Immune response triggered by the ingestion of polyethylene microplastics in the dipteran larvae, *Chironomus riparius*

Carlos J.M. Silva, Sónia Beleza, Diana Campos, Amadeu M.V.M. Soares, Ana L. Patrício Silva, João L.T. Pestana, Carlos Gravato

PII: S0304-3894(21)00364-2

DOI: https://doi.org/10.1016/j.jhazmat.2021.125401

Reference: HAZMAT125401

To appear in: *Journal of Hazardous Materials*

Received date: 5 November 2020
Revised date: 8 February 2021
Accepted date: 9 February 2021

Please cite this article as: Carlos J.M. Silva, Sónia Beleza, Diana Campos, Amadeu M.V.M. Soares, Ana L. Patrício Silva, João L.T. Pestana and Carlos Gravato, Immune response triggered by the ingestion of polyethylene microplastics in the dipteran larvae, *Chironomus riparius*, *Journal of Hazardous Materials,* (2020) doi:https://doi.org/10.1016/j.jhazmat.2021.125401

Immune response triggered by the ingestion of polyethylene microplastics in the dipteran larvae, *Chironomus riparius*

Carlos J. M. Silva[1], Sónia Beleza[1], Diana Campos[1], Amadeu M. V. M. Soares[1], Ana L. Patrício Silva[1], João L. T. Pestana[1*], Carlos Gravato[2]

[1]CESAM - Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2]Faculty of Sciences & CESAM, University of Lisbon, Campo Grande 1749-016, Lisboa, Portugal

*jpestana@ua.pt

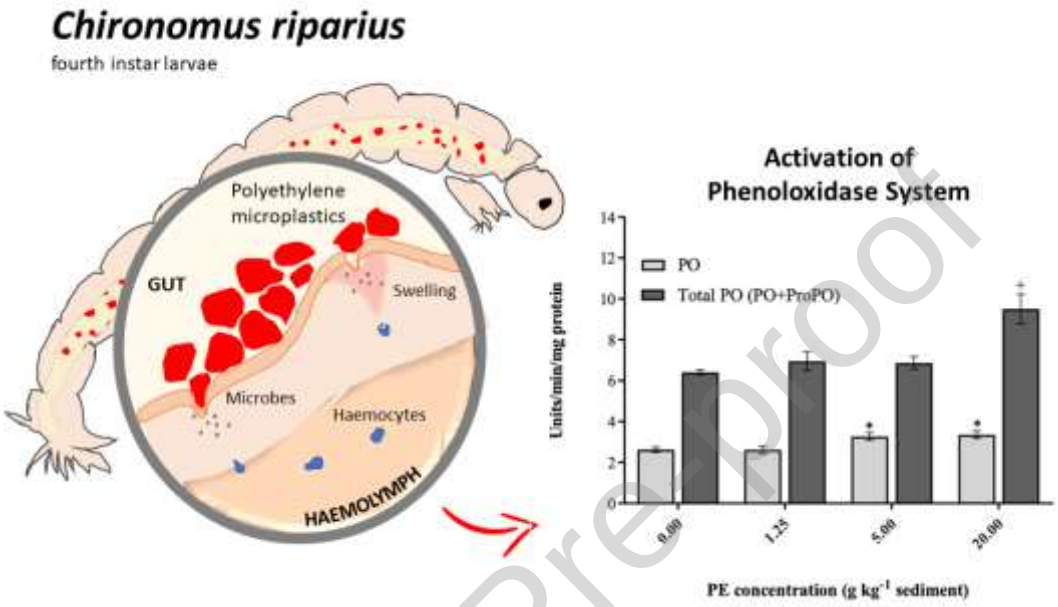
Abstract

The activation of insects' immune system due to the ingestion of microplastics (MPs) has only been evidenced by the upregulation of specific genes. The activation of phenoloxidase (PO) system is one of the primary responses involved in insects' innate immunity when facing parasites and pathogens, and ingestion of MPs can trigger a similar process. This study aimed at addressing the activities of basal PO and total PO (PO+ prophenoloxidase - proPO), in *Chironomus riparius* larvae (a model species in ecotoxicology) exposed to sediments spiked with polyethylene microplastics (PE-MPs; size-range 32-63 µm; concentrations: 1.25; 5; to 20 g kg^{-1}) for 48 h.

The ingestion of PE-MPs by larvae triggered a significant increase of basal PO activity at 5 and 20 g PE-MPs kg^{-1}, by 26 and 29%, respectively, whereas total PO increased significantly in the latter (+48%), suggesting *de novo* synthesis of proPO by organisms. Considering the particle size, the immune response's activation is probably linked to damage in the epithelial cells of the gut lumen. This research work provides the first evidence on the activation of the insect's innate immune system after ingestion of MPs

and underlines the PO activity as a good indicator of the immune response induced by MPs' ingestion.

Graphical abstract



Keywords: Innate immune system, phenoloxidase, biomarkers, invertebrates, plastic pollution

1. INTRODUCTION

The ingestion of microplastics (MPs) by freshwater benthic invertebrates, particularly detritivore-collector species, such as chironomid larvae and lumbriculids, has been previously observed and characterised (Scherer et al., 2017, 2019; Ziajahromi et al., 2018; Silva et al., 2019, 2021a). Although MPs ingestion rarely results in significant mortality in such invertebrates, it can reduce somatic growth (Silva et al., 2019) and developmental rates (Ziajahromi et al., 2018; Silva et al., 2019; Silva et al., 2021b). The observed impairments on organisms' life-history traits suggest that MPs' ingestion can reduce feeding and/or alter resource trade-offs between maintenance and growth

processes. In fact, ingestion of MPs seems to cause gut clogging or abdominal distention, particularly in chironomids which ingest high quantities of MPs (Nel et al., 2018; Silva et al., 2019), which could have affected feeding and digestion processes; thus, resulting in a deficit on energy acquisition/assimilation (Avio et al., 2015; Ziajahromi et al., 2018). Such energy constraints can partially explain why chironomids failed to prevent oxidative stress and damage (Silva et al., 2021a). In addition, the potential inflammation processes and activation of immune responses in organisms that ingest and accumulated MPs may also increase the energy requirements and add additional pressure on energy reserves that are being affected by suppressed feeding activity (Silva et al., 2021a, 2021b).

The activation of the immune responses due to exposure to MPs have only been suggested by the upregulation of genes related to the immune system as observed in mussels and oysters (Détrée & Gallardo-Escárate, 2017, 2018; Gardon et al., 2020), and with the increased phagocytic activity of particles smaller than 10 μm as observed in polychaeta (Browne et al., 2013; Wright et al., 2013) and bivalves (Avio et al., 2015; Pittura et al., 2018). However, the pathway through which MPs elicit these sub-cellular responses is poorly documented, particularly in sediment-dwelling invertebrates that ingest a significant amount of MPs (Wright et al., 2013; Ziajahromi et al., 2018; Silva et al., 2019, 2021a; Khosrovyan & Kahru, 2020; Stanković et al., 2020). This study hypothesised that the retention or blockage of MPs in organisms' gut, particularly of particles larger than 10 μm and cannot be phagocyted, may cause mechanical/proteolytic damage in the epithelial cells of the gut lumen. Since the gut epithelium is an immunologically active tissue that produces PO (Wilson et al., 2001) is likely that the damage signals (by-products generated during mechanical/proteolytic damage) will trigger the activation of PO system (Krautz et al., 2014).

The activation of phenoloxidase (PO) is one of the mechanisms involved in invertebrates innate immune system, ultimately leading to encapsulation followed by melanisation of pathogens, parasitoids, damaged tissues (Davis and Engström, 2012; González-Santoyo & Córdoba-Aguilar, 2012), and potentially MPs. Phenoloxidase system is controlled by an inactive zymogen prophenoloxidase (proPO), mostly synthesised in haemocytes (Cerenius & Söderhäll, 2004) and activated to PO by numerous microbial surface components, such as peptidoglycans, β-1,3-glucan, lipopolysaccharide, and zymosan (Smith et al., 1984; Eleftherianos & Revenis, 2011) via controlled proteolysis controlled by the action of a serine protease cascade (Eleftherianos & Revenis, 2011). The PO activation ultimately leads to a melanisation process (González-Santoyo & Córdoba-Aguilar, 2012). Phenoloxidase activity has been used as a biomarker of the immune response of several invertebrate species to organic contaminants, generally measured in haemolymph samples of organisms (Lee et al., 1998; Lilley et al., 2012; Yousefi-Lardeh & Zibaee, 2020). However, more recently, whole-body samples of chironomids were also used to assess the immune response of larvae to bioinsecticides (Bordalo et al., 2020) and ultraviolet filters (Muñiz-González & Martínez-Guitarte, 2020). Moreover, PO has also been used as a proxy for disease resistance of several invertebrate species (Cotter & Wilson, 2002; Aladaileh et al., 2007; van Ooik et al., 2007, 2008; Lilley et al., 2012).

Therefore, this study aimed to assess if microplastics (foreign and inert particles) ingested by insects trigger an immune response through the *in vivo* activation of phenoloxidase system. For this purpose, *Chironomus riparius* (Diptera: Chironomidae) was used as test species because its larvae are known to ingest and retain, in their gut, a considerable number of MPs (Silva et al., 2019, 2021a). *C. riparius* larvae were exposed for 48 h to irregularly-shaped polyethylene microplastics (PE-MPs; 32 to 63

µm), one of the most found in freshwater sediments, and considering concentrations previously determined in field-sediments (9 g kg^{-1}; Hurley et al., 2018) up to a worst-case scenario (20 g kg^{-1}). Moreover, in order to confirm that the ingestion of MPs triggered a true PO system by larvae, PO inhibition was estimated using an inhibitor of true PO, phenylthiourea (PTU). The activity of PO measured in whole-body samples was also optimised and characterised using *in vitro* activation by chymotrypsin as well as the response and sensitivity to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and zymosan (Zs).

2. MATERIALS & METHODS

2.1. *Chironomus riparius* culture conditions

Chironomus riparius were cultivated and maintained under controlled conditions at the Department of Biology, University of Aveiro. Organisms were reared in glass aquaria containing fine inorganic sediment (<1 mm), previously burnt at 500 °C (5h), and American Society for Testing and Materials (ASTM) hard water medium (ASTM, 1980). The culture room was kept at 20 ± 1 °C and under a photoperiod of 16 h light under constant aeration. Food was provided every two days in the form of macerated commercial food fish TetraMin® (Tetrawerke, Melle, Germany).

2.2. Detection and characterisation of phenoloxidase (PO)

2.2.1. Homogenisation and isolation of the samples

Fourth instar *C. riparius* larvae (twelve-days old) were selected and used to characterise the PO system. For all determinations, each sample consisted of fifteen larvae that were cultivated in a separate container, being collected in the twelfth day, rinsed, quickly dried on filter paper, being subsequently frozen in liquid nitrogen and stored at -80 °C until further analysis. Briefly, samples were homogenised by sonication (Branson

SONIFIER 250) on ice and using 0.2 M phosphate buffer pH=7.4 (3200 µL). Homogenates were then centrifuged for 20 minutes at 9000 g (4 °C). After centrifugation, the supernatant was collected and reserved on ice.

2.2.2. Basal PO and total PO activity: activation of proPO using chymotrypsin

Five samples comprising 30 µL each of the above-mentioned supernatant were incubated for 10 minutes in the absence (50 µL of Milli-Q water); and presence of chymotrypsin (CAS Number: 9004-07-3 from Sigma-Aldrich) (50 µL of 0.5 mg chymotrypsin mL^{-1}), and 100 µL of 0.2 M phosphate buffer pH=7.4 at 25 °C. After incubation, 100 µL of sodium cacodylate (CAS Number: 6131-99-3 from Sigma-Aldrich) (CAC, 10 mM) buffer (pH 7.4) and 20 µL of L-DOPA (CAS Number: 59-92-7 from Sigma-Aldrich) (200 mM) were added (Pang et al., 2004), to start the reaction. Reaction blanks were added and consisted of phosphate buffer in substitution of the sample. Half of the replicates (of the same sample) were incubated in the presence of chymotrypsin, whereas the other half was incubated in its absence. After the addition of L-DOPA, the absorbance was instantly read at 490 nm (time 0) and monitored for up to 6 hours. Each sample was tested four times (technical replicates) for higher accuracy. All microplate readings were performed on Thermo Scientific Multiskan Spectrum microplate reader. The enzymatic activity was expressed as units (U), with one unit defined as 0.001 OD (Optical Density) change per mg protein (Pang et al., 2010).

2.2.3. Inhibition of basal PO and total PO by phenylthiourea (PTU)

Larval basal PO was also analysed in the presence of a widely applied PO inhibitor – the phenylthiourea (PTU), which inhibition achieves a high percentage when facing a true PO (Asokan et al., 1997; Pang et al., 2005). Five samples (30 µL each) were added in quadruplicate (four technical replicates for each sample) and incubated in the absence (50 µL of Milli-Q water) and presence of chymotrypsin (50 µL of 0.5 mg chymotrypsin

mL^{-1}). Each condition was then tested in the absence and presence of PTU. To test PO inhibition by PTU, 100 µL of PTU solution (0.1 mg mL^{-1} PTU in 2 M phosphate buffer pH 7.4) (Hellio et al., 2007), and incubated for 10 min at 25 ºC. In the samples tested for the absence of PTU, the same volume of phosphate buffer (2 M, pH 7.4) was added before incubation. After incubation, 100 µL CAC buffer (10 mM, pH 7.4) were added to the samples, followed by 20 µL of L-DOPA (200 mM) (Pang et al., 2004). Reaction blanks consisted of the replacement of the sample by 30 µL of phosphate buffer. The incubation and remaining procedure were performed as explained above for each condition (e.g., presence and absence of PTU). After the addition of L-DOPA, the absorbance was instantly measured (time 0) at 490 nm and monitored for up to 6 hours. PO activity was expressed as U min^{-1} mg $protein^{-1}$.

2.2.4. Effects of the addition of zymosan (Zs) and lipopolysaccharides (LPS) on PO activity

Four samples consisting of 30 µL of homogenate were added to a microplate containing 50 µL of Milli-Q water in the absence (100 µL of 2 M phosphate buffer pH=7.4) and presence of zymosan (CAS Number: 58856-93-2 from Sigma-Aldrich) (100 µL of 0.1 mg mL^{-1} zymosan in 2 M phosphate buffer pH=7.4). The microplate was then incubated at 25 °C for 10 min. After incubation, 100 µL CAC buffer (10 mM, pH 7.4) and 20 µL of L-DOPA (200 mM) (Pang et al., 2004) were added. Reaction blanks were performed by replacing the sample volume with phosphate buffer (30 µL) followed by incubation as explained above. Four samples (30 µL of the homogenate) were added to a microplate performing four technical replicates per sample, followed by the addition of Milli-Q water (50 µL) in the absence (100 µL of 2M phosphate buffer pH=7.4) and presence of LPS solution (100 µL of LPS solution- 1 mg mL^{-1}, in 2M phosphate buffer pH=7.4) and incubated for 10 min at 25 °C. After incubation, CAC buffer (100 µL of 10

mM, pH 7.4) and L-DOPA (20 µL of 200 mM L-DOPA) were added (Pang et al., 2004). Reaction blanks were performed by replacing the volume of the sample with an equal volume of phosphate buffer followed by incubation as explained above. After the addition of L-DOPA, the absorbance was instantly measured (time 0) at 490 nm and monitored for up to 48 hours. PO activity was expressed as U min^{-1} mg $protein^{-1}$.

2.3. Basal PO and total PO after ingestion of microplastics

2.3.1. Short-time exposure to polyethylene microplastics (PE-MPs) contaminated sediment

The exposure of fourth instar *Chironomus riparius* larvae to PE-MPs followed the procedures of previous studies (Silva et al., 2019, 2021a). Briefly, *C. riparius* larvae (12-day old) were exposed for 48 h to three PE-MP concentrations (1.25 - 5 - 20 g kg^{-1} dry sediment) of PE-MPs pool of small-sized particles (32-63 µm; CAS Number: 9002-88-4 from Sigma-Aldrich UK). The PE-MP concentrations and size range were based on previous studies in which cellular and organismal level effects were described thus allowing for an integrative discussion of the obtained results (Silva et al., 2019, 2021a). Each PE-MP condition plus a control treatment (uncontaminated inorganic sediment) was prepared to contain seven replicates. Each replicate consisted of fifteen larvae exposed to PE-MPs. For exposure, PE-MPs were directly mixed (except for control treatment) in the sediment (50 g of fine sediment <1mm) and 150 mL of ASTM hard water. During the experiment, no food was offered to the larvae. After the exposure period (48 h), larvae were gently collected from the sediment, briefly rinsed (Milli-Q water) to minimize the possibility of having microplastics adhered to the larval cuticle. After rinsing, larvae were instantly dried (using filter paper), they were weighed (fresh weight. – FW) and subsequently frozen in liquid nitrogen, being stored at –80 °C until further analysis.

2.3.2. Determination of basal PO and total PO in PE-MPs exposed larvae

Samples were homogenised by sonication (on ice) using 0.2 M phosphate buffer pH=7.4 (3200 µL), and homogenates were then centrifuged for 20 minutes at 9000 g (4 °C). After centrifugation, the supernatant was collected and reserved on ice. Each sample (30 µL) was incubated 10 minutes in the absence of chymotrypsin (using 50 µL of Milli-Q water) and in the presence of chymotrypsin (using 50 µL of 0.5 mg mL^{-1}chymotrypsin) for the determination of basal PO and total PO (PO+ProPO) activity, respectively. After incubation, 100 µL of sodium cacodylate (CAC, 10 mM) buffer (pH 7.4) and 20 µL of L-DOPA (200 mM) were added to start the reaction. Reaction blanks were added and consisted of phosphate buffer (0.2 M, pH= 7.4) in substitution of the sample. Half of the replicates (of the same sample) were incubated in the presence of chymotrypsin, whereas the other half was incubated in its absence. After the addition of L-DOPA, the absorbance was instantly read at 490 nm (time 0) and monitored for up to 6 hours. Each sample was tested four times (technical replicates) for higher accuracy. The enzymatic activity was expressed as U, being one unit defined as 0.001 OD (Optical Density) change per mg protein (Pang et al., 2010).

2.4. Protein determination

The determination of the protein levels on each sample followed the method described by Bradford (Bradford, 1976), adapted for microplate reader.

2.5. Data analysis

Data analyses were performed using one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test or using *t-tests* to identify significant differences between each condition tested and the respective control or between two conditions of the assay, respectively. The type of analysis is also signalised in the figure caption. The homoscedasticity and normality of data were assessed using Bartlett's and Brown–

Forsythe tests and residual probability plots. The significance level was set at $p \leq 0.05$ for all statistical tests. GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA) was used for statistical analyses.

3. RESULTS

3.1. Basal PO and proPO activation by chymotrypsin

Values of PO activity (2.97 ± 0.92 Units/min/mg protein) on larvae of *Chironomus riparius* were significantly lower ($t_{(8)}$=5.568, p=0.0005) than the activity of total PO (9.34 ± 2.39 Units/min/mg protein) determined in the presence of 0.5 mg mL^{-1} chymotrypsin (Figure 1). This significant increase in the activity of total PO (PO+proPO) by chymotrypsin compared to basal PO (+218 ± 9%) suggests activation of ProPO by chymotrypsin through proteolysis of proPO (Figure 1).

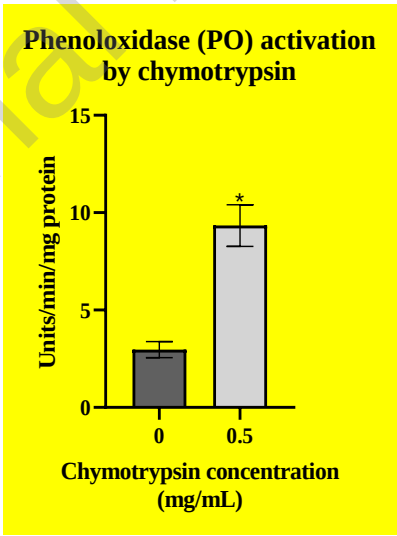


Fig. 1. Basal Phenoloxidase (PO) activity in the absence of chymotrypsin and total PO (PO+ProPO) after activation by 0.5 mg mL^{-1} chymotrypsin in fourth instar larvae of *Chironomus riparius*. Values are presented as mean of five samples ± SEM. * symbolizes a significant difference compared to control (0) treatment at $p < 0.05$ (t-test).

3.2. Inhibition assay of basal PO activity by phenylthiourea (PTU)

The PTU significantly inhibited basal PO activity ($t_{(8)}$=3.571, p<0.0073) and total PO ($t_{(8)}$=8.358, p<0.0001) compared to control (Figure 2A and B). The presence of 0.1 mg mL^{-1} PTU inhibited ~50 ± 10% the activity of basal PO and more than 95 ± 1%, showing that all total PO measured in larvae of *Chironomus riparius* is due to a true PO activity (Figure 2B).

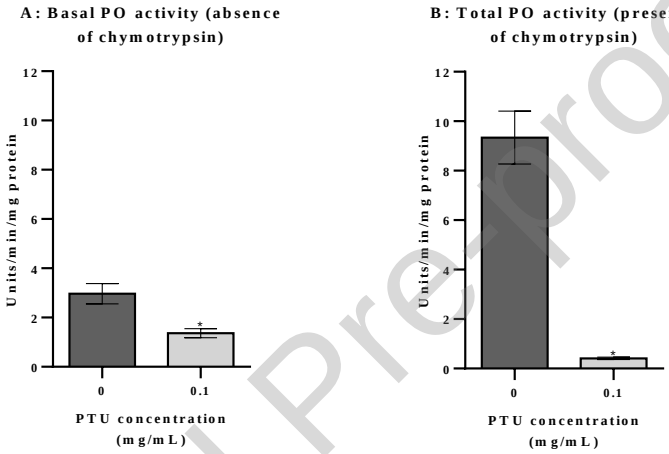


Fig. 2. Effect of PTU (0.1mg mL^{-1}) on A: basal phenoloxidase (PO) (absence of chymotrypsin) and B: total PO (PO+ProPO; 0.5 mg mL^{-1} chymotrypsin) activities of *Chironomus riparius*. Values are presented as the mean of five samples ± SEM. * symbolizes a significant difference compared to control (0) treatment at $p < 0.05$ (t-test).

3.3. Effects of zymosan (Zs) and lipopolysaccharides (LPS) on basal PO and proPO activation

Significant increases of basal PO were observed on larvae of *C. riparius* after *in vitro* treatment with 1 mg mL^{-1} LPS ($t_{(6)}$=5.849, p=0.0011; Figure 3A) and 0.1 mg mL^{-1} Zs ($t_{(6)}$=3.156, p=0.0197; Figure 3B) compared to respective control. Basal phenoloxidase activity increased 100 ± 13 % in the presence of LPS and 52 ± 23 % in the presence of

zymosan, suggesting activation of PO system in the presence of bacterium and fungi components (Figure 3A, B).

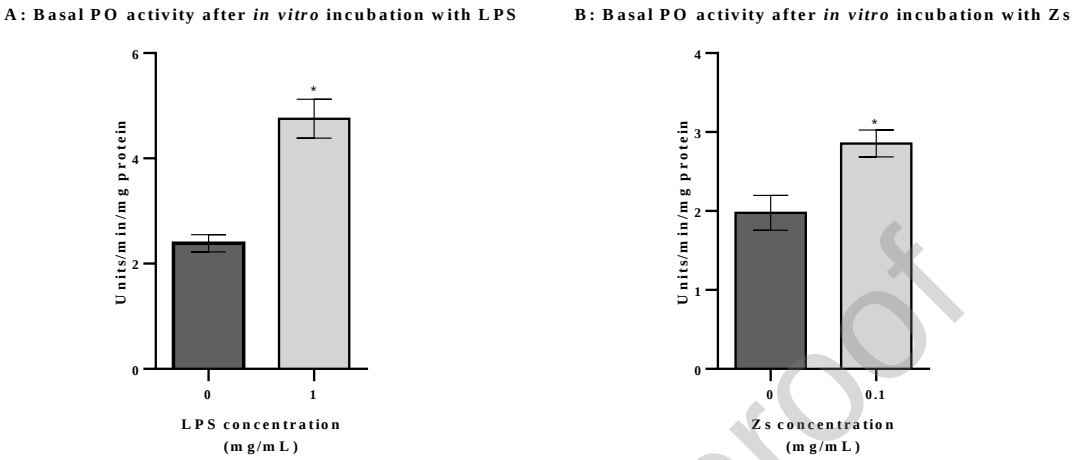


Fig. 3. Basal phenoloxidase (PO) activity on homogenates of *Chironomus riparius* larvae after *in vitro* exposure to 1mg mL^{-1} lipopolysaccharides (LPS) (A) and 0.1 mg mL^{-1} zymosan (Zs) (B). Values are presented as the mean of four samples ± SEM. * symbolizes a significant difference compared to control (0) treatment at $p < 0.05$ (t-test).

3.4. Basal PO and total PO activities in *Chironomus riparius* larvae exposed to PE-MPs

Basal phenoloxidase activity was significantly increased in larvae exposed to 5 and 20 g kg^{-1} PE-MPs compared to control ($F_{(3,23)}$= 5,251, p=0.0066, Figure 4), reaching increases of 26 ± 8 % and 29 ± 7 %, respectively. The activity of total PO was significantly increased (48 ± 9 %) in larvae exposed to the highest concentration of PE-MPs (20 g kg^{-1}) tested compared to total PO activity of control ($F_{(3,23)}$= 9,071, p= 0.0004, Figure 4). Moreover, and considering all treatment conditions, the total PO activity was always significantly higher than basal PO activity ($t_{(6)}$=6.130, p=0.0009). The activity of total PO observed in larvae of control and exposed to 1.25 and 5 g kg^{-1} was 6,41±0,33; 6,95±1,22 and 6,87±0,77 Units/min/mg protein, respectively,

representing activation of +177; and +108 compared to basal PO activity, respectively. The activity of total PO observed in larvae exposed to 20 g kg^{-1} was 9,50± 1,92 Units/min/mg protein representing activation of +182 % compared to basal PO activity. The increased total PO activity (Figure 4) shows the activation of almost all existent proPO (+182%) and suggests its *de novo* synthesis (+48%).

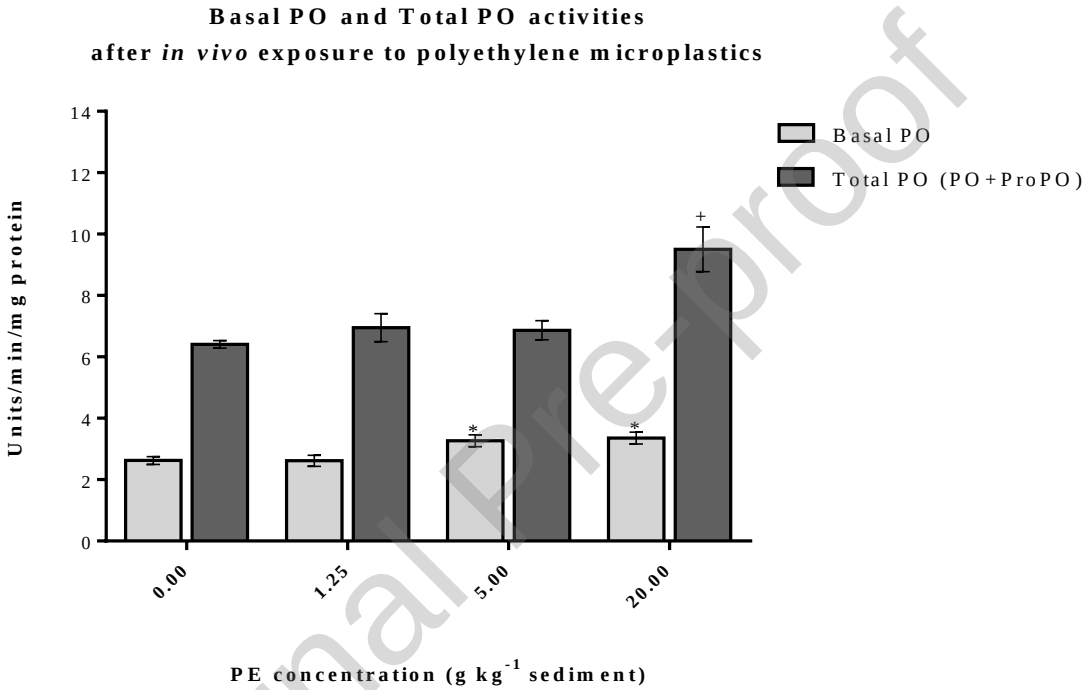


Fig. 4. Activities of basal phenoloxidase (PO) and total phenoloxidase (PO+proPO) in larvae of *Chironomus riparius* after *in vivo* exposure to PE-MPs (0, 1.25, 5 and 20 g kg^{-1} dry sediment). Values are the mean of seven replicates performed in quadruplicated ± SEM and expressed as Units/min/mg protein. * represents significant statistical differences (p<0.05) from control (PO) and (+) represents statistical differences from control (total PO).

4. DISCUSSION

4.1. Phenoloxidase system in *Chironomus riparius*

The present study showed that phenoloxidase activity in *C. riparius* larvae (whole-body homogenates) was activated by chymotrypsin. In fact, *in vitro* activation of proPO by chymotrypsin was previously observed using whole-body samples of larvae of *Chironomus riparius* (Bordalo et al., 2020). However, the characterisation of this activity was not carried out by studying the effect of specific inhibitors of true type o-diphenoloxidase. Our results show that the typical o-diphenoloxidase inhibitor – PTU – significantly inhibited the activity of not only total PO (> -95 %) but also PO activity (~ -50%), which confirms the occurrence of PO system in *C. riparius* (Hellio et al., 2007) and that the measured PO activities of whole-body samples of larvae result mainly from a true type of o-diphenoloxidase. Phenoloxidase system is a key element of the immune response of many insects (González-Santoyo & Córdoba-Aguilar, 2012), including *C. riparius* (Lilley et al., 2012). As in all insect species (Ashida & Brey, 1995), *C. riparius* larvae hold a latent pro-enzyme (proPO). ProPO is activated by a cascade of enzymatic reactions that include pattern recognition proteins, proteinases, and regulatory factors (Cerenius & Söderhäll, 2004). Moreover, this PO system was significantly increased in *C. riparius* larvae homogenates by the presence of LPS (+100.35 %) and Zs (+52.04 %) *in vitro*, confirming that phenoloxidase present in the homogenates is activated in the presence of bacterial and fungal PAMPs. This fact is in good agreement with a previous work that reported increased PO activity on whole-body samples of larvae of *C. riparius* exposed to bioinsecticides based on bacterial and fungal components (Bordalo et al., 2020). Other studies using haemolymph of insects exposed to bacteria, fungi, and viruses also showed an increased PO activity (Götz et al., 1987; Stączek et al., 2020). The use of whole-body measurement of phenoloxidase allows for simultaneous

determination of several other parameters since it only needs a small volume aliquot of the homogenate and also reduces the number of organisms needed for the measurements of phenoloxidase activity.

4.2. Effects of PE-MPs on *C. riparius* PO system and potential physiological costs

The novelty of this study was the unequivocal activation of the immune system of larvae of *C. riparius* expressed in the PO system activation, probably triggered after mechanical damage caused by PE-MPs in their gut. Fourth instar *C. riparius* larvae are known to present a significant number of PE-MPs in their gut after 48 h exposure, which can be dependent on PE-MP concentration in the sediment if the particles size is compatible with larval mouth apparatus for their ingestion (up to ~2400 particles/organism, with the size range of 32-64 µm) (Silva et al., 2021a). The presence (and previously suggested potential accumulation) of such PE-MPs in the larval gut triggered the activation of the immune system of *C. riparius* observed as increased activity of PO in exposed organisms (PO system activation) to PE-MPs concentrations equal or higher than 5 g kg^{-1}. The activation of this immune response occurred in larvae that ingested large amounts of PE-MPs (>2000 PE-MPs/larvae) (Silva et al., 2021a). Furthermore, the total PO's increased activity was observed on larvae exposed to 20 g kg^{-1}, suggesting the possibility of *de novo* synthesis of proPO. Additional ProPO synthesis indicates a possible attempt to refuel the zymogen levels, capable of being readily activated in the active form (PO) to be involved in the melanisation process. Likewise, increased levels of proPO after infection have already been reported in insects (Gillespie et al., 2000). The here observed activation of PO together with possible *de novo* synthesis of ProPO may indicate a stronger stimulation of immune response on larvae exposed to the highest concentration of PE-MPs (20 g kg^{-1}). This possibility is in

good agreement with the maximum ingestion of PE-MPs (up to ~2400 PE-MPs/larvae) previously observed, related to potential gut passage block or retention (Silva et al., 2021a).

The activity of phenoloxidase and activation of the inactive form ProPO can be associated with encapsulation of foreign particles and/or immune response after mechanical damage to the epithelial cells of the gut lumen of larvae. The encapsulation of foreign bodies, like MPs into nodules, i.e., granulocytomas, has been observed after short exposure to MPs (size: < 10 μm) in the mussel *Mytilus edulis* (Von Moos et al., 2012). However, the MPs ingested by *C. riparius* were generally too large to cross the gut lumen and be encapsulated in the haemolymph (size range 32-63 μm). Thus, the observed immune response translated in basal and total PO activation after the ingestion of PE-MPs may be linked to damage to the gut epithelium, which is a recognised immunologically active tissue (Wilson et al., 2001; Krautz et al., 2014). Mechanical damage to gut epithelium can also activate other components of the immune system, namely, haemocytes, which are involved in wound repair (Rowley & Powell, 2007; Grizanova et al., 2014). Several other studies have described immune responses mainly through increased phagocytic activity after exposure to MPs (Von Moos et al., 2012; Wright et al., 2013; Avio et al., 2015; Rodriguez-seijo et al., 2017). Moreover, a potential damage in epithelial gut' cells can also represent an entrance door for pathogens present in the gut, allowing them to enter the main body cavity. In response to this entrance, the immune system can also be activated, and the pathogens generally phagocytosed by immune cells (Rowley & Powell, 2007).

The damage of tissues inside the intestinal tract of invertebrates induced by microplastics has been associated with increased oxidative stress (Magara et al., 2019). The activation of PO system for the melanisation process involves the production and

release of quinones, phenols, and reactive oxygen species (ROS) by hemocytes and can even harm the host if produced in excess (Siva-Jothy et al., 2005; Saad and Siva-Jothy, 2006; González-Santoyo & Córdoba-Aguilar, 2012; Stahlschmidt et al., 2015). The increased production of ROS as a product of PO activation may partially explain the alteration in antioxidant capacity and subsequent oxidative damage observed in *C. riparius* larvae exposed to similar-sized PE-MPs (Silva et al., 2021a). This possibility has already been suggested after the reported oxidative damage that was linked to possible gut passage blocking and probable prolonged residence time of inert particles inside *C. riparius* larval gut (Scherer et al., 2017; Silva et al., 2021a).

It is thus clear that induction of the immune challenge triggered by MPs can further increase the energy constraints suffered by organisms since energy is diverted from growth/reproduction to maintenance, i.e., to sustain multiple energetically expensive processes related to melanogenesis and other cellular responses (Olsen et al., 2015; Wolowczuk et al., 2008) and thus may imply physiological costs (Siva-Jothy et al., 2005).

Physiological costs of immune responses and increased phenoloxidase activity were shown before as the reduced growth of the damselfly *Coenagrion puella* exposed to non-pathogenic bacteria (Janssens & Stoks, 2014), and the reduced longevity of the beetle, *Tenebrio molitor* after insertion of a inert sterile nylon monofilament (Armitage et al., 2003).

The activation of the innate immune response induced by MPs ingestion (here confirmed by the PO activation); the possible *de novo* synthesis of prophenoloxidase; the production of ROS and associated mechanisms of infection tolerance (e.g., glutathione synthesis to cope with increased ROS) are all energy-demanding processes that can carry physiological costs and affect life-history traits of the organisms. In fact,

reductions in growth, altered development rates or in reproduction have been observed before in *Chironomus tepperi* (Ziajahromi et al., 2018), *Chironomus riparius* (Scherer et al., 2019; Silva et al., 2019; Khosrovyan et al., 2020; Stanković et al., 2020), and *Hyalella azteca* (Au et al., 2015) after exposure to MPs at similar concentrations as tested here. These effects are, at least in part, a likely consequence of the costs associated with the activation of immune response after mechanical damage induced by MPs.

5. Conclusions

This investigation provides the first evidence of the activation of insects' innate immune system in response to foreign agents such as MPs. The ingestion and retention of MPs in *C. riparius* larval gut induced the immune response through the activation of phenoloxidase (PO) system. However, the size of the PE-MPs ingested by *C. riparius* larvae was considerably large to cross biological barriers; thus, the activation of immune response – PO activation and the subsequent melanin production is probably linked to mechanical/proteolytic damage of the gut epithelium rather than endocytosis.

PO activation and melanisation processes are linked to ROS production, consequent oxidative stress, and increased energy expenditure, which might explain the deleterious effects in life-history traits observed in *C. riparius*. Changes in the immune system, energy metabolism and antioxidant defences induced by MPs might increase the susceptibility to further deal with pathogens, other pollutants, and abiotic stressors, compromising population fitness under natural conditions.

Additional studies, including co-exposure of different polymer-type MPs and pathogens, can be interesting to investigate immune responses under realistic exposure scenarios and its physiological consequences for invertebrates. It is also of great importance to further understand which damage signals might be involved in the

activation of PO isoforms in *C. riparius* after ingestion of MPs using omics approaches and analysis of gene expression (Reverse Transcription Polymerase Chain Reaction - RT-PCR) to confirm/corroborate *de novo* synthesis of prophenoloxidase and ratio of basal PO and total PO. Finally, the innate immune responses would be important sensitive endpoints to assess the effects of nanoplastics that, besides their potential physical damage to gut epithelium, might also be internalised by cells and accumulate in different tissues.

Acknowledgements

Thanks are due to FCT/MCTES for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020), through national funds, and to research project (POCI-01-0145-FEDER-030361) funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/ MCTES.

CS was supported by Portuguese Science Foundation (FCT) through scholarship PD/BD/128134/2016 and ALPS was supported by CEECIND/01366/2018; whereas JLTP and CG were supported by the FCT research contracts IF/01420/2015 and IF/01401/2014, respectively; all under POCH funds, co-financed by the European Social Fund and Portuguese National Funds from MEC. DC has a research contract within the project ComPET (nr. 5662).

References

Aladaileh, S., Rodney, P., Nair, S. V., & Raftos, D. A. (2007). Characterization of phenoloxidase activity in Sydney rock oysters (*Saccostrea glomerata*). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *148*(4), 470–480. https://doi.org/10.1016/j.cbpb.2007.07.089

Armitage, S.A.O., Thompson, J.J.W., Rolff, J., Siva-Jothy, M.T. (2003). Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. *J. Evol. Biol.*, *16*, 1038–1044. https://doi.org/10.1046/j.1420-9101.2003.00551.x

Ashida, M., & Brey, P. T. (1995). Role of the integument in insect defense: Pro-phenol oxidase cascade in the cuticular matrix. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(23), 10698–10702. https://doi.org/10.1073/pnas.92.23.10698

Asokan, R., Arumugam, M., & Mullainadhan, P. (1997). Activation of prophenoloxidase in the plasma and haemocytes of the marine mussel *Perna viridis* Linnaeus. *Developmental and Comparative Immunology*, *21*(1), 1–12. https://doi.org/10.1016/S0145-305X(97)00004-9

Avio, C. G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'Errico, G., Pauletto, M., Bargelloni, L., & Regoli, F. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution*, *198*, 211–222. https://doi.org/10.1016/j.envpol.2014.12.021

Bordalo, M. D., Gravato, C., Beleza, S., Campos, D., Lopes, I., & Pestana, J. L. T. (2020). Lethal and sublethal toxicity assessment of *Bacillus thuringiensis* var. *israelensis* and *Beauveria bassiana* based bioinsecticides to the aquatic insect Chironomus riparius. *Science of the Total Environment*, *698*, 134155.

https://doi.org/10.1016/j.scitotenv.2019.134155

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *254*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Browne, M. A., Niven, S. J., Galloway, T. S., Rowland, S. J., & Thompson, R. C. (2013). Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Current Biology*, *23*(23), 2388–2392. https://doi.org/10.1016/j.cub.2013.10.012

Cerenius, L., & Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, *198*, 116–126. https://doi.org/10.1111/j.0105-2896.2004.00116.x

Cotter, S. C., & Wilson, K. (2002). Heritability of immune function in the caterpillar *Spodoptera littoralis*. *Heredity*, *88*(4), 229–234. https://doi.org/10.1038/sj.hdy.6800031

Davis, M. M., and Engstrom, Y. (2012). Immune response in the barrier epithelia: lessons from the fruit fly *Drosophila melanogaster*. *J. Innate Immun.*, *4*, 273–283. doi: 10.1159/000332947

Détrée, C., & Gallardo-Escárate, C. (2017). Polyethylene microbeads induce transcriptional responses with tissue-dependent patterns in the mussel *Mytilus galloprovincialis*. *Journal of Molluscan Studies*, *83*(2), 220–225. https://doi.org/10.1093/mollus/eyx005

Détrée, C., & Gallardo-Escárate, C. (2018). Single and repetitive microplastics exposures induce immune system modulation and homeostasis alteration in the edible mussel *Mytilus galloprovincialis*. *Fish and Shellfish Immunology*,

83(September), 52–60. https://doi.org/10.1016/j.fsi.2018.09.018

Eleftherianos, I., & Revenis, C. (2011). Role and importance of phenoloxidase in insect hemostasis. *Journal of Innate Immunity*, *3*(1), 28–33. https://doi.org/10.1159/000321931

Gardon, T., Morvan, L., Huvet, A., Quillien, V., Soyez, C., Le Moullac, G., & Le Luyer, J. (2020). Microplastics induce dose-specific transcriptomic disruptions in energy metabolism and immunity of the pearl oyster *Pinctada margaritifera*. *Environmental Pollution*, *266*. https://doi.org/10.1016/j.envpol.2020.115180

Gillespie, J. P., Bailey, A. M., Cobb, B., & Vilcinskas, A. (2000). Fungi as elicitors of insect immune responses. *Archives of Insect Biochemistry and Physiology*, *44*(2), 49–68. https://doi.org/10.1002/1520-6327(200006)44:2<49::AID-ARCH1>3.0.CO;2-F

González-Santoyo, I., & Córdoba-Aguilar, A. (2012). Phenoloxidase: A key component of the insect immune system. *Entomologia Experimentalis et Applicata*, *142*(1), 1–16. https://doi.org/10.1111/j.1570-7458.2011.01187.x

Götz, P., Enderlein, G., & Roettgen, I. (1987). Immune reactions of *Chironomus* larvae (Insecta: Diptera) against bacteria. *Journal of Insect Physiology*, *33*(12), 993–1004. https://doi.org/10.1016/0022-1910(87)90013-8

Grizanova, E. V., Dubovskiy, I. M., Whitten, M. M. A., & Glupov, V. V. (2014). Contributions of cellular and humoral immunity of *Galleria mellonella* larvae in defence against oral infection by *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, *119*, 40–46. https://doi.org/10.1016/j.jip.2014.04.003

Hellio, C., Bado-Nilles, A., Gagnaire, B., Renault, T., & Thomas-Guyon, H. (2007). Demonstration of a true phenoloxidase activity and activation of a ProPO cascade

in Pacific oyster, *Crassostrea gigas* (Thunberg) in vitro. *Fish and Shellfish Immunology*, *22*(4), 433–440. https://doi.org/10.1016/j.fsi.2006.06.014

Hurley, R., Woodward, J., & Rothwell, J. J. (2018). Microplastic contamination of river beds significantly reduced by catchment-wide flooding. *Nature Geoscience*, *11*(4), 251–257. https://doi.org/10.1038/s41561-018-0080-1

Janssens, L., Stoks, R. (2014). Reinforcing effects of non-pathogenic bacteria and predation risk: from physiology to life history. *Oecologia*, *176*, 323–332. https://doi.org/10.1007/s00442-014-3030-7

Khosrovyan, A., & Kahru, A. (2020). Evaluation of the hazard of irregularly-shaped co-polyamide microplastics on the freshwater non-biting midge *Chironomus riparius* through its life cycle. *Chemosphere*, *244*, 125487. https://doi.org/10.1016/j.chemosphere.2019.125487

Krautz, R., Arefin, B., & Theopold, U. (2014). Damage signals in the insect immune response. *Frontiers in Plant Science*, *5*, 1–11. https://doi.org/10.3389/fpls.2014.00342Lee, S. Y., Kwon, T. H., Hyun, J. H., Choi, J. S., Kawabata, S. I., Iwanaga, S., & Lee, B. L. (1998). In vitro activation of pro-phenol-oxidase by two kinds of pro-phenol- oxidase-activating factors isolated from hemolymph of coleopteran, *Holotrichia diomphalia* larvae. *European Journal of Biochemistry*, *254*(1), 50–57. https://doi.org/10.1046/j.1432-1327.1998.2540050.x

Lilley, T. M., Ruokolainen, L., Pikkarainen, A., Laine, V. N., Kilpimaa, J., Rantala, M. J., & Nikinmaa, M. (2012). *Impact of Tributyltin on Immune Response and Life History Traits of Chironomus riparius : Single and Multigeneration E ff ects and Recovery from Pollution*. https://doi.org/10.1021/es300536t

Magara, G., Khan, F. R., Pinti, M., Syberg, K., Inzirillo, A., & Elia, A. C. (2019). Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (*Mytilus edulis*). *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *82*(10), 616–625. https://doi.org/10.1080/15287394.2019.1633451

Muñiz-González, A. B., & Martínez-Guitarte, J. L. (2020). Combined effects of benzophenone-3 and temperature on gene expression and enzymatic activity in the aquatic larvae *Chironomus riparius*. *Science of the Total Environment*, *698*, 134292. https://doi.org/10.1016/j.scitotenv.2019.134292

Nel, H.A., Dalu, T., Wasserman, R.J. (2018). Sinks and sources: assessing microplastic abundance in river sediment and deposit feeders in an Austral temperate urban river system. *Science of the Total Environment*, *612*, 950-956. https://doi.org/10.1016/j.scitotenv.2017.08.298

Olsen, T. B., Christensen, F. E. G., Lundgreen, K., Dunn, P. H., & Levitis, D. A. (2015). Coelomic transport and clearance of durable foreign bodies by starfish (*Asterias rubens*). *Biological Bulletin*, *228*(2), 156–162. https://doi.org/10.1086/BBLv228n2p156

Pang, Q., Liu, X., Zhao, B., Jiang, Y., Su, F., Zhang, X., Nie, M., Zhang, M., & Sun, H. (2010). Detection and characterization of phenoloxidase in the freshwater planarian *Dugesia japonica*. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *157*(1), 54–58. https://doi.org/10.1016/j.cbpb.2010.05.002

Pang, Q., Zhang, S., Shi, X., Su, F., & Wu, D. (2005). Purification and characterisation of phenoloxidase from amphioxus *Branchiostoma belcheri tsingtauense*. *Fish and Shellfish Immunology*, *19*(2), 139–148. https://doi.org/10.1016/j.fsi.2004.12.001

Pang, Q., Zhang, S., Wang, C., Shi, X., & Sun, Y. (2004). Presence of prophenoloxidase in the humoral fluid of amphioxus *Branchiostoma belcheri tsingtauense*. *Fish and Shellfish Immunology*, *17*(5), 477–487. https://doi.org/10.1016/j.fsi.2004.05.003

Pittura, L., Avio, C. G., Giuliani, M. E., d'Errico, G., Keiter, S. H., Cormier, B., Gorbi, S., & Regoli, F. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: Combined chemical and physical hazards to the mediterranean mussels, *Mytilus galloprovincialis*. *Frontiers in Marine Science*, *5*(APR). https://doi.org/10.3389/fmars.2018.00103

Rodriguez-seijo, A., Lourenço, J., Rocha-santos, T. A. P., Costa, J., Duarte, A. C., Vala, H., & Pereira, R. (2017). Histopathological and molecular effects of microplastics in *Eisenia andrei* Bouché. *Environmental Pollution*, *220*, 495–503. https://doi.org/10.1016/j.envpol.2016.09.092

Rowley, A. F., & Powell, A. (2007). Invertebrate immune systems–specific, quasi-specific, or nonspecific? *The Journal of Immunology*, *179*(11), 7209–7214. https://doi.org/10.4049/jimmunol.179.11.7209

Sadd, B. M., & Siva-Jothy, M. T. (2006). Self-harm caused by an insect's innate immunity. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1600), 2571–2574. https://doi.org/10.1098/rspb.2006.3574

Scherer, C., Brennholt, N., Reifferscheid, G., & Wagner, M. (2017). Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. *Scientific Reports*, *7*(17006), 1–9. https://doi.org/10.1038/s41598-017-17191-7

Scherer, C., Wolf, R., Völker, J., Stock, F., Brennhold, N., Reifferscheid, G., & Wagner, M. (2019). Toxicity of microplastics and natural particles in the freshwater dipteran *Chironomus riparius*: Same same but different? *Science of The*

Total Environment, *xxxx*, 134604. https://doi.org/10.1016/j.scitotenv.2019.134604

Silva, C. J. M., Patrício, A. L., Gravato, C., & Pestana, J. L. T. (2019). Ingestion of small-sized and irregularly shaped polyethylene microplastics affect *Chironomus riparius* life-history traits. *Science of the Total Environment*, *672*, 862–868. https://doi.org/10.1016/j.scitotenv.2019.04.017

Silva, C. J. M., Patrício Silva, A. L., Campos, D., Machado, A. L., Pestana, J. L. T., & Gravato, C. (2021a). Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by *Chironomus riparius* (Diptera) larvae. *Journal of Hazardous Materials*, *402*, 123775. https://doi.org/https://doi.org/10.1016/j.jhazmat.2020.123775

Silva, C. J. M., Patrício Silva, A. L., Campos, D., Soares, A.M.V.M., Pestana, J. L. T., & Gravato, C. (2021b). *Lumbriculus variegatus* (oligochaeta) exposed to polyethylene microplastics: biochemical, physiological and reproductive responses. *Ecotoxicology and Environmental Safety*, *207*, 111375. https://doi.org/10.1016/j.ecoenv.2020.111375

Siva-Jothy, M. T., Moret, Y., & Rolff, J. (2005). Insect immunity: an evolutionary ecology perspective. In *Advances in Insect Physiology* (Vol. 32, Issue 05). https://doi.org/10.1016/S0065-2806(05)32001-7

Smith, V. J., Söderhäll, K., & Hamilton, M. (1984). β 1,3-Glucan induced cellular defence reactions in the shore crab, *Carcinus maenas*. *Comparative Biochemistry and Physiology -- Part A: Physiology*, *77*(4), 635–639. https://doi.org/10.1016/0300-9629(84)90176-2

Stączek, S., Zdybicka-Barabas, A., Pleszczyńska, M., Wiater, A., & Cytryńska, M. (2020). *Aspergillus niger* α-1,3-glucan acts as a virulence factor by inhibiting the

insect phenoloxidase system. *Journal of Invertebrate Pathology*, *171*(February). https://doi.org/10.1016/j.jip.2020.107341

Stahlschmidt, Z. R., Acker, M., Kovalko, I., & Adamo, S. A. (2015). The double-edged sword of immune defence and damage control: Do food availability and immune challenge alter the balance? *Functional Ecology*, *29*(11), 1445–1452. https://doi.org/10.1111/1365-2435.12454

Stanković, J., Milošević, D., Savić-Zdraković, D., Yalçın, G., Yildiz, D., Beklioğlu, M., & Jovanović, B. (2020). Exposure to a microplastic mixture is altering the life traits and is causing deformities in the non-biting midge *Chironomus riparius* Meigen (1804). *Environmental Pollution*, *262*. https://doi.org/10.1016/j.envpol.2020.114248

van Ooik, T., Pausio, S., & Rantala, M. J. (2008). Direct effects of heavy metal pollution on the immune function of a geometrid moth, *Epirrita autumnata*. *Chemosphere*, *71*(10), 1840–1844. https://doi.org/10.1016/j.chemosphere.2008.02.014

van Ooik, T., Rantala, M. J., & Saloniemi, I. (2007). Diet-mediated effects of heavy metal pollution on growth and immune response in the geometrid moth *Epirrita autumnata*. *Environmental Pollution*, *145*(1), 348–354. https://doi.org/10.1016/j.envpol.2006.03.008

Von Moos, N., Burkhardt-Holm, P., & Köhler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environmental Science and Technology*, *46*(20), 11327–11335. https://doi.org/10.1021/es302332w

Wilson, K., Cotter, S. C., Reeson, A. F., & Pell, J. K. (2001). Melanism and disease

resistance in insects. *Ecology letters*, *4*(6), 637-649.

Wolowczuk, I., Verwaerde, C., Viltart, O., Delanoye, A., Delacre, M., Pot, B., & Grangette, C. (2008). Feeding our immune system: Impact on metabolism. *Clinical and Developmental Immunology*, *2008*. https://doi.org/10.1155/2008/639803

Wright, S. L., Rowe, D., Thompson, R. C., & Galloway, T. S. (2013). Microplastic ingestion decreases energy reserves in marine worms. *Current Biology*, *23*(23), 1031–1033. https://doi.org/10.1016/j.cub.2013.10.068

Yousefi-Lardeh, L., & Zibaee, A. (2020). Biochemical characterization of a hemolymph phenoloxidase and its endogenous inhibitor in the larvae of an invasive moth, *Cydalima perspectalis* Walker (Lepidoptera: Crambidae). *Journal of Asia-Pacific Entomology*, *23*(4), 890–900. https://doi.org/10.1016/j.aspen.2020.07.018

Ziajahromi, S., Kumar, A., Neale, P. A., & Leusch, F. D. L. (2018). Environmentally relevant concentrations of polyethylene microplastics negatively impact the survival, growth and emergence of sediment-dwelling invertebrates. *Environmental Pollution*, *236*, 425–431. https://doi.org/10.1016/j.envpol.2018.01.094

CRediT authorship contribution statement

Carlos J.M. Silva: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Data curation. **Sónia Beleza:** Conceptualization, Investigation, Writing - review & editing. **Ana L. Patrício Silva**: Funding acquisition, Project administration, Formal analysis, Data curation, Writing - review & editing. **Diana Campos**: Investigation, Formal analysis, Writing - review & editing, Data curation. **Amadeu M.V.M. Soares**: Funding acquisition, Writing - review & editing, Formal analysis. **João L.T. Pestana**: Conceptualization, Funding acquisition, Investigation, Project administration, Formal analysis, Data curation, Supervision, Writing - review & editing. **Carlos Gravato**: Conceptualization, Funding acquisition, Investigation, Formal analysis, Data curation, Supervision, Writing - review & editing.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Phenoloxidase was characterized in *Chironomus riparius* larvae whole-body homogenates
- Phenoloxidase activity was induced by ingestion of microplastics in *C. riparius* larvae
- Immune responses can be sensitive indicator of the sub lethal effects induced by microplastics ingestion