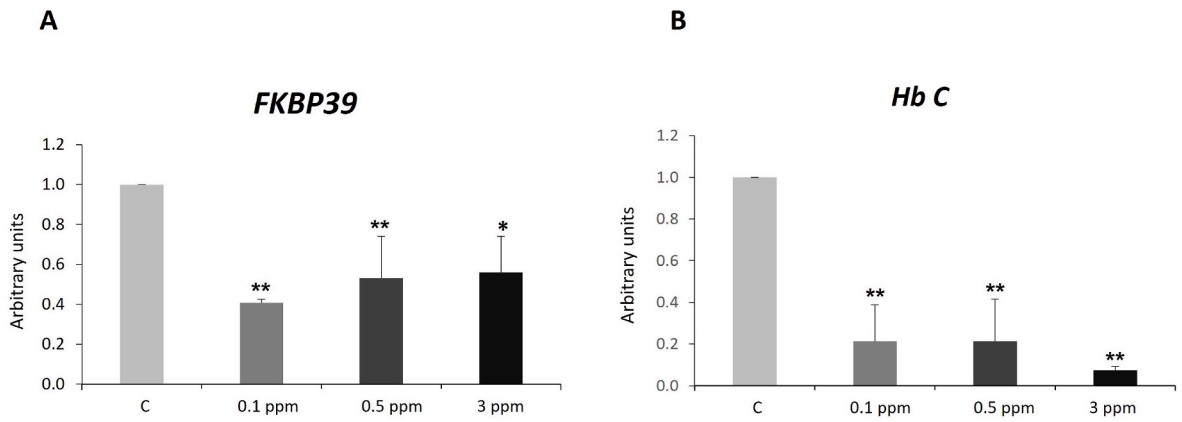

Fig. 5. Expression of *FKBP39* (A) and *Hemoglobin C* (Hb C) (B) in larvae after exposure to 0.1, 0.5, and 3 ppm PS NPs at 24 h. Differences were considered significant: $*p \leq 0.05$ and $**p \leq 0.01$.

and emergence of *C. tepperi* (Ziajahromi et al., 2018). In this sense, PS NPs seem to inhibit the expression of *FKBP39* and this could affect the metamorphosis of *C. riparius*. Recently, this *FKBP39* gene is beginning to be used in response to nanomaterials in the aquatic invertebrate *C. riparius* (Martín-Folgar et al., 2023; Kalman et al., 2023; Carrasco-Navarro et al., 2021). The results obtained expand a range of different particles, different sizes, and concentrations suggesting that different micro/nano particles in the environment could have important effects on metamorphosis. This clearly requires further research as it could have a wide effect on populations and ecosystems exposed to nanomaterials.

Hemoglobin (Hb) is an oxygen binding protein that increases the oxygen carrying capacity of hemolymph, having an elevated level of polymorphism (Osmulski and Leyko, 1986; Weber and Vinogradov, 2001). The Hbs in invertebrates reveals their adaptation to hostile environmental conditions, specifically in *Chironomus*, these proteins have physiological functions in O_2 transport and storage in larvae living in contaminated and hypoxic sediments (Osmulski and Leyko, 1986; Walshe, 1951; Weber, 1965) and in the uptake of O_2 from water. Under hypoxic conditions they may maintain aerobic metabolism. Hbs are synthesized in the larval fat body (Saffarini et al., 1991; Bergtrom et al., 1976). Despite its peculiar characteristics, there are no toxicological studies focusing on *Chironomus* Hbs as a sublethal endpoint (Choi and Roche, 2004). We propose that the expression of Hb genes can be considered as environmental biomarkers in *Chironomus*. In this study, *Hb C* expression in larvae exposed to PS NPs was inhibited in a dose-dependent manner (Fig. 5B). Similar results were obtained with silver nanoparticles (AgNPs) that induced down-regulated expression of *Hb* genes in Zfe (Cui et al., 2016). These results suggest that PS NPs block transcription of *Hb C*.

3.7. Immune system

The immune system of mammals and insects is based on different responses that are activated sequentially. These organisms share physicochemical barriers, innate response, and sensitization of the immune system. Insects have a highly developed innate response, but do not have an acquired immune system. The innate immune response is activated when the antigen crosses these physicochemical barriers. Innate immunity is divided into humoral and cellular defenses (Strand, 2008). In invertebrates, the humoral response is the main one and its activity consists of eliminating nodules by melanization to eliminate invading microorganisms (Marmaras et al., 1996; Marmaras and Lampropoulou, 2009; Nappi and Christensen, 2005). This process is triggered by activation of the prophenoloxidase (ProPO) system. The ProPO enzyme requires proteolytic activation carried out by proPO-activating protease (PAP). PAP is found in an inactive form (pro-PAP). When required for the melanization process, an immunoprotein is synthesized that mediates PAP activation (Satyavathi et al., 2014) (Fig. 6A). PAP activates ProPO and a cascade is initiated that ends with melanin production (Sheehan et al., 2018) (Fig. 6A). We examined a gene related to the humoral immune response, ProPO. We observed an increase in ProPO expression after exposure to different concentrations of PS NPs (Fig. 6B).

These results agree with results with *C. riparius* larvae exposed to MPs in which activation of the innate immune system of these organisms was demonstrated. Ingestion and retention of MPs in the gut of

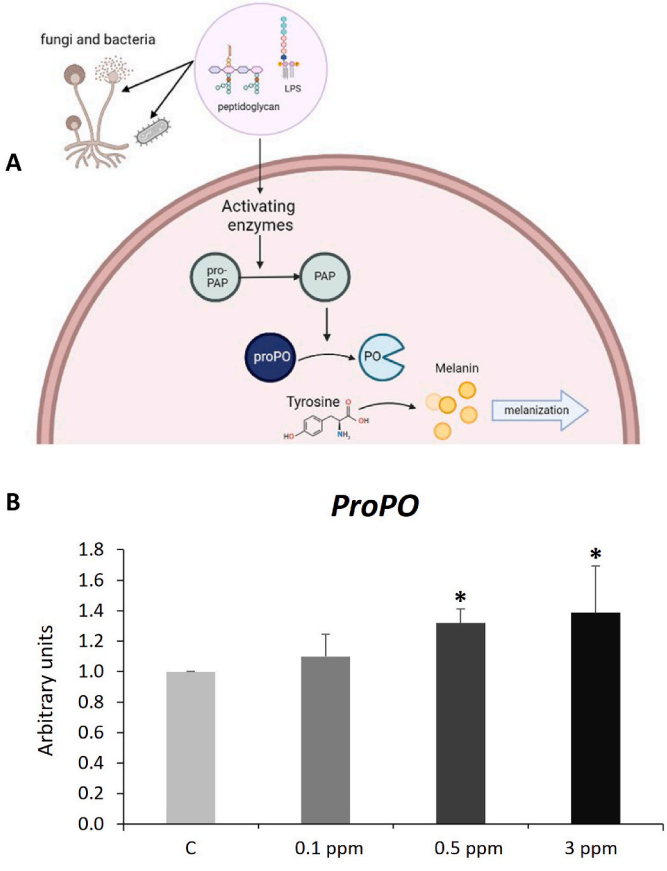

Fig. 6. A. Schematic of the immune pathway (humoral response) and the gene evaluated in *C. riparius* after exposure to PS NPs. Biorender generated image. **B.** Expression of gene from the immune system *PropO* in this invertebrate after exposure to 0.1, 0.5, and 3 ppm NPs at 24 h. Differences between PS NPs treated and untreated samples were considered significant at $* p \leq 0.05$ and $**p \leq 0.01$.

C. riparius larvae activated the immune response through activation of the phenoloxidase (PO) system (Silva et al., 2021). Previous studies, suggest that PO activation and melanization processes are related to the production of ROS by hemocytes, oxidative stress, and increased energy expenditure by the immune system (Sadd and Siva-Jothy, 2006; González-Santoyo and Córdoba-Aguilar, 2012; 423 Dolezal et al., 2019). The modification of the immune system, energy metabolism, and antioxidant defenses induced by nano/microparticles could make invertebrates more susceptible to microorganisms and xenobiotics, endangering populations in aquatic ecosystems (Silva et al., 2021). Our results show for the first time that nanoplastics affect the immune system of *C. riparius*. This result is in agreement with a study in shrimp (*Macrobrachium nipponense* and *Litopenaeus vannamei*) exposed to MPs (Liu et al., 2020; Wang et al., 2021a,b), in intertidal mud crab (*Macrophthalmus japonicus*) after exposure to plasticizers: Bisphenol A and di (2-etilhexil) phtalate (DEHP) (Park et al., 2019), in *C. riparius* after exposure to benzophenone-3 (Muñiz-González and Martínez-Guitarte, 2020) and ibuprofen (Muñiz-González, 2021), and in crabs after exposure to herbicide (glyphosate) (Hong et al., 2019). Alternatively, it is possible that the NPs "confuse" the immune system of *C. riparius* and that this organism responds as if it were being infected by a microorganism. This result highlights the relevance of the invertebrate immune system and its response to external attacks. ProPo gene is postulated as a good biomarker of expression to evaluate the effects of micro- and nanomaterials in plastics and plastic products.

3.8. PS NPs genotoxicity

Genotoxic damage produced by PS NPs in larvae of *C. riparius* was evaluated using the comet assay (single cell gel electrophoresis). This assay was performed under alkaline conditions, as it allows the detection of single and double stranded DNA breaks, incomplete repair activity, and alkali labile sites (Singh et al., 1988). Two parameters obtained after the comet assay, the percentage of DNA in the tail and the olive moment, were selected for damage quantification. The percentage of DNA in the tail provides information about the total DNA damage produced by the genotoxic compound whereas the olive moment relates to total damage to the average fragment size (Martínez-Paz et al., 2014). As shown in Supplementary Table S2, statistically significant differences were found in all the comet parameters measured: % DNA in tail and olive moment. The results showed a statistically significant differences in the % DNA in tail and olive moment at all the concentrations (Fig. 7). Our results demonstrate that PS NPs are able to cause DNA damage in the insect nuclear genome, in terms of strand breaks and alkali labile sites. DNA strand breaks induced by PS NPs exposure are sensitive markers of genotoxicity damage. These results are in accordance with previous work with fish (Guimarães et al., 2021) and cells (Cortés et al., 2020). In addition, previous studies with other species exposed to NPs have also shown genotoxicity through the comet assay in whole organisms such as the marine mussel (Gonçalves et al., 2022), *Drosophila melanogaster* (Turna Demir et al., 2022), and in human cell lines (Roursgaard et al., 2022). Genotoxicity is one of the most important toxicity parameters when assessing environmental risks. Our results provide valuable information on the genotoxic effects of PS NPs on arthropods. The data support the need for studies on the genotoxicity of contaminants, which allow us to relate genotoxic activity to other biological responses. This information gives us a better understanding of the adverse effects in aquatic environments.

4. Conclusions

For the first time, the potential effects of PS NPs on the transcription of genes involved in different pathways and their genotoxic effect in an aquatic organism *C. riparius*, were analyzed. In the present study, significant changes in the transcription of genes related to cellular stress, oxidative stress, DNA damage, ecdysone pathway, development, oxygen transport, and immunity were observed in *C. riparius* larvae exposed to environmentally relevant concentrations of PS NPs and short exposure time.

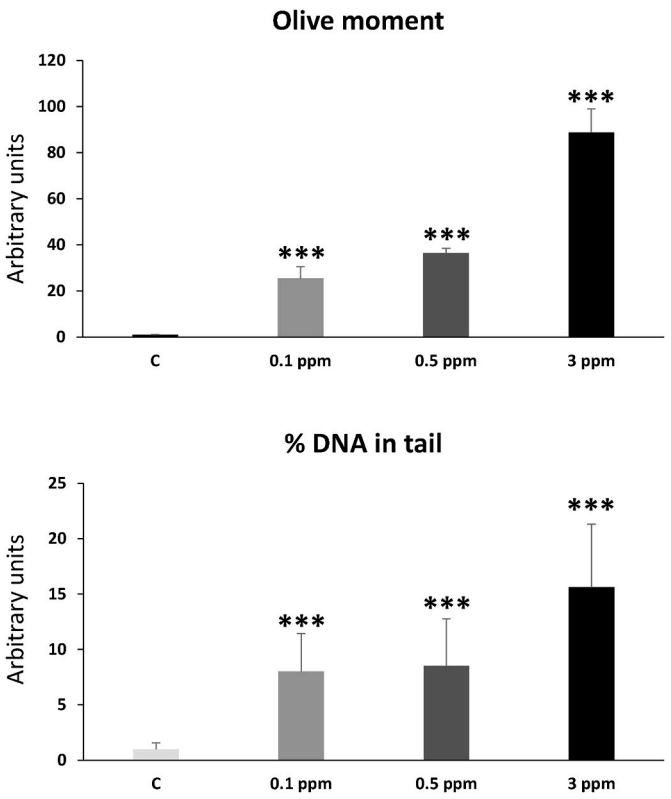

Fig. 7. DNA damage measured by Tail DNA% (A) and Olive moment (B) values in *C. riparius* larvae exposed to PS NPs. Differences between PS NPs samples were considered significant at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The decrease in the expression of genes involved in cellular stress (*hsp90, hsp60,* and *hsp27*) suggests problems in the stress response, in protein folding, in the apoptotic response, etc.; and the activation of *hsp70* suggests a stress situation, as a consequence of the NPs, or an anti-apoptosis response. The marked response of the four *hsps* studied postulates them as excellent biomarkers of NPs exposure.

PS NPs produce a marked genotoxic effect that has been corroborated by the comet assay, possibly due to the inhibition of the antioxidant response (*SODMn* gene) and the downward trend in the expression of genes involved in DNA repair (*xrcc1, ATM, DECAY,* and *NLK*). Taken together these results indicate that PS NPs produce a strong genotoxic effect in *C. riparius*.

The modified expression of endocrine (*EcR, E74, E93*) and developmental (*FKBP39*) genes suggests that development, metamorphosis or even reproduction could be perturbed in *C. riparius* by PS NPs. In addition, the NPs affect the expression of the *Hb C* gene, a protein involved in oxygen transport, which raises questions about its mechanism of action, but makes this gene a possible biomarker of NP toxicity. Ultimately, activation of the *ProPO* gene reveals an effect of NPs on the humoral immune response in *C. riparius*.

The data from this study highlight the risk of PS NPs in aquatic invertebrates, specifically in *C. riparius*, and the need to further evaluate their effects at the molecular and cellular level by including more avenues of study that will allow us to know exactly how these nanomaterials, present in aquatic ecosystems, exert their toxic effects and their possible transgenerational effects.

Credit author statement

Raquel Martín Folgar: Conceptualization, Analysis, Research,

Writing - review and editing Resources. Celia Sabroso: Methodology and Analysis. Mónica Torres-Ruiz: Methodology, Research, Writing - review and Characterization of carbon nanomaterials. Ana I. Cañas-Portilla: Methodology, Writing – review and Supervision. M[a] Carmen González: Methodology, Analysis, Research. Helena Dorado. Methodology and Analysis. Nacho Velasco: Methodology and Analysis. Monica Morales: Conceptualization, Methodology, Writing of original draft, Preparation, Supervision.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140552.

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