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Orally administered nano-polystyrene caused vitellogenin alteration and oxidative stress in the red swamp crayfish (*Procambarus clarkii*)

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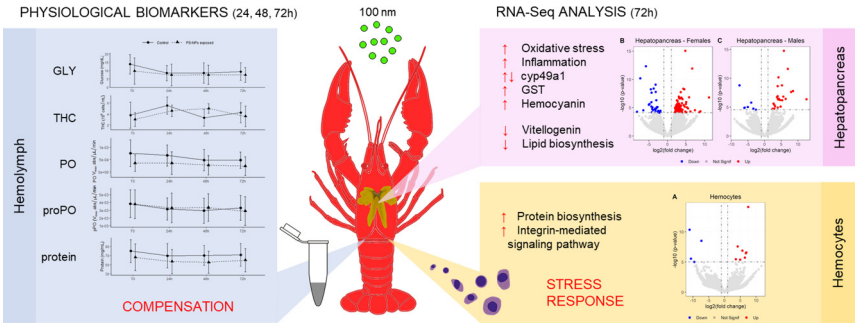
HIGHLIGHTS

- Crayfish did not exceed generic physiological stress thresholds.
- Few key differentially expressed genes were found in hemocytes and hepatopancreas.
- NPs induced oxidative stress in the hepatopancreas of *P. clarkii*.
- Vitellogenin expression was downregulated in female crayfish.
- RNA-Seq is a powerful tool in ecotoxicological studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanoplastics (≤100 nm) represent the smallest fraction of plastic litter and may result in the aquatic environment as degradation products of larger plastic material. To date, few studies focused on the interactions of micro- and nanoplastics with freshwater Decapoda. The red swamp crayfish (*Procambarus clarkii*, Girard, 1852) is an invasive species able to tolerate highly perturbed environments. As a benthic opportunistic feeder, this species may be susceptible to plastic ingestion. In this study, adult *P. clarkii*, at intermolt stage, were exposed to 100 μg of 100 nm carboxylated polystyrene nanoparticles (PS NPs) through diet in a 72 h acute toxicity test. An integrated approach was conceived to assess the biological effects of PS NPs, by analyzing both transcriptomic and physiological responses. Total hemocyte counts, basal and total phenoloxidase activities, glycemia and total protein concentration were investigated in crayfish hemolymph at 0 h, 24 h, 48 h and 72 h from PS NPs administration to evaluate general stress response over time. Differentially expressed genes (DEGs) in the hemocytes and hepatopancreas were analyzed to ascertain the response of crayfish to PS NP challenge after 72 h. At a physiological level, crayfish were able to compensate for the induced stress, not exceeding generic stress thresholds. The RNA-Sequencing analysis revealed the altered expression of few genes involved in immune response, oxidative stress, gene transcription and translation, protein degradation, lipid metabolism, oxygen demand, and reproduction after PS NPs exposure. This study suggests that a low concentration of PS NPs may induce mild stress in crayfish, and sheds light on molecular pathways possibly involved in nanoplastic toxicity.

1. Introduction

In 2019, global plastics production almost reached 370 million tons (PlasticsEurope, 2020). Plastic pollution has been recognized as a severe human pressure on aquatic ecosystems and a major water quality problem (Koelmans et al., 2015; Rochman et al., 2013; Wagner et al., 2014). To date, the bulk of micro- (MP) and nanoplastic (NP) research efforts

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have been focused on the marine environment, with global oceans considered as the ultimate sink for contamination (Eerkes-Medrano et al., 2015; Lambert and Wagner, 2018; Wagner et al., 2014). Nevertheless, given that most of the plastic is used and disposed of on land, both terrestrial and freshwater environments can be subject to plastic pollution, including then act as long-term reservoirs (Horton et al., 2017). Horton and colleagues assessed that in the EU between 473,000 and 910,000 metric tons of plastic waste are released and retained annually within continental environments, which is 4 to 23 times the amount estimated to be released to the ocean (Horton et al., 2017). Several recent monitoring studies have established that MPs are ubiquitously found in waters and shore sediments of rivers and lakes all over the world (Atwood et al., 2019; Hurley et al., 2018; Lambert and Wagner, 2018; Lebreton et al., 2017; Leslie et al., 2017; Mani et al., 2015; Piehl et al., 2019; Sighicelli et al., 2018). Therefore, understanding MPs and NPs dynamics in terrestrial and freshwater systems seems of critical importance to allow for a more comprehensive assessment of hazards and risks posed by these pollutants to ecosystems (Hurley and Nizzetto, 2018; Ter Halle et al., 2017). NPs represent the smallest fraction of plastic litter and are referred to as particles under 1 μm (da Costa et al., 2016; Gigault et al., 2018) or 100 nm (Alimi et al., 2018; Besseling et al., 2019; EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2016; Koelmans et al., 2015) in size. NPs may be directly released in the environment from domestic products (Hernandez et al., 2019, 2017), as well as from the breakdown of larger plastic items (Ekvall et al., 2019; Gigault et al., 2016; Lambert and Wagner, 2016; Wahl et al., 2021). To date, no established protocols for the quantification of NPs in wild environmental samples are available (Pinto da Costa et al., 2019; Renner et al., 2018). Nonetheless, NPs numeric abundance is predicted to potentially become 17 orders of magnitude higher than microplastic particle concentrations (Besseling et al., 2019). These discoveries caused concern about the impacts of plastic particles on freshwater biota. Ecological effects of NPs on freshwater organisms have been evaluated across several levels of organization, from infra-organismic responses, such as oxidative stress (Jeong et al., 2016), altered lipid metabolism (Cedervall et al., 2012; Li et al., 2020b), and mobilization (Auclair et al., 2020) and glucose metabolism (Brun et al., 2019), to organismic endpoints like mortality, impaired reproductive capacity (Jeong et al., 2016; W. Zhang et al., 2020), histopathological and behavioral changes (Brun et al., 2019; Cedervall et al., 2012; Chae et al., 2018), which then potentially affect population dynamics. A rapidly growing body of "omics" data on MPs and NPs is available in literature, aiming at providing a detailed description of the cellular pathways underlying plastic particles detoxification (Gu et al., 2020; W. Liu et al., 2020; Liu et al., 2021; Magni et al., 2019; W. Zhang et al., 2020). To date, only few studies focused on decapod crustaceans, despite their ecological and economic relevance. These investigations suggested that decapods may be able to fragment MPs into nanosized particles through digestion, and highlighted NP effects on survival, growth, molting, nutrition values and energy metabolism, immunity and antioxidant defense (Bergami et al., 2020; Dawson et al., 2018; Li et al., 2020b, 2020a; P. Yu et al., 2018).

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) is a benthic freshwater crustacean widely distributed all over the world. Native to Mexico and South-Central America, this species has been extensively harvested since the 1950s and successfully translocated all over the world for aquaculture purposes (Gherardi, 2006; Hobbs et al., 2008; Manfrin et al., 2019). As a result of these translocations, today *P. clarkii* is the most cosmopolitan crayfish (Gherardi, 2006). Because of its high environmental tolerance, this species has been used for a long time as a bioindicator of environmental pollution from heavy metals (Fernández-Cisnal et al., 2018, 2017; Gherardi et al., 2002; Goretti et al., 2016; Osuna-Jiménez et al., 2014; Y. Zhang et al., 2019), cyanotoxins (Tricarico et al., 2008) and organic compounds (Vioque-Fernández et al., 2007a). Being a benthic opportunistic feeder, showing a pronounced borrowing activity this species may be susceptible to plastic ingestion from soil (Lv et al., 2019; D. Zhang et al., 2020).

In this study, common biochemical and cellular measures of crustacean condition, including hemolymph glycemia (Lorenzon, 2005; Manfrin et al., 2016) and total protein content, total hemocyte count (THC, Giulianini et al., 2007), as well as immune-related enzymes (prophenoloxidase proPO, and phenoloxidase PO) activities (Cerenius and Söderhäll, 2004) were assayed to estimate the immunological toxicity of polystyrene (PS) NPs on *P. clarkii*. Furthermore, transcriptome sequencing was performed on crayfish hemocyte and hepatopancreas samples following NP exposure for 72 h. This integrated approach allowed us to figure out at the same time if crayfish were able to maintain homeostasis and which specific pathways were involved in the compensatory mechanisms. This study will be useful to shed light on the potential effects of plastic nanoparticles on crayfish.

2. Materials and methods

2.1. Animal collection and housing conditions

Adult crayfish *Procambarus clarkii* (wet weight: 32.2 ± 15.5 g; total length, from the tip of the rostrum to the tip of telson: 102.2 ± 13.9 mm, $N = 38$) were sampled in Brancolo Channel ("Brancolo's reclamation area", 45°46′N, 13°30′E, GO, Italy) in October 2018. They were acclimatized for one month in three 120 L glass aquaria (120 × 40 × 50 cm) provided with closed-circuit filtered and thoroughly aerated tap water (pH 8.35 ± 0.1, electric conductivity 305 ± 2.1 μS, and temperature 21.0 ± 1.0 °C). Polyvinyl chloride tubings were placed within each tank as shelters. The photoperiod was set to 12:12 (L:D) and individuals were fed daily ad libitum with commercial food (Sera granular, Heisenberg, Germany). Water was completely changed twice a week. Only apparently healthy crayfish were selected for the experiment. Specimens were starved for 24 h prior to the start of the experiment to empty their digestive systems.

2.2. Polystyrene nanoplastics characterization

Yellow-Green fluorescent polystyrene nanoparticles (Fluoresbrite® YG Carboxylate Microspheres, 0.10 μm) with a density of 1.05 g/cm^3 were purchased from Polysciences (Polysciences Inc., Warrington, PA), supplied as a 2.5% aqueous suspension with a concentration of 4.55 × 10^{13} particles/mL. For particle characterization, a 2 mg/L solution in MilliQ water was prepared. Primary particle diameter identification was achieved by transmission electron microscopy (TEM, EM 208, Philips, Eindhoven, The Netherlands). Intensity-weighted size distribution and hydrodynamic diameter (*Z*-average), as well as polydispersity index (PdI), were acquired by Dynamic Light Scattering (DLS) using a Malvern Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Measurements were conducted at 26.5 °C and performed in triplicate.

2.3. Nanoplastic-supplemented food

Artificial agar-based food was prepared with a specified amount of artificial food (Sera granular, Heisenberg, Germany) and a supplement of nanoparticles. The standard food preparation contained one regular granule (6.3 ± 0.7 mg) of artificial food, which was suspended in 400 μL of MilliQ water in a 1.5 Eppendorf tube. After vortexing, the supernatant was removed. 4 μL of nanoplastic solution 2.5% solids (1.82e^{11} particles) were added to the rehydrated granules, which correspond to 100 μg PS NPs accounting for a 1.6 ± 0.2% of food dry weight (the agar content was not considered part of the diet). A solution of agar 3× (25–30 μL for each feeding unit, Amresco, Solon, OH, USA) was added as a thickener. The mixture was then dried at room temperature overnight to obtain small pellets. One single artificial granule was administered to each exposed crayfish at the beginning of the experiment. The dose of 100 μg (equivalent to 1.4 × 10^{11} particles/L) was selected considering a polluted freshwater environment scenario, that possibly record microplastic concentrations up to 10^2 particles/L

(Triebskorn et al., 2019). As suggested by Besseling et al. (2019), nanofragmentation of MPs can ultimately result in nanoparticle concentrations of 14 orders of magnitude higher. Additionally, the relatively low tested dose of 100 μg was chosen to evaluate the sub-lethal effects of PS NPs on *P. clarkii*, based on acute toxicity thresholds available in literature on other crustacean species (Heinlaan et al., 2020; Li et al., 2020a; Liu et al., 2019b).

2.4. Feeding assay

A total of 24 intact *P. clarkii* (12 females and 12 males) with an average body weight of 32.2 ± 11.0 g and a mean total length of 106 ± 10.5 mm were selected for the experiment. Crayfish were randomly divided into exposed ($N = 12$) and control (N = 12) groups. Two custom-made glass thanks filled with 4 L tap water were used for the experiment, one for each group (Fig. S1). Aquaria were provided with three almost entirely separated cells (12 × 14 × 15 cm) to keep individuals isolated and prevent aggressive behaviors. During the 72 h experiment, water recirculated in a closed loop but was not replaced, and the experimental conditions were kept stable with a photoperiod of 12:12 (L:D) and a temperature of 21 °C. Crayfish were fed once at T0 (0 h) with one pellet of normal (control group) or nanoplastic spiked (exposed group) food, with no additional feeding provided for the rest of the trial. After 72 h crayfish were anesthetized by hypothermia and sacrificed. Four replicates of the experiment were conducted. Four individuals (2 control and 2 exposed animals) from the fourth replica were excluded from subsequent analysis, as they escaped from exposure tanks.

2.5. Samples collection

Hemolymph was withdrawn from exposed and control animals by pericardial cavity puncture with a 1-ml syringe (26-gauge needle) before the exposure (as baseline sample, T0) and also at 24 (T1), 48 (T2), 72 (T3) hours after the food pellets provision. Hemolymph samples were taken between 10 and 12 AM at about the same time each day to compare the same circadian status for all the experimental crayfish. Approximately 200 μL of hemolymph from each animal were collected in a sterile Eppendorf tube without using anticoagulant and promptly placed on ice. Plasma was isolated from hemolymph through centrifugation (10,000 ×*g* for 1 min at 4 °C) and immediately stored at −20 °C for subsequent analysis. For transcriptomic analysis, the remaining hemocyte pellet after plasma isolation at T3 sampling time was saved and stored at −80 °C in 200–500 μL of TRIzol RNA isolation solution (Invitrogen, Thermo Fisher Scientific, Inc.). At the end of the experiment, crayfish were dissected, hepatopancreas samples were collected and immediately stored in 300 μL of TRIzol at −80 °C for RNA extraction.

2.6. General stress parameters

2.6.1. Glycemia

Plasma glucose was determined using the Glucose Colorimetric Assay Kit (Catalog No. 10009582; Cayman Chemical, Ann Arbor, MI, USA). Standard curves were prepared at concentrations of 0, 2.5, 5, 7.5, 10, 15, 20 and 25 mg/dL of glucose following manufacturer's instructions. Standard curves R^2 values were of 0.9985 or greater. Standard and sample absorbances were measured at 510 nm in an Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland) equipped with the software Tecan i-control (version 1.7.1.12). Absorbance values were corrected by subtracting measurements from control reactions without sample. Glucose levels were interpolated from standard curves and reported as mg/dL. All standards and samples were assayed in duplicate.

2.6.2. Total hemocyte count

After collection, a drop of hemolymph was immediately placed on a hemocytometer for total hemocyte count (THC) assay. For each time point (0, 24 h, 48 h, 72 h), all crayfish were checked. The number of hemocytes was determined using a Bürker counting chamber placed under an Olympus BX50 microscope (Olympus, Tokyo, Japan). Each sample was photographed at 10× magnification using an Olympus DP12 camera (Olympus, Tokyo, Japan). The pictures were then uploaded in the free software ImageJ (more information available at http://rsb.info.nih.gov/ij/) implemented with the Cell Counter plug-in allowing for the manual cell counting and hemocyte quantification.

2.6.3. Basal and total plasmatic phenoloxidase activities

Phenoloxidase (PO) activity was monitored spectrophotometrically as the formation of dopachrome from 3,4-dihydroxyDL-phenylalanine (DL-DOPA, Sigma-Aldrich) as reported previously by Giglio et al. (2018), with minor modifications. Briefly, 20 μL of plasma were mixed with either 180 μL of DL-DOPA (3 mg/mL in PBS) or 180 μL of a solution of DL-DOPA (3 mg/mL in PBS) and SDS (1 mg/mL) in a microtiter plate (Biorad), for the determination of basal and total plasmatic PO (proPO) enzyme activity, respectively. SDS has been described as a good chemical activator of PO from its inactive zymogen, prophenoloxidase (proPO, Radha et al., 2013). Absorbance was measured kinetically at 25 °C at 492 nm over 60 min at 5-min intervals in an Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland) equipped with the software Tecan i-control (version 1.7.1.12). Four technical repetitions were performed for each plasma sample. The enzyme activity was measured as the slope (absorbance vs time) of the reaction curve during the linear phase of the reaction. Absorbance values were blank (reagents) subtracted. The slope of the reaction curve at V_{max} was plotted as absorbance per μL of hemolymph per min.

2.6.4. Hemolymph protein concentration

Total Protein content was assessed in plasma samples. The analysis was performed by measuring samples absorbance at 280 nm following the Protein A280 method for NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, DE, USA).

2.7. Transcriptomic analysis

2.7.1. Total RNA isolation

Total RNA was extracted from *P. clarkii* hemocytes and hepatopancreas. Tissues in TRIzol were mechanically homogenized using a Dremel homogenizer (Dremel® 300-1/55, USA) for about 2–3 min or a Mini-Beadbeater (BioSpec Products, Bartlesville, Oklahoma) using glass beads (0.5 mm diameter, Scientific Industries Inc., Bohemia, New York) for hepatopancreas and hemocyte samples, respectively. RNA purification was performed using a Direct-zol™ RNA MiniPrep (Catalog No. R2052; Zymo Research, Irvine, CA, USA) spin column system according to the manufacturer instructions with minor modifications. RNA quality and concentration were assessed with NanoDrop™ 2000 Spectrophotometer (Thermo Scientific; Thermo Fisher Scientific Inc.), agarose gel electrophoresis and Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) using the Qubit® RNA HS Assay Kit (Catalog No. Q32852; Invitrogen; Thermo Fisher Scientific, Inc.). RNA was successfully extracted from 18 out of 19 hepatopancreas samples (18 digestive glands from 7 males and 11 females). However, extracted RNA from female hemocytes showed inadequate quality standards for library preparation, therefore only male hemocyte samples were used ($N = 7$).

2.7.2. Library generation, Illumina de novo sequencing

Illumina libraries were constructed from 25 (18 hepatopancreas and 7 hemocyte) samples using QuantSeq™ 3′ mRNA-Seq Library Prep Kit FWD (Catalog No. 15; Lexogen GmbH, Vienna, Austria) according to the manufacturer's instructions using 65–525 ng of total RNA for each sample as input. The quality of purified libraries was evaluated by examining the size distribution and the absence of primer species using an Agilent High Sensitivity DNA Kit (Catalog No. 5067-4626 and 5067-4627; Agilent Technologies, Palo Alto, CA, USA) for the 2100 Bioanalyzer

System. Libraries concentrations ranged from 1.9 to 8.2 ng/μL, with a typical size of 200–600 base pairs (bp). High-throughput sequencing was performed as single-end 100 bp sequencing using a NovaSeq™ 6000 platform (Illumina, San Diego, CA, USA) at CBM S.c.r.l. (Area Science Park, Trieste, Italy) based on standard protocols. The RNA sequencing data are available online at NCBI sequence read archive (SRA) under the BioProject ID: PRJNA691574.

2.7.3. Raw data analysis

The raw demultiplexed reads in FASTQ format provided by the sequencing center were quality assessed and trimmed using the Software CLC Genomics Workbench v20.0.03, developed by QIAGEN (Hilden, Germany). The trimming procedure included the removal of adaptor sequences, low-quality bases (quality score threshold = 0.05), ambiguous nucleotides, poly(A) and poly(G) sequence stretches and short reads (read length < 75 nucleotides). Additionally, 2 and 15 nucleotides at the 3′ and 5′ terminus of the reads, respectively, were discarded to remove a compositional nucleotide bias observed in a preliminary screening. Finally, trimmed reads underwent an additional filtering procedure to remove residual non-mRNA contamination through a workflow set up ad-hoc using the *Workflow* tool (CLC Genomics Workbench v12.0.3). For a detailed description see Appendix 1.2.

2.7.4. Differential gene expression analysis

Clean reads from each library were aligned to a reference transcriptome previously assembled in our laboratory, the Laboratory of Applied and Comparative Genomics at the University of Trieste, from Illumina RNA-sequencing data (depth 2 × 100 bp) from 12 tissues of *P. clarkii* (brain, eyestalk, green glands, ventral ganglia, heart, hepatopancreas, gills, hemocytes, muscle, Y-organ and epidermis, ovary and testis) (Unpublished results), with the *RNA-seq analysis* tool included in the CLC Genomics Workbench. The mapping parameters were set as follows: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.5, similarity fraction = 0.9. Mapping outcomes, as read counts per gene per sequencing library, were analyzed via the *Differential expression for RNA-seq* tool (CLC Genomics Workbench), considering three separated groups of samples (i.e., hemocytes, male hepatopancreas and female hepatopancreas) and thus comparing each experimental condition with the paired control. To identify significant differentially expressed genes (DEGs) the adjusted *p*-value (false discovery rate - FDR) and absolute fold change cutoffs were set at 0.05 and 2, respectively. Finally, a functional gene ontology (GO) enrichment analysis of DEGs was performed through a hypergeometric test to identify significantly enriched features.

2.8. Statistical analysis

All statistical data analyses were performed using R version 4.0.2 software (R Core Team, 2020). The significance of treatment effects was assessed for each physiological variable (hemolymph plasmatic PO and proPO activity, glucose concentration, protein concentration, THC) using Linear Mixed effects Models (LMM). LMM assuming Gaussian distributions of residual error were fitted using lmer function from the lmerTest package (v. 3.1.2, Kuznetsova et al., 2017) in R. The analysis tested 3 fixed factors: "Sex" (2 levels, males and females), "Treatment" (2 levels, exposed and control) and "Time" (4 levels, T0, T24 h, T48 h, T72 h). To avoid pseudo-replication due to repeated sampling of crayfish from different trials over time, "Individual" (19 levels) was incorporated as a random factor (random intercept) nested in "Trial" (Replicate 1, 2, 3, or 4) in the model designs. Fully models comprising all fixed effects, together with their interactions and random effects, were first tested. The goodness-of-fit of all the models was assessed using maximum likelihood for model fits (Zuur et al., 2009). Final models were obtained by backward elimination of non-significant variables from the full model using the step function from the lmerTest package (Kuznetsova et al., 2017) and were presented using REML estimation (Zuur et al., 2009). Model assumptions, in terms of linearity, normality, and homogeneity of variances, were evaluated by visual inspection of residuals' plots, and verified by Shapiro and Fligner-Killeens tests. Hypothesis testing was conducted by performing a type III Wald chi-squared test using the Anova function from the car package (v. 3.0.9, Fox and Weisberg, 2019). Post hoc Tukey tests for pairwise comparisons were conducted using the function pairs, after the least-square means were calculated using the function lsmeans (from the R package lsmeans v. 2.30.0, Lenth, 2016). The conditional coefficients of determination were calculated for all the models using the function r.squaredGLMM implemented in the package MuMIn (v. 1.43.17, Barton, 2020). An alpha level of 0.05 was used for all statistical tests.

3. Results

3.1. Particle characterization

TEM images showed well-distributed particles with uniform size and spherical morphology (Fig. S2). Primary particles' nominal size of 100 nm was confirmed by TEM imaging with an average diameter of 103 ± 3.18 nm (average ± S.D., $N = 440$). PS NP particle hydrodynamic diameter (D_h), and heterogeneity (polydispersity index, pdi) were characterized by Dynamic Light Scattering (DLS) at a concentration of 2 mg/L in MilliQ water, resulting in a *Z*-average diameter size (±S.D.) of 113.9 ± 0.91 nm (Fig. S2) and an average polydispersity index (± S.D.) of 0.02 ± 0.01.

3.2. Physiological biomarkers

3.2.1. Glycemia

The best-fit model for plasma glucose included Treatment and Time as fixed explanatory variables and id nested in Trail as a random intercept. Glycemia levels did not differ between treatments ($\chi^2 = 0.97$, df = 1, 69, $P > 0.05$; Table S3), neither was found a significant interaction between treatment and exposure time when comparing glucose concentrations over all time points (i.e., T0, T24 h, T48 h, and T72 h post-exposure) among control and exposed groups. The model revealed only a significant main effect of time ($\chi^2 = 11.25$, df = 3, 69, $P = 0.01$; Table S3), with a general reduction of glycemia from T0 to T72 h and a significant decline between T0 and T24 h (Mean ± S.D.; T0: 11.7 ± 7.13 mg/dL; T24 h: 7.95 ± 5.57 mg/dL; post hoc test $P = 0.027$, Fig. 1A). A full description of the results can be found in the Appendix (Tables S1, S2, and S3).

3.2.2. Total hemocyte counts

The final mixed model for THC comprised Treatment, Time and Sex as fixed effects, and included Treatment:Time and Time:Sex as significant interactions ($\chi^2 = 9.77$, df = 3, 72, $P = 0.021$; $\chi^2 = 9.53$, df = 3, 72, $P = 0.023$; Table S6). A random intercept for id was also taken into account. Despite a moderate inter-individual variability, PS NP exposure was followed by an increase of THC (Fig. 1B). At 48 h, exposed crayfish showed statistically significant higher THC levels compared to controls (Mean ± S.D.; ctrl: 3.34 ± 1.5 × 10^6 cells/mL; exposed: 5.01 ± 0.89 × 10^6 cells/mL; post hoc test P = 0.023; Table S4, Fig. 1B). At 72 h, PS NP exposed individuals demonstrated a different behavior based on sex: free hemocytes' concentration remained stable in males, while markedly decrease in females (post hoc test P = 0.021, Fig. 1D). Thence, 3 days post-administration males showed statistically higher THC levels than females (Mean ± S.D.; females: 2.71 ± 1.35 × 10^6 cells/mL; males: 4.87 ± 1.90 × 10^6 cells/mL; post hoc test $P = 0.020$, Fig. 1C, D). Total hemocyte count mean values for control and exposed individuals are summarized in Table S4; model estimates, 95% confidence intervals, and *p*-values, as well as the outputs of the analysis of deviance, are reported in Tables S5 and S6.

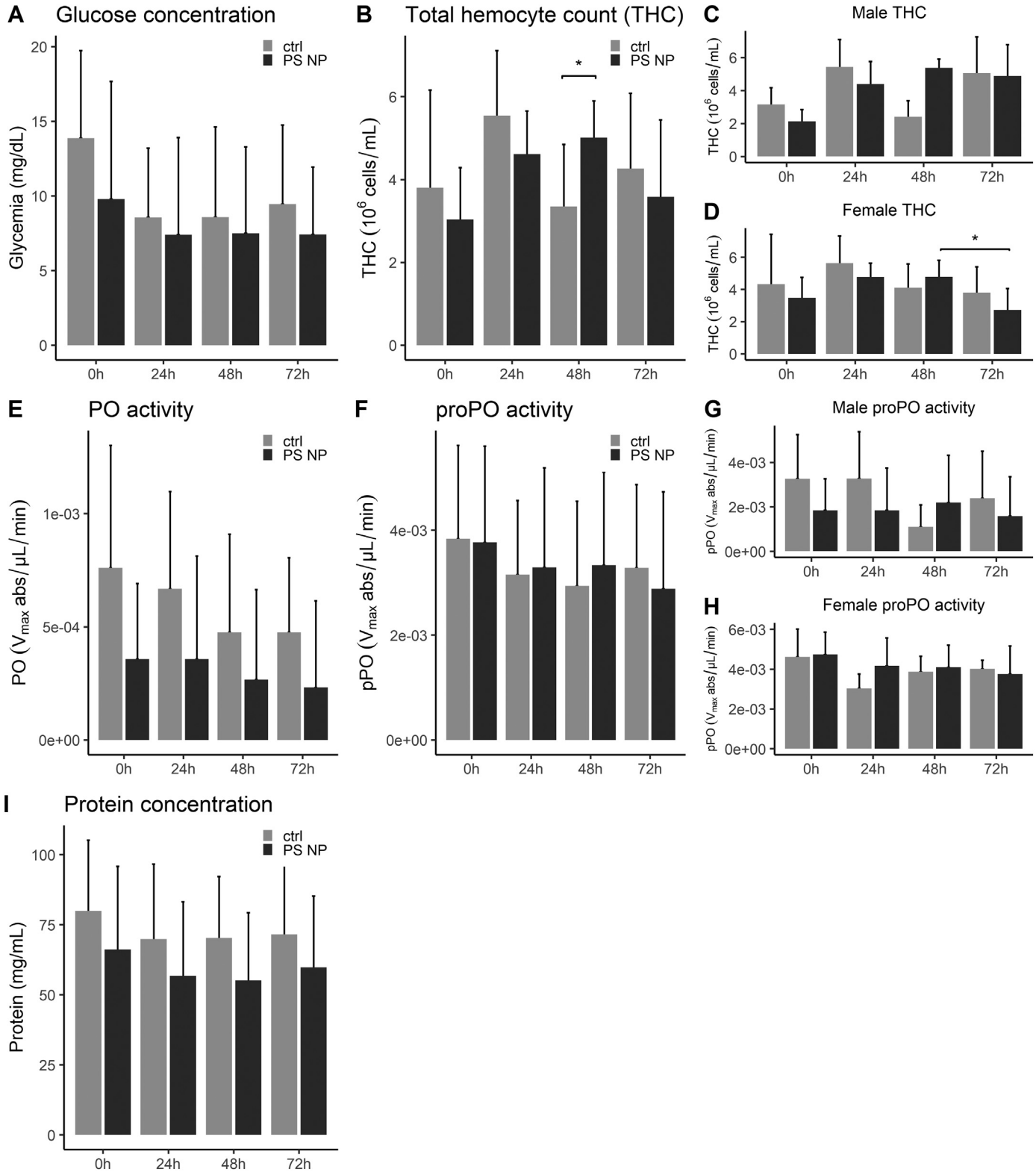

Fig. 1. Effects of PS NP exposure on physiological parameters of *P. clarkii*. Data are presented for all individuals ($N = 19$) as mean ± S.D., over all time points (T0, T24 h, T48 h, and T72 h post-exposure). When mixed models revealed a statistical significance for sex, data are also showed for males ($N = 8$) and females ($N = 11$) separately. Asterisks represent significant differences between groups ($^*P < 0.05$).

3.2.3. Basal and total plasmatic phenoloxidase activities

The results showed no significant differences in either basal PO ($\chi^2 = 2.70$, df = 1, 65, $P > 0.05$; Table S9) or total plasmatic PO ($\chi^2 = 0.11$, df = 1, 64, P > 0.05; Table S12) activities among control and exposed groups, despite significant overall variation over time was found for both phenoloxidase ($\chi^2 = 16.2$, df = 3, 65, $P = 0.001$, Table S9) and prophenoloxidase ($\chi^2 = 23.2$, df = 3, 64, $P < 0.001$, Table S12) activities. Although a high inter-individual variability was

observed, PO activity appeared to slightly decrease in all specimens over time (Fig. 1E). Meanwhile, total phenoloxidase activity was significantly determined by sex ($\chi^2 = 6.95$, df = 1, 64, $P = 0.008$; Table S12), with an enzyme activity higher in females than in males (Mean ± S.D.; females: $4.93 \pm 4.29 \times 10^{-4}$ abs/μL/min; males: $3.26 \pm 4.05 \times 10^{-4}$ abs/μL/min; post hoc test $P = 0.018$, Fig. 1G, H). Although non-significant, a minor proPO activity increase after 48 h was recorded both in males and in females. Phenoloxidase and prophenoloxidase enzyme activity summary statistics can be found in Tables S7 and S10, while mixed models and ANOVAsummaries are reported in Tables S8, S9 and S11, S12, respectively.

3.2.4. Hemolymph protein concentration

As with glycemia and phenoloxidase enzyme activity, no clear effect of Treatment was found in protein concentration after 72 h of exposure ($\chi^2 = 1.25$, df = 1, 73, P > 0.05; Table S15, Fig. 1I). A full description of protein concentration statistics is given in the Appendix (Tables S13, S14, S15).

3.3. Transcriptomic responses of Procambarus clarkii to nanoplastic exposure

3.3.1. Illumina sequencing

A total of 126,179,823 raw reads were obtained from Illumina-based RNA-seq (Table 1), with a mean of 5,047,193 raw reads per sample and an average length of 97.5 ± 1.23 bp (Mean ± S.D.). After trimming, 1,078,492 to 6,572,996 clean reads per sample (mean length of 83.1 ± 0.07 bp) were generated. The Q30 (Q score ≥ 30) was 98.2% and mean GC contents was 40.8% (Table 1). Clean reads were mapped back to the reference transcriptome and the read counts for each gene were obtained from the mapping results. A total of 48 million reads (80.2%) mapped to the reference transcriptome (Table 1). Detailed information on sequencing outputs and mapping results are available in the Appendix (Table S16).

3.3.2. Analysis of differentially expressed genes (DEGs)

A preliminary Multidimensional Scaling (MDS) analysis was performed to visualize the level of similarity between samples based on gene expression profiles (Fig. 2). MDS revealed a clear separation of samples by tissue type along the first dimension (Dim1), as expected. Although not as clearly marked, further segregation between the two sexes in hepatopancreas samples was visible along the second dimension (Dim2). A weak clustering effect due to treatment was observable for females in the hepatopancreas, while no evident segregation by treatment was observable within other groups of samples (Fig. 2). Differential expression analysis was performed between PS NP exposed crayfish and control counterparts in hemocytes (Hem, $N = 7$) and hepatopancreas samples according to sex (Hep_F, $N = 10$; Hep_M, $N = 7$). One sample, belonging to the female hepatopancreas group (R3T3), was excluded from DEG analysis because it was considered an outlier as its gene expression pattern greatly differed from other specimens of the same group, possibly because of an undetermined individual peculiarity. In general, the alterations induced by PS NPs were of a limited entity, both in terms of up- and down-regulation, even though a tissue-dependent effect was detectable. DEG analysis highlighted a

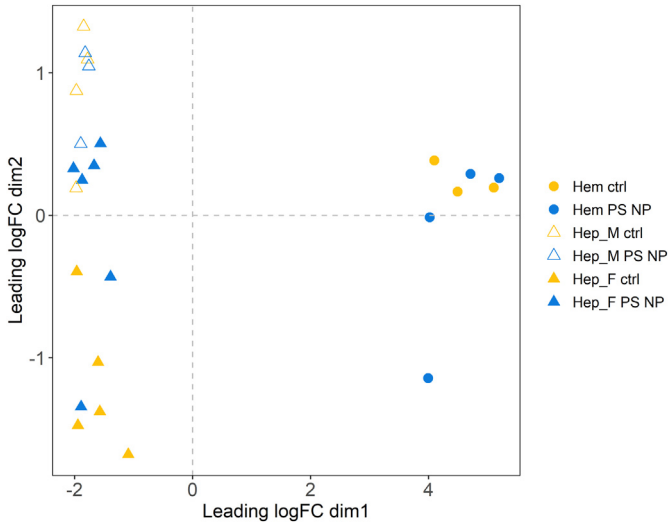

Fig. 2. Multidimensional scaling (MDS) plot of *P. clarkii* samples with total counts used as expression value parameter. Distances correspond to leading log-fold-changes, i.e., the average (root-mean-square) of the largest absolute log-fold-changes, between each pair of samples. Different colors correspond to different experimental groups (Control, yellow; PS NP exposed, blue). Shapes define different groups of samples: hemocytes (Hem, circle), hepatopancreas of male specimens (Hep_M, empty triangle), and hepatopancreas of female specimens (Hep_F, full triangle). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

total of 12, 98, and 32 DEGs in Hem, Hep_F, and Hep_M groups, respectively. Overall, the NP treatment mostly resulted in upregulation, as evidenced by the disproportion between positively and negatively regulated genes (8 vs 4 in Hem, 65 vs 33 in Hep_F, and 25 vs 7 in Hep_M). A complete list of DEGs, including the fold ratio, the adjusted *p*-value, and annotation information can be found in the Appendix (Table S17), while they are visually displayed by volcano plots and heatmaps in Fig. 3. Only 3 transcripts, *cytochrome P450 49a1* alongside two unknown genes, were differentially expressed in both sexes in the hepatopancreas, even though their regulation patterns differed between sexes (Fig. S3). A functional Gene Ontology (GO) enrichment analysis was performed by hypergeometric test for all different groups of DEGs but was inconclusive, likely due to the low number of genes and relatively low rate of annotated genes as a consequence of dealing with a non-model organism.

4. Discussion

4.1. Physiological biomarkers

Numerous studies have reported hyperglycemia mediated by CHH release following exposure to several stressors in crustaceans (Bonvillain et al., 2012; Celi et al., 2013; El-Bakary and Sayed, 2011; Lorenzon et al., 2004; Manfrin et al., 2016), although to the best of our knowledge, the effects of NPs have never been investigated in this species. MPs and NPs have been described to induce different glycemic responses in fish. Brun and colleagues reported a significant reduction in whole-body glucose levels in zebrafish (*Danio rerio*) larvae following

Table 1
General information on RNA-Seq output and mapping rates for hemocytes and hepatopancreas libraries.

Terms	Hemocytes	Hepatopancreas – males	Hepatopancreas – females	All
Number of libraries	7	7	11	25
Number of raw sequencing reads	43,763,356	32,742,172	49,674,295	126,179,823
Number of clean sequencing reads	19,895,749	16,021,331	24,550,051	60,467,131
% GC content	39.7	41.0	41.3	40.8
Q20	100	100	100	100
Q30	97.8	98.4	98.3	98.2
Mapping rate (%)	67.4%	86.6%	84.3%	80.2%

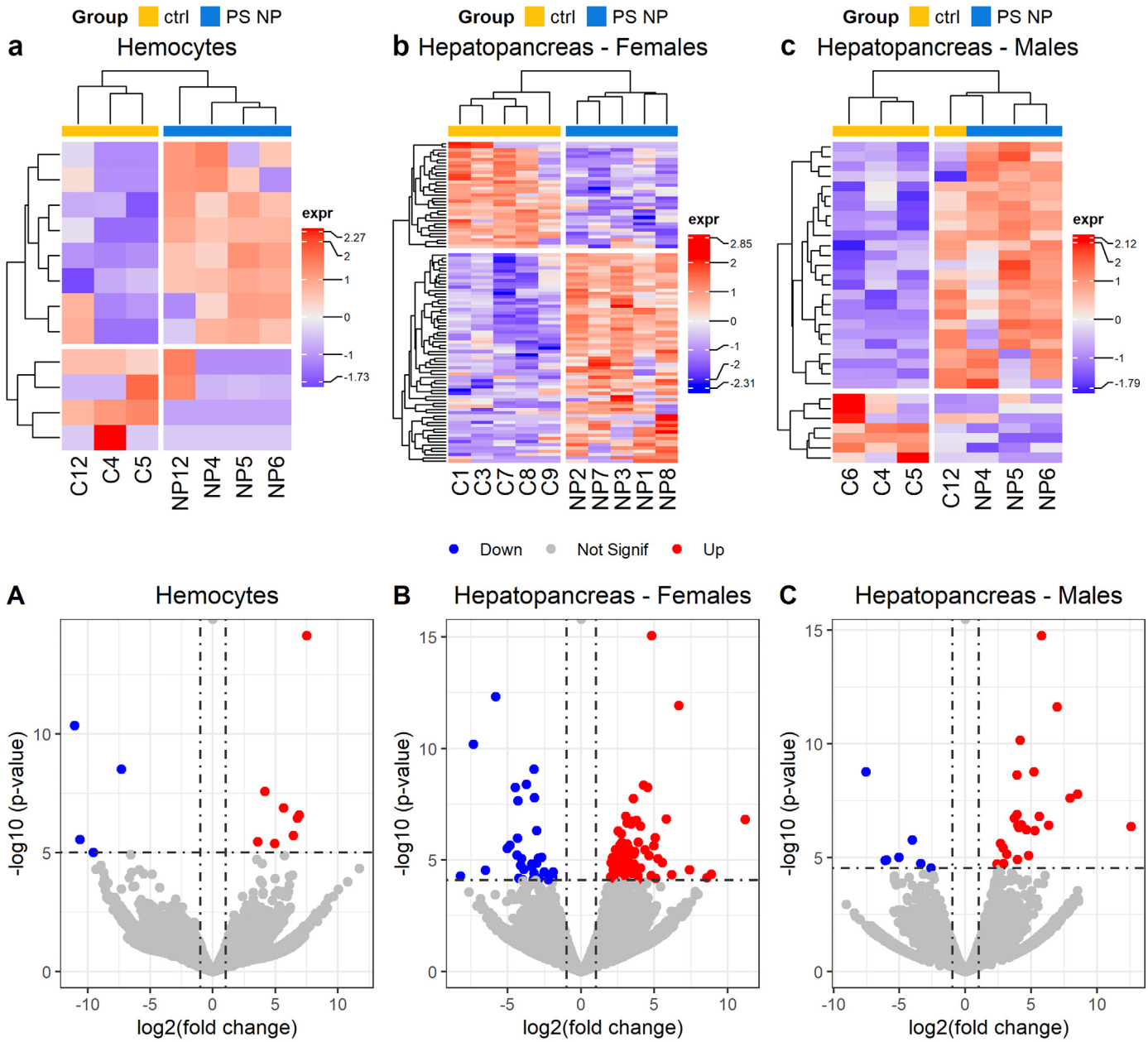

Fig. 3. Visual identification of DEGs (FDR ≤ 0.05 and |fold change| > 2) identified in *P. clarkii* exposed to PS NPs, relative to the controls, for each sample group analyzed: hemocytes (A, a), female hepatopancreas (B, b), and male hepatopancreas (C, c). The hierarchical clustering is based on Euclidean distance and complete linkage of normalized expression values (log CPM - counts per million -). Clustering indicates similar expression patterns among the samples of the control group (C, yellow) or nanoplastic-exposed group (NP, blue) (columns) and among genes (rows). Colors represent the normalized gene expression levels from light blue (low) to red (high). In the volcano plots, red (upregulation) and blue (downregulation) dots indicate DEG transcripts in the nanoplastic-exposed group, respectively, and dots in grey color show no significant differential expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a 2-day exposure to 20 mg/L of PS NPs (Brun et al., 2019). Differently, a significant hyperglycemic response was recorded in Nile tilapia (*Oreochromis niloticus*) after exposure to microplastics (1–100 mg/L) for 15 days in a dose-dependent manner (Hamed et al., 2019). In this study, the lack of a significant glycemic response to plastic exposure could either be due to the moderate physiologic stress induced by the relatively low dose (100 μg) of PS NPs administered or to the recovery of the animals to glycemia homeostatic levels within 24 h after ingestion (Lorenzon et al., 2004).

Crustaceans' hemocytes are crucial players in the host immune response. THC is sensitive to various environmental pressures, and a reduction in circulating hemocytes, termed hemocytopenia, has been reported in several crustacean species under stress conditions (Celi et al., 2013; Johnson et al., 2011; Lorenzon et al., 2008; Wei et al., 2020). In this study, PS NP exposure did not cause hemocytopenia but conversely was followed by a significant increase of THC 48 h after exposure. A similar increase in total hemocyte count was previously observed by Giulianini et al. in *Pontastacus leptodactylus* injected with 200 μL of 0.9 μm carboxylated polystyrene latex beads after 24 h (Giulianini et al., 2007). The authors reported that maximum THC increases were registered at 1–2 h after injection and clearly showed phagocytic activity of crayfish hemocytes against exogenous particles. Nanoparticles, particularly metal-based NPs, have recently gained great interest as antimicrobials, drug delivery vehicles, and immunostimulants in aquaculture (Shaalan et al., 2016; Swain et al., 2014). Several peer-reviewed papers have reported the enhancement

of crayfish and shrimp immune responses as a result of nanoparticles mediated supplementary diets (Ishwarya et al., 2019, 2018; Juarez-Moreno et al., 2017; Kandasamy et al., 2013; Muralisankar et al., 2016, 2014; Sivaramasamy et al., 2016; Sun et al., 2016; Tello-Olea et al., 2019). Authors addressed that Au, Cu, Zn and Ag-based and chitosan NPs, within a certain range of doses, were all able to increase THC, phenoloxidase enzyme activity, nutritional indices, and development and to raise survival rate after virus infection in decapod species, without causing toxicity in terms of mortality, antioxidant and metabolic enzymes activity or lipid peroxidation. Even though this study relied on a relatively small sample number, these results may suggest that a low dose of PS NPs promotes immune stimulation but do not cause toxicity in crayfish after 72 h of exposure, with minor differences between sexes.

Phenoloxidase, primary involved in the melanization cascade, is one of the most common measures of invertebrate immunity. Overall, elevated PO levels identify a stress response, while low levels are associated with immunocompromised animals (Coates and Söderhäll, 2020). To cope with MP and NP-induced stress, PO activity initially increased under short time and low concentration exposure in decapod crustaceans (Li et al., 2020a; Liu et al., 2019a). Prophenoloxidase expression was also enhanced by low doses of NPs (Li et al., 2020a). However, higher concentrations of particles over long time exposure caused a decrease in PO activity and proPO expression, showing an exceed capacity of the immune defense system (Li et al., 2020a; Liu et al., 2019a). The lack of a clear enzyme response in *P. clarkii* may be due to the lower exposure time and dose of PS NPs administered to crayfish, compared to the mentioned studies (5–40 mg/L for 28 d, Li et al., 2020a; 0.04–40 mg/L for 21 d, Liu et al., 2019a).

The assessment of hemolymph protein levels is a useful tool for monitoring the physiological status of crustaceans exposed to different environmental conditions or stressors (Coates and Söderhäll, 2020; Lorenzon et al., 2011). We did not find significant alterations of total protein content in *P. clarkii* after PS NP exposure. Our findings agree with other laboratory studies that tested acute stress on crustaceans (Celi et al., 2013; El-Bakary and Sayed, 2011), and may confirm the idea that hemolymph protein concentration can be considered a chronic, rather than an acute response, biomarker as previously reported by Bonvillain et al. (2012), who evidenced that fluctuations in protein concentrations in *P. clarkii* were associated with chronic stress in different laboratory and field studies (Bonvillain et al., 2012).

This poor response could be explained in the light of the relatively low concentration of PS NPs (100 μg) used for this study in comparison to PS NP toxicity thresholds available in literature for crustacean species. Indeed, a 96 h half-lethal concentration (LC50) value of 396 mg/L (95% CI, 26.0–638 mg/L) was calculated for 75 nm PS NPs in juvenile shrimp *Macrobrachium nipponense* (Li et al., 2020a). In *Daphnia* species, Liu and colleagues reported a 48 h LC50 of approximately 77 mg/L for 75 nm PS NPs (95% CI, 32.4–127 mg/L, Liu et al., 2019b). Moreover, Heinlaan et al. determined an effective concentration for 50% immobilization (EC50) of 22.0 ± 0.7 mg/L and 13 ± 1.4 mg/L for 26 nm and 100 nm non dialyzed carboxylated PS NPs, respectively, which further rose to >100 mg/L for dialyzed (removal of antimicrobial additive sodium azide) particles (Heinlaan et al., 2020).

4.2. Transcriptomic analysis

4.2.1. Hemocytes

The differential gene expression analysis in hemocyte samples revealed that just a little number of genes underwent significant expression shifts in response to PS NP exposure. Three out of 8 upregulated DEGs in hemocytes (i.e., *transcription factor btf3*, *pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16*, and *putative ribosomal protein S23e*) were involved in gene transcription and translation (Table S17). Since proteins catalyzed most cellular processes, the regulation of their levels through changes in gene expression is fundamental to respond to stress stimuli (Holcik and Sonenberg, 2005).

In previous studies, MP exposure induced upregulation of genes associated with translation, ribosomal and spliceosomal functions in the marine copepod *Tigriopus japonicus* (6 μm PS MPs; 0.23 mg/L for two-generation exposure, C. Zhang et al., 2019) and in developing zebrafish (10–45 μm PE MPs; 5 mg/L for 48 h, LeMoine et al., 2018). Besides, proteomic analysis of the zebra mussel (*Dreissena polymorpha*) gills revealed an upregulation of protein involved in ribosomal structure and function (including 40S ribosomal protein S23) after 6 days of MP exposure (1–10 μm; 4×10^6 MP/L, Magni et al., 2019). Interestingly, the authors related the over-production of this class of proteins with their involvement in the formation of stress granules (SG), given the concurrent alteration of other RNA-binding proteins (i.e., eukaryotic translation initiation factors). Here, as we did not find a significant difference in the expression of other specific proteins involved in the formation of SG, (e.g., eukaryotic translation initiation factors, translational silencers, polysome-associated proteins, and cytoplasmic polyadenylation element-binding protein, Anderson and Kedersha, 2009) we assumed that the upregulation of these genes must be traced back to an overall higher protein biosynthesis.

In response to PS NP toxicity, two cytoskeleton-associated DEGs were also identified in hemocytes. The genes annotated as *dystonin-like* and *integrin beta-4-like* were upregulated in PS NP exposed crayfish, suggesting a primary involvement of hemocytes in the immune response (Table S17). Indeed, dystonins can interact with the hemidesmosomal transmembrane β-integrins to form cell-extracellular matrix junctions (Jefferson et al., 2004; Koster et al., 2003). In turn, hemocyte-surface associated integrins, and specifically the integrin β subunit, have been suggested to have a role in many cell-mediated innate immune responses, such as microbial agglutination (Huang et al., 2015; Zhang et al., 2012), hemocytes degranulation, activation of proPO activating system and phagocytosis (Chai et al., 2018; Lin et al., 2013; Wang et al., 2014; Xu et al., 2018). In accordance with these results, Gu and colleagues observed an alteration of phagosome processes and integrin-mediated signaling pathway in phagocytes of zebrafish exposed to nano-sized polystyrene (Gu et al., 2020). Furthermore, altered cytoskeletal dynamics have been previously revealed by proteomic analysis in gills of the zebra mussel (Magni et al., 2019) and oocytes of female oysters (*Crassostrea gigas*) (Sussarellu et al., 2016) after MP exposure.

4.2.2. Hepatopancreas

4.2.2.1. Acute phase and inflammation-related genes. In the hepatopancreas of *P. clarkii*, a group of DEGs coding for acute phase and inflammatory response proteins was identified. Specifically, the expression of *serum amyloid A-5 protein-like*, *activating transcription factor 4*, *hemocyte homeostasis-associated protein*, and the antimicrobial peptide *anti-lipopolysaccharide factor ALF9* were significantly upregulated after PS NP exposure in females (Table S17). Similarly, *C-type lectin-2*, *macrophage mannose receptor 1-like*, and the *anti-lipopolysaccharide factor ALF4* were over-expressed in males (Table S17).

C-type lectins (CTLs), including mannose receptors (Man et al., 2018), are pattern recognition receptors (PRRs) responsible for pathogen detection and immune system activation (Cerenius and Söderhäll, 2018). In *P. clarkii*, CTLs have been described to mediate hemocyte binding (opsonization), to promote encapsulation (Zhang et al., 2011) and phagocytosis of bacteria (Chen et al., 2013; Zhang et al., 2016, 2013) and to trigger the proPO activating system (Wang et al., 2011). CTLs have been also reported to influence the expression of antimicrobial peptides (Bi et al., 2020; Sun et al., 2017) and other immune effector genes (Luo et al., 2019). Here, an up-regulation of antimicrobial peptides (AMPs) ALF4 and ALF9 was observed in PS NP-group. The promoted expression of AMPs by carboxy-modified PS NPs has been earlier described by Bergami and colleagues in Antarctic sea urchin (*Sterechinus neumayeri*) coelomocytes, under in vitro conditions (Bergami et al., 2019). AMPs are regulated by Toll-like receptors (TLR) signaling pathway and may involve ATF4, a member of the ATF/CREB

(activating transcription factor/cyclic AMP response element binding protein) transcription factor family (Huang et al., 2017; Lan et al., 2016). Previous proteomic analysis revealed that CTLs were significantly upregulated by MP exposure in *T. japonicus* (C. Zhang et al., 2019) and *Litopenaeus vannamei* (Duan et al., 2020), in accordance with our findings (Rosa et al., 2013; Smith and Dyrynda, 2015). Several other studies conducted on the Mediterranean mussel *Mytilus galloprovincialis*, indicated an antimicrobial response to micro- and nanoplastics in hemocytes and hepatopancreas (Auguste et al., 2020; Détrée and Gallardo-Escárate, 2018, 2017; Sendra et al., 2020). Our results, in accordance with literature, suggest that NPs may be perceived as non-self by crayfish immune system in the hepatopancreas, and through different pattern recognition receptor signaling pathways, they can trigger inflammatory response.

4.2.2.2. Detoxification-related genes. In this study, four genes involved in xenobiotic detoxification pathways were modulated in the hepatopancreas of male and female crayfish exposed to PS NPs compared to the control. Among them, *cytochrome P450 49a1* was significantly upregulated in females, while it resulted downregulated in males. Additionally, *glutathione S-transferase*, *carboxylesterase 4A-like*, and *thiopurine S-methyltransferase-like* were over-expressed in males (Table S17).

Cytochrome P450 monooxygenases (P450), glutathione S-transferase (GST), and carboxylesterases (CES) are widely used biomarkers of phase I and II detoxification of endogenous and exogenous lipophilic compounds in environmental toxicology studies (Barata et al., 2004; Fernandes et al., 2002; Han et al., 2017; Porte and Escartín, 1998; Vioque-Fernández et al., 2007b). A recent RNA-Seq analysis revealed a significant alteration of cytochrome P450 and glutathione metabolism KEGG pathways in *D. pulex* exposed to PS NPs (70 nm; 1 mg/L for 96 h, Liu et al., 2021). Furthermore, P450 enzymes have been reported to respond to NP insult both in vitro and in vivo (Fröhlich et al., 2010; Wu et al., 2019). A study by Fröhlich and colleagues reported that carboxyl PS NPs (20–60 nm) were able to reach high intracellular concentrations inhibiting the catalytic activity of P450 isoenzymes and increasing the effect of known P450s inhibitors in vitro (Fröhlich et al., 2010). A comprehensive in vivo study demonstrated that the expression levels of P450s resulted in a two-phase modulation after chronic exposure of *Daphnia pulex* to 75 nm PS NPs: P450s were induced by low concentrations (0.1–0.5 mg/L) and inhibited at high concentrations (1–2 mg/L) of NPs (Wu et al., 2019). An antioxidant response mediated by GST and other antioxidant enzymes triggered by MPs and NPs is widely reported in literature in several crustacean species (Chae et al., 2019; Jeong et al., 2017, 2016; Z. Liu et al., 2020). In this study, NP exposure induced the over-expression of several genes involved in detoxification, with a more marked response in male crayfish as compared to females. Our results further support the hypothesis that P450 system may play a role in detoxification from NPs, even though the molecular pathway underlying cytochrome's response remains unclear. The distinct expression of cytochrome P450 49a1 may indicate a different response of the two sexes to PS NP exposure. Even though P450s mode of action in NP response has not been yet clarified, GST triggering is likely to be linked to the detoxification of reactive and oxygen radicals. Concerning CES4 and TPMT, no additional literature has been found to hypothesize their direct involvement in PS NP detoxification.

4.2.2.3. Oxidative stress-related genes. Oxidative stress is one of the most common hurdles encountered by cells and organisms (Lushchak, 2011). The removal of non-functional oxidized cytosolic proteins is an essential part of the antioxidant defenses of cells (Grune, 2000). The ubiquitin-proteasome system (UPS) and autophagy are the two major degradation pathways maintaining cellular protein homeostasis (Kwon and Ciechanover, 2017). In the hepatopancreas of female crayfish, the UPS was altered by PS NP exposure. Indeed, six transcripts annotated as *E3 ubiquitin-protein ligase*, *26S proteasome non-ATPase regulatory subunit 10*, *ubiquitin carboxyl-terminal hydrolase*, and *aminopeptidase N* resulted upregulated (Table S17). Besides, the upregulation of the *activating transcription factor 4* (ATF4) (Table S17), illustrated in Section 4.2.2.1, may be linked to the promotion of transcription of genes involved in autophagy (B'Chir et al., 2013), and the resistance to oxidative stress in the unfolded protein response (UPR) directed by PERK (Fusakio et al., 2016; Harding et al., 2003). The alteration of the intracellular protein degradation system has been reported in crustaceans exposed to several stressors (Götze et al., 2017; Hansen et al., 2008; Jiao et al., 2019; Xu et al., 2017; Zhao et al., 2017). Nevertheless, to the best of our knowledge, this is the first investigation reporting such a clear response of the UPS to NPs in Crustacea. Actually, only a recent proteomic analysis has described a similar response to MPs in the gills of zebra mussel (Magni et al., 2019). It has been outlined that intracellular proteolysis shows a biphasic response to oxidative stress: moderate stress promotes upregulation of the ubiquitination system and proteasome activity, while UPS is inactivated by sustained oxidative stress (Shang and Taylor, 2011). Thus, our results suggest that UPS was regulated under polystyrene NP exposure as an antioxidant defense in order to maintain cellular integrity.

Furthermore, molecular chaperones transcription was altered in crayfish: *sacsin-like* was upregulated in females, while *heat shock protein HSP 90-alpha* was downregulated in males (Table S17). HSP90 and sacsin display several regions of sequence similarities and thus share molecular chaperone ability function and the capacity to interact with the proteasome (Anderson et al., 2011; Imai et al., 2003). Specifically, HSP90 has been proposed to play a principal role in the assembly and maintenance of the 26S proteasome (Imai et al., 2003), while sacsin is involved in protein quality control in the UPS and chaperone-mediated autophagy (Morani et al., 2019). The higher relative expression of sacsin in NP exposed female crayfish is in line with the upregulation of the other proteasome components described above. Another explanation of sacsin overexpression may lie in its recently suggested role in mitochondrial dynamics and bioenergetics, promoting mitophagy following mitochondrial damage (Morani et al., 2019). Indeed, RNA-seq revealed two other DEGs involved in mitochondrial dynamics, namely *peptidyl-prolyl cis-trans isomerase F* (PPIF) and *MICOS complex subunit mic25-a* (Mic25/CHCHD6), which were respectively up- and downregulated in exposed females (Table S17). PPIF is a major modulator of the mitochondrial permeability transition pore (MPTP) (Gutiérrez-Aguilar and Baines, 2015) and it is reported to mediate Ca^{2+} overload- and oxidative damage-induced cell death (Baines et al., 2005) through the transient or permanent depolarization and rearrangement of the cristae (Azzolin et al., 2010). Mic25/CHCHD6 is a subunit of the mitochondrial contact site and cristae organizing system (MICOS), which is deputed to create crista junctions, in order to maintain cristae morphology, and to form contact sites with the mitochondrial outer membrane (An et al., 2012; Ding et al., 2015; Muñoz-Gómez et al., 2015). Overall, several authors reported that MPs and NPs are able to alter cellular membrane integrity by inducing lipid peroxidation in a concentration-dependent manner (Jeong et al., 2018; Li et al., 2020a; Lin et al., 2019; P. Yu et al., 2018). In *Brachionus koreanus*, Jeong and colleagues unearthed NP-induced damage to mitochondrial membrane integrity, which is in line with our results (Jeong et al., 2016).

Lastly, one transcript annotated as *arylsulfatase B-like* (ARSB-like) resulted also downregulated in females (Table S17). In mammals, ARSB deficiency has been associated with pathological processes (Bhattacharyya et al., 2016). In particular, it has been reported that ARSB downregulation inhibits mitochondrial membrane potential and oxygen consumption (Bhattacharyya et al., 2016) as well as leads to increased reactive oxygen species (ROS) production and activation of the MAPK signaling pathway (Q. Wang et al., 2019). Although its specific role in crayfish is unknown, it seems reasonable to speculate the ARSB contribution to oxidative stress. Mounting evidence support the idea that oxidative stress is one of the molecular mechanisms underlying

the toxicity of NPs (Hu et al., 2020). PS NPs caused the over-production of ROS and activates the downstream mitogen-activated protein kinases MAPK signaling pathway in *D. pulex* (75 nm PS NPs; 0.1–2 mg/L for 48 h, Z. Liu et al., 2020), *B. koreanus* and *Paracyclopina nana* (50 nm PS NPs; 0.1–20 mg/L for 24 h, Jeong et al., 2018, 2017, 2016). The gene expression of antioxidant enzymes, including GST which was found to be up-regulated in male crayfish in this study, displayed an initial rise and a subsequent decline with increasing NPs dose in *D. pulex* (Z. Liu et al., 2020) and *M. nipponense* (75 nm; 5–40 mg/L for 28 d, Li et al., 2020a), suggesting that high nanoplastic concentrations can overwhelm antioxidant systems and induce loss of compensatory mechanisms, but lower concentration induces antioxidant response. Despite our investigation used a concentration that lies the lower range of the ones used in cited NP toxicity studies, a mild antioxidant response was visible in crayfish. This seems to be interconnected with ER stress, protein degradation, and possibly mitochondrial dysfunction, which are known to be frequently related (Chaudhari et al., 2014; Liang et al., 2016).

4.2.2.4. Lipid metabolism and oogenesis-related genes. Environmental stress can affect the optimal allocation of energy: under moderate stress, basal maintenance takes priority over other processes, including growth, reproduction, or storage and it can negatively affect the organism's fitness (Sokolova, 2013). Crustacean hepatopancreas is the principal organ for lipid synthesis (González-Baró and Pollero, 1993) and storage (Cheng et al., 1998). In the current study, a dysfunction of the lipid metabolism pathway emerged in females (Table S17). Three genes coding for proteins involved in glycerolipids biosynthesis were downregulated: *long-chain-fatty-acid—CoA ligase* (ACSBG2), *glycerol-3-phosphate acyltransferases 3* (GPAT3), and *phosphoethanolamine N-methyltransferase-like* (Athamena et al., 2011; Mashek et al., 2007; Pellon-Maison et al., 2009; J. Yu et al., 2018). Conversely, *gamma-butyrobetaine dioxygenase-like* (BBOX1), which play a role in the carnitine biosynthetic pathway (Lindstedt and Lindstedt, 1970; Vaz et al., 1998), was upregulated. Changes in carnitine concentration affect the rate of mitochondrial β-oxidation and therefore energy metabolism (Clark et al., 2017). The upregulation of BBOX1, in association with the downregulation of ACSBG2, GTPA3, and phosphoethanolamine *N*-methyltransferase likely reflects an increased demand for energy in the PS NP exposed crayfish as compared to control. Besides, a reduced biosynthesis of phosphatidylcholine, as a consequence of phosphoethanolamine *N*-methyltransferase downregulation, can affect membrane fluidity.

Consistently, four transcripts all annotated as *vitellogenin* (Vtg) were downregulated in the hepatopancreas of female crayfish (Table S17), while male crayfish did not show a marked response in this regard. Vtg biosynthesis is the first step of vitellogenesis and occurs both in the ovary and in the hepatopancreas of *P. clarkii* (Cai et al., 2016; Shen et al., 2014). Given that vitellus is the main energy reserve for the developing embryos, alteration in the vitellogenesis balance may result in significant reproductive impairment (Arambourou et al., 2020). Evidence of reproductive dysfunction in rotifers, bivalves, crustaceans, and fish exposed to MPs and NPs have been focusing on a reduced fecundity (Cole et al., 2015; Heindler et al., 2017; Jeong et al., 2017; Lee et al., 2013; Sussarellu et al., 2016) and fertilization success (Tallec et al., 2018), fewer total offspring and offspring performance (Au et al., 2015; Cong et al., 2019; Ziajahromi et al., 2017) including increased embryonic malformations (Besseling et al., 2014; Cui et al., 2017), and a delayed reproduction time (Jaikumar et al., 2019; Jeong et al., 2016; Liu et al., 2019a). Micro- and nanoplastics have been suggested to cause endocrine disruption directly or indirectly through leaching of plastic additives and/or associated chemicals (Amereh et al., 2019; Chen et al., 2019; Mak et al., 2019; Rochman et al., 2017a, 2014; J. Wang et al., 2019). A study by Wang and colleagues reported downregulation of Vtg expression in the liver of female medaka after PS MP exposure, while an upregulation was noticed in males (J. Wang et al., 2019). Similarly, Rochman et al. found downregulation of Vtg expression in the liver of female medaka exposed to virgin or weathered MPs, but no effect was reported in males (Rochman et al., 2014). Another study by Rochman et al. (2017b), revealed a lower vitellogenin protein content in the liver of *Acipenser transmontanus* females fed with clams previously exposed to different MP polymers virgin or spiked with polychlorinated biphenyls for 28 days. Even though a histopathologic analysis of crayfish ovaries was not conducted in this study, a significantly lower expression of vitellogenin transcripts in both ovary and hepatopancreas was previously associated with a clear inhibition of ovarian growth in *P. clarkii* exposed to the insecticide atrazine (Silveyra et al., 2018), confirming the primary role of vitellogenin in oogenesis for this species.

A very good illustration of a significant shift in energy allocation induced by microplastic exposure was provided by Sussarellu and colleagues in Pacific oysters (Sussarellu et al., 2016). After 2-month exposure to MPs during gametogenesis (2–6 μm; 0.023 mg/L), oyster energy flows were relocated to organism maintenance and structural growth at the expense of reproduction, leading to impacts on reproductive health indices (i.e., quantity and quality of gametes produced, Sussarellu et al., 2016). Similarly, a proteomic analysis on the zebra mussel showed that MPs interfered with glycolysis and the Krebs cycle (Magni et al., 2019). In *D. magna* neonates, the glycometabolic changes enriched by PS NP exposure were suggested to increase energy production as a way to counteract NP toxicity (Liu et al., 2021). Likewise, we suppose that the altered lipid metabolism in crayfish has to do with an increased energy demand for the innate immune response, and also to maintain cellular homeostasis. Altogether, our findings are in line with the data available in literature and may suggest that *P. clarkii*'s reproduction efficiency could be threatened by the exposure to nanoplastics. Undoubtedly, further phenotypic data collection is required to support our inference.

4.2.2.5. Hemocyanin. Hemocyanin (Hc) expression was upregulated in the hepatopancreas of female and male crayfish (Table S17). Hc is the main respiratory pigment of arthropods, primary synthetized in hepatopancreas and then released in hemolymph (Gellissen et al., 1991; Qin et al., 2018). Although having a primary role in oxygen transport, *P. clarkii* Hc exhibited antibacterial capacity by promoting phagocytosis and exerting phenoloxidase activity (Qin et al., 2018). In the current study, the overexpression of Hc may indicate either that challenged crayfish had a greater need for tissue oxygenation, or that Hc took part in the immune response and homeostasis maintenance following NP exposure. Consistently with our results, crabs exposed to MPs showed an enhanced Hc expression in the hepatopancreas (Liu et al., 2019a), and an altered Hc hemolymph content, albeit the magnitude and the direction of change (i.e., an increase or a decrease) varied with the exposure duration or dose level, as well as with the different particle surface coatings (COOH or NH_2) (Liu et al., 2019a; Watts et al., 2016).

Along with Hc, pseudohemocyanins (PHc) belong to the arthropod hemocyanin superfamily, which further comprises phenoloxidases and the insect hexamerins (Burmester, 2015, 2002). PHcs have lost the ability to bind copper and have been proposed to be involved in molting and reproduction as storage proteins (Burmester, 1999). Here, PHc underwent a different expression regulation based on sex, and particularly it was found to be upregulated and downregulated in females and males, respectively. The limited existing knowledge on PHc function in crustaceans, together with the diverse direction of the alteration between males and females in the current study, prevent us from inferring the possible role of PHcs in response to NP in crayfish.

4.2.2.6. Transcription and translation-related genes. Just like in hemocytes, PS NPs caused an alteration in gene transcription and translation in the hepatopancreas of both males and females. Two RNA helicases, *NFX1-type zinc finger-containing protein 1-like*, *la-related protein 6-like* (LARP6), and an RNA polymerase (RPABC5) were all upregulated in

the hepatopancreas of females, while *serine/arginine repetitive matrix protein 1-like* was downregulated (Table S17). In males, the *transcription activator BRG1-like*, also part of the ATP-dependent helicases superfamily, was downregulated, while the *RNA-binding protein squid-like* was upregulated (Table S17). Ribosomal proteins remain consistently upregulated in all groups of samples analyzed (Table S17). As stated above, an enrichment in ribosomal proteins, both from a transcriptomic and proteomic perspective, was pointed out in several other investigations of microplastic exposure in zebrafish (LeMoine et al., 2018), the zebra mussel (Magni et al., 2019), and marine copepod *T. japonicus* (C. Zhang et al., 2019).

5. Conclusion

The present study represents the first attempt to unravel the effects of polystyrene nanoparticles at both transcriptomic and physiological levels in a freshwater decapod species. In an integrated approach, RNA sequencing data were complemented by physiological responses, revealing that after 72 h exposure to relatively low concentrations of PS NPs, the studied species was able to face the induced stress, not exceeding generic stress thresholds. Our results evidence the power of RNA-Seq analysis in ecotoxicology to disclose minor physiological, immunological, and molecular alterations induced by environmental contaminants such as nanoplastics. In the red swamp crayfish, we reported that PS NPs can trigger transcriptomic pathways linked to immune response, induce oxidative stress and interfere with gene transcription and translation, protein degradation, lipid metabolism, oxygen demand, and potentially reproduction. Particularly, a quite clear transcriptomic response to NPs emerged as a strong downregulation of vitellogenin expression in female crayfish, which can lay the basis for a deeper exploration about the potential impacts of polystyrene nanosized particles at a population level. Nonetheless, further studies investigating phenotypic and ecological effects of nanoplastics on decapod crustaceans are needed to support our findings.

CRediT authorship contribution statement

Francesca Capanni: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Samuele Greco:** Software, Formal analysis, Resources, Data curation, Writing – review & editing. **Noemi Tomasi:** Formal analysis, Investigation, Writing – review & editing. **Piero G. Giulianini:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Chiara Manfrin:** Conceptualization, Methodology, Resources, Writing – review & editing, 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.

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