Linking sub-individual and supra-individual effects in *Daphnia magna* exposed to sub-lethal concentration of chlorpyrifos

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ABSTRACT

The main objective of the present study was to investigate possible links between sub-individual and supra-individual levels (i.e. population level) biomarkers in *D. magna* exposed to sublethal concentrations of the insecticide chlorpyrifos (CPF). To achieve the aim, 8d old individuals were exposed for 96 hrs to two environmentally relevant concentrations of CPF (50 and 250 ng/L). Sub-individual level effects were investigated by measuring the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes, as well as by measuring the acetylcholinesterase (AChE) inhibition. In addition, the effects at supra-individual level were assessed by using a video-tracking system and analyzing changes in swimming capabilities (i.e. percentage of activity time, distance moved, and velocity). Our data have shown that daphnids exposed to both CPF concentrations were in a condition of stress which was highlighted by changes in both sub- and supra-individual biomarkers. Moreover, our results highlighted that the lowest tested CPF concentration did not modulate the antioxidant and detoxifying enzymes, whereas, an inhibition of AChE and a decrease of some parameters related to swimming behavior (distance moved and velocity) were noted. On the contrary, significant changes in all the sub-individual biomarkers were measured at the highest tested concentration. In addition, organisms recovered the movement capability (distance moved) and also activate a mechanism of avoidance (increased swimming velocity). On the other hand, a reduction in the percent of active time was measured and this was attributed to the energy spent by organisms to activate antioxidant and detoxifying enzymes and the mechanism of avoidance. Based on these results, our study suggests the existence of a link between sub- and supra-individual levels, as the

activation or non-activation in the antioxidant and detoxifying enzymes activities can led to different modifications of the swimming behaviour in *D. magna*.

KEYWORDS insecticide, oxidative stress, swimming behaviour, video tracking, *Daphnia magna*

Capsule: Effects of chlorpyrifos on biomarker responses and swimming behaviour

1. INTRODUCTION

The ecotoxicological effects of chemical compounds are currently evaluated by means of standardised toxicity tests which are performed on organisms considered representative of the exposed ecosystems (Hood, 2005; Stadler, 2011). For the aquatic compartment, they include tests on algae, invertebrates and fish (the three levels of the trophic chain of the aquatic ecosystems) which are mainly focused on assessing acutely lethal concentrations (e.g., median lethal concentration, LC50) and chronic sub-lethal effects on developmental or reproductive endpoints. According to Amiard-Triquet (2012), in these tests a number of biochemical and physiological processes are completely overwhelmed as they do not allow organisms to cope with contaminants as they do in the field. However, at sub-lethal concentrations (which are commonly measured in the aquatic environments) these mechanisms are functional, and many of them respond on the scale of days or weeks (Amiard-Triquet et al., 2011). The measurement of these sub-individual responses is the basis of the use of biomarkers in ecotoxicology as early warning indicators of potential risk (Forbes et al., 2006). All these mechanisms that are frequently involved in tolerance towards chemical stressors (adaptive mechanism) are energetically expensive, and thus may interfere with the allocation of energy, thereby governing the success of reproduction and growth of individuals and population and, in ultimate analysis, on the relative fitness (Sokolova et al., 2012). Thus the adaptive benefit of being tolerant may have negative counterparts in the long term period. In addition, the stress induced by chemical exposure can also have consequences at the higher hierarchical levels of the bio-ecological organization, from organism, population, up to the community levels (Parolini et al., 2017). For instance, at organism level, the presence of toxicants can lead to several behavioral changes (Boyd et al., 2002) such as the increase of the average speed (i.e., escape from contamination through the so called chemical avoidance), or the decreased swimming activity (protection reaction) (Wolf et al., 1998). Looking at the definition of biomarkers given by Depledge (Depledge and Fossi, 1994), behavioral changes can be included in this category (Forbes et al., 2006). In aquatic toxicology,

behavioral responses of species have been used since the 80s as a method of monitoring and to measure potential environmental stress (Cairns and Gruber, 1980; Kramer et al., 1989; Diamond et al., 1990; Gerhardt et al., 1998; Van der Schalie et al., 2001). Nevertheless, only in recent years, with the improvement of video tracking technologies offering a better quantification of behavioral patterns, these studies are receiving the due attention (Asher, 2009; Little and Brewer, 2001; Amiard-Triquet, 2009; Sloman and McNeil, 2012).

At higher ecological hierarchy, impaired behavior can have detrimental consequences at the population level through altered interactions with other members of the same species and at the community level through changes in competitive or predator/prey interactions. Ultimately, altered behavior can affect ecosystem structure itself (Reichmuth et al., 2009; Duquesne and Küster, 2010). In a review of Faimali and coworkers (2017), it is reported that aquatic vertebrate and invertebrate behavior such as predator-prey interactions, avoidance, and spatial movement have been impacted by toxicants at low concentrations and, for that, have a great potential as ecologically relevant end-points for contributing in ecological risk assessment mainly in the weight of evidence approach (Berninger et al., 2011; Boyd et al., 2002; Dodson and Hanazato, 1995; Gerhardt, 2007; Stanley et al., 2007; Valenti et al., 2009).

On these bases, it is evident that the investigation on how the responses to chemical stress are spread through the different levels of the ecological hierarchy is one of the challenges of modern ecotoxicology (Amiard-Triquet, 2009). In fact, the knowledge of the links between responses measured at a particular hierarchical level and those measured at the adjacent ones would be very effective in the risk assessment procedures, particularly for improving the use of biomarkers as early warning indicators of risk. Indeed, results obtained from studies at biochemical, molecular, cellular and even at organism level do not automatically allow predictions of stress responses at higher levels, such as population and community. For instance, it is difficult to determine whether the biomarker response indicates that an organism has been exposed to a chemical (and is dealing with it successfully) or whether it is being impaired by such exposure (Forbes, 2006). For these reasons, in the last two decades, the integration of several biomarkers at different levels of biological organization has been discussed as a tool to assess the extent of disturbances of a biological system and to quantify its actual state (Broeg et al., 2005; McCarthy and Munkittrick, 1996; Attrill and Depledge, 1997; Allen and Moore, 2004). For instance, Hagger and coworkers (2008) proposed a biomarker response index (BRI) to grade the level of biological impact of contaminants. However, more recently, the number of studies highlighting the link between sub-individual biomarkers responses and behavioral changes is constantly increasing (Ren et al., 2007; Baatrup, 2009;

Ballesteros et al. 2009; Gravato and Guilhermino, 2009; Almeida et al., 2010, Mesquita et al, 2011; Oliveira et al., 2012; Silva et al. 2013; Van Praet et al., 2014; Sabullah et al., 2015; Goodchild et al., 2016; Parolini et al., 2017). The present study is mainly aimed at highlighting the link of stress signals across two levels of bio-ecological hierarchy due to the exposure to chlorpyrifos (CPF). CPF is an organophosphorus insecticide widely used worldwide (George et al., 2014), with specific mode of action on aquatic invertebrates and vertebrates (Kavitha et al., 2008), which is frequently present in aquatic environments at concentration ranging from 0.01 to 1.95 µg/L (Palma et al., 2009).

Particularly, we focused the attention on the stress transition from the sub-individual to the supra-individual levels by measuring changes in molecular and behavioral biomarkers in *Daphnia magna* exposed to two sublethal concentrations of this organophosphorus compound. Regarding biomarkers (sub-individual level), we measured the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes, as well as the acetylcholinesterase (AChE) inhibition. At supra-individual level, we analyzed the changes in swimming behavior of *D. magna* individuals due to CPF exposure by a video tracking approach, focusing on percentage active time, distance moved and active velocity.

2. MATERIALS AND METHODS

2.1 Test chemical and reagents

CPF (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate; purity >99.7%) and the reagents used for biomarker analyses were purchased from Sigma-Aldrich. All solvents (residue analysis grade; Merck Darmstadt, Germany) used for chemical analyses were checked by gas chromatography (GC) before use.

2.2 Test species

Daphnia magna Straus individuals were derived from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy). They were maintained (30 individuals/L) in commercial mineral water (San Benedetto® - conductivity 415 µS cm^{-1} at 20 °C; pH 7.42; 301 mg/L HCO^{3-}, 48.6 mg/L Ca^{2+}; 28.2 mg/L Mg^{2+}). The daphnids were cultured in 400 mL beakers (40 individuals/L of San Benedetto® water) and fed *ad libitum* three times a week with a suspension of the unicellular green algae *Raphidocelis subcapitata* (8×10^6 cells ind^{-1} day^{-1} until they were 8-day old, then 16×10^6 cells ind^{-1} day^{-1}) and the yeast *Saccharomyces cerevisiae* (15×10^6 cells mL^{-1}). The culture medium was renewed every two days. Culture medium, as well as the solutions used for the exposures, were maintained at 20.0 ± 0.5 °C under a 16h light: 8h dark photoperiod, which are conditions ensuring

continuous parthenogenetic reproduction (Frey, 1982). Fourth generation were reared before the starting of the exposure experiments. Eight-day old *D. magna* individuals with dimensions allowing the video tracking of their swimming activity (personal observation) were utilized.

The algae were cultured in 2 L flask filled with ISO 8692/89 medium at 20.0 ± 2 °C under continuous light and shaken through aeration. Algae were harvested during their exponential growth and let for sedimentation in the dark at 4 °C for a week. At the end of sedimentation, the density of algal suspension was determined through a Burker chamber under a brightfield light microscope.

2.3 Test conditions

All the experiments were performed in beakers of 400 ml under static conditions. Eight-day old *D. magna* individuals (in group of 20 specimens) were exposed for 96 hours to 50 and 250 ng/L of CPF (nominal concentrations). The stability in water of CPF during the overall exposure period (96h) was measured by GC-MS and no significant degradation was noticed. Exposures were performed on 8-day old individuals because our preliminary analyses have shown that at this age they reached the minimum dimension allowing the video tracking of their swimming activity (see also Parolini et al., 2018). Exposure concentrations were identified by considering both the EC_{50} (48-h) of CPF to *D. magna* (EC_{50mean} = 500 ng/L) (Pesticide Properties DataBase; Tomlin, 1994; Moore et al., 1998; Kikuchi et al., 2000; Printes and Callaghan, 2003; Palma et al., 2009) and the range of concentrations measured in surface waters (Palma et al., 2009). Individuals were not fed during the experiments. Stock solutions of CPF (0.01 µg/mL and 0.1 µg/mL) were prepared in dimethylsulfoxide (DMSO) and the final concentrations of DMSO was under the level suggested by the OECD guidelines (OECD, 2004). Water solutions of CPF were prepared by spiking water with the stock solutions in DMSO in order to reach the two concentrations of exposure. Four independent experimental replicates were performed. Two negative control beakers (CTRL) containing each one 20 individuals were carried out during the period of exposure in all experimental replicate. Similarly, two control beakers containing 0.0005% of DMSO (DMSO) were also included to verify any carrier solvent effects.

2.4 Analysis of molecular biomarkers

The biomarker suite applied in the present study was performed on homogenates from a pool of all the alive *D. magna* individuals found in each jar at the end of the 96-h of static exposure (CTRL, DMSO, 50 ng/L and 250 ng/L). After video tracking (see the next paragraph), individuals were moved to a 1.5 mL Eppendorf tube, frozen in dry ice and stored at -80 °C until the biochemical analyses. As it cannot be excluded the complete removal of CPF from the outer carapax, individuals were washed

trice with 0.5 mL of homogenization buffer to prevent potential bias caused by *in vitro* interactions. After washing, individuals (17-20 individuals per beaker) were homogenized using a pestle in 100 mM potassium phosphate buffer with the addition of 100 mM KCl, 1 mM EDTA, protease inhibitors 1:100 v/v and 1 mM dithiothreitol (pH 7.4). The homogenates were centrifuged at 15.000 x g for 15 minutes at 4 °C, then the supernatant was collected and immediately processed to determine the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and the inhibition of acetylcholinesterase (AChE) through spectrophotometric methods. All the enzymatic activities were measured in triplicate per each pool. SOD activity was assessed by measuring the inhibition of the reduction of cytochrome c (10 μM) caused by the superoxide anion produced by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 μM) reaction for 1 min at λ = 550 nm (Mc Cord and Fridovich, 1969). We added 25 μL of supernatant to 1.5 mL of reaction mixture. Results were expressed as SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The CAT activity was assessed according to Aebi (1974) by measuring the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (100 mM; pH 7) for 1 min at λ = 240 nm. We added 50 μL of supernatant to 3 mL of reaction mixture. The GPx activity was assessed according to Livingstone et al. (1992) monitoring for 1 min the consumption of NADPH at λ = 340 nm using H_2O_2 (0.2 mM) as substrate in potassium phosphate buffer (50 mM, pH 7) including glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). We added 50 μL of supernatant to 1 mL of reaction mixture. The GST activity was assessed monitoring the reaction of reduced glutathione (1 mM) in phosphate buffer (100 mM; pH 7.4) and CDNB (1 mM) for 1 min at λ = 340 nm (Habig et al., 1974). We added 20 μL of supernatant to 1 mL of reaction mixture. AChE activity was measured following the method described by Jemec et al. (2007), with slight modifications. The reaction mixture (1.5 mL) was prepared in potassium phosphate buffer (100 mM, pH 7.4) with the addition of acetylthiocholine chloride (1 mM) and 5,5' dithiobis-2-nitrobenzoic acid (0.5 mM). Then, 100 μL of supernatant was added to the mixture and the reaction was monitored for 15 min at λ = 412 nm. AChE activity was expressed as nmoles of acetylcholine chloride hydrolyzed min^{-1} mg $protein^{-1}$ (ε = 13,600 M^{-1} cm^{-1}). The activity of all the enzymes was normalized on protein concentration determined with the Bradford method, using bovine serum albumin (BSA) as a standard.

2.5 Analysis of behavioral biomarkers

Video tracking analyses were performed on all the alive individuals at the end of the 96h exposures into 24-well plates. Each well contained 1 individual in 3 mL of culture medium, which was tracked individually. After a brief acclimation (3 minutes), video recordings were carried out by placing the

24-well plate with 17-20 animals on a light panel, and the movement of each individual was tracked three times for 15 seconds. The three 1080p Full HD videos acquired for each individual was analyzed using the software LoliTrack v.4 (Loligo Systems, Tjele, Denmark). This software was calibrated to measure the following endpoints: swimming velocity (mm/s), distance moved (mm) and active/inactive time (%). Tracking was based on differences in contrast between objects (animals) and background (water) without use of markers. When the object appeared against a contrasting background, the software assigned a coordinate pair (x, y) to the centroid of the contrasting object. Each well in the 24-well plates was defined as an arena, and each individual was considered as a single object. Data were reported as the mean of the three replicates per each single individual.

2.6 Statistical analysis

The effects of CPF exposure on the activity of antioxidants, GST and AChE, as well on the swimming activity endpoints of 8-day old *D. magna* individuals were investigated by using a one-way Analysis of Variance (ANOVA), after controlling for normal distribution and homoscedasticity of data. Each single endpoint was considered as dependent variable, while the treatments as predictor. When a significant effect of treatment was found, a Fisher LSD *post-hoc* test was applied to point out significant differences between treatments. Significance was set at $P < 0.05$. Statistical analyses were performed by using STATISTICA 7.0 software package (StatSoft, Inc., 2004) and R 3.1.2 software (R core team 2015).

3. RESULTS AND DISCUSSION

At the end of the exposure period, no significant difference in mortality/immobilization was found among the treated and untreated samples ($p > 0.05$). In the following paragraphs the results obtained both for the molecular and behavioral biomarkers are presented and discussed.

3.1 Molecular biomarkers (sub-individual level)

In invertebrates, enzyme activities and other sub-cellular components are commonly used as biomarkers to identify causal mechanisms potentially responsible for effects at higher levels of bio-ecological organization. These include various defense enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), and the acetylcholinesterase (AChE). SOD represent the first defense against free radicals, intervening by dismuting the most reactive and dangerous molecules, such as the superoxide anion, into ions that are less reactive (Shi et al., 2010), CAT and GPx decomposes the hydrogen peroxide into water and

oxygen (Halliwell and Gutteridge, 2007). The GST catalyzes the conjugation of glutathione with diverse electrophilic molecules and contributes to the prevention of oxidative damage by conjugating glutathione to breakdown products of lipid peroxidation (Ketterer et al., 1983). In case the activities of these enzymes are not sufficiently adequate, the organism can be exposed to high levels of pro-oxidant molecules, which are produced during the metabolic pathways of contaminants (including pesticides) and this can lead to oxidative stress and consequent damage to lipids, proteins and DNA (Trypuć, 2017).

The measure of the acetylcholinesterase (AChE) activity is also frequently utilized as a useful biomarker to indicate that organisms have been exposed to a cholinesterase-inhibiting compound (such as organophosphate, carbamate insecticides, metals or detergents) at a sufficiently high level to elicit a significant effect (Lionetto et al., 2011). The inhibition of AChE suggests an over-accumulation of the acetylcholine, causing prolonged electrical activity at nerve endings and ultimately leading to death.

In the first part of our study, we measured changes in the activities of all the previously described biomarkers in 8-day old *D. magna* individuals after 96h of exposure to 50 ng/L and 250 ng/L of CPF (Figure 1). This allowed us to get an overall picture about the effects at sub-individuallevel. Since the activity of CAT was significantly increased in DMSO treated specimens with respect to CTRL, we compared the effects of CPF both to CTRL and DMSO. A significant effect of the treatments was found for all the molecular biomarkers: SOD ($F = 9.723$; $p < 0.01$), CAT ($F = 58.310$; $p < 0.01$), GPx ($F = 35.041$; $p < 0.01$) and GST ($F = 9.113$; $p < 0.01$), AChE ($F = 6.483$; $p < 0.05$). Whilst the lowest tested concentration did not cause a significant modulation of antioxidant and detoxifying enzymes ($p > 0.05$ in all the cases, except of a significant reduction of CAT compared to DMSO), the exposure to 250 ng/L of CPF induced a 2- to 4-fold significant increase of SOD, CAT, GPx and GST. Similarly, CPF exposure had a significant effect on AChE activity of individuals, which showed an inhibition accounting for the 22% compared to DMSO). However, no significant differences were found between the two tested concentrations.

- *SOD activity*

The enhancement in SOD activity of CPF-treated *D. magna* individuals suggested that this pesticide induced superoxide radicals ($O_2^{\cdot -}$) with the increase in concentrations. In an analogous experiment on *D. magna* exposed to CPF, Song and coworkers (2017), highlighted the dependency of the SOD activity in function of both the experiment duration and the exposure concentration. These authors, found that after 24h of exposure the SOD activity in 6-24 h old specimens of *D. magna* did not

significantly change in all the experimental treatments (range 360-5,720 ng/L). However, after 48h of exposure, SOD activity showed an increasing trendfirst (reaching a peak at 720 ng/L of exposure) followed by a decrease according to the increase of the concentrations. This may be explained by the oxidation of SOD cysteine due to superoxide anions or their transformation to hydrogen peroxide (Dimitrova et al., 1994). Our observation on SOD activity somewhat confirmed and widened these findings; in fact, SOD activity increased with the increase of CPF concentrations. In addition, the higher time of exposure (96h) also increased the SOD activity. In fact, even if our highest tested concentration (250 ng/L) was less than 720 ng/L we observed an increase of SOD. In the study of Song et al. (2017), after 48h the peak of SOD was 59.33 U mg protein^{-1} whereas in our study we obtained a value of 94.5 U mg protein^{-1} after 96h.

- *CAT and GPx activity*

As previously stated, a clear induction of CAT and GPx activity was observed with the increase of CPF concentrations. CAT and GPx concur for the removal of H_2O_2, which is metabolized to O_2 and water. However, GPx is also considered an efficient enzyme in protection against lipid peroxidation (Winston and Di Giulio, 1991). CAT activity is directly regulated by the concentration of H_2O_2 (Fornazier et al., 2002). Our results showed that the trends of both CAT and GPx was consistent with the changes of SOD activity. This suggests that both enzymes are involved in the protective response by the Daphnia's antioxidant systems to counteract the adverse effects of hydrogen peroxide. Our results agree with the findings of Basopo and Ngabaza (2015) who measured an enhanced activity of CAT and GPx in the freshwater snail *Helisoma duryi* exposed to 25 ng/L of CPF.

- *GST activity*

GST is involved in the detoxification processes of different organic xenobiotics including CPF (Ecobichon, 1996). Exposure to CPF has been demonstrated to induce GST in chickens and rats (Vodela and Dalvi, 1995). In freshwater invertebrates, the experimental evidences on the role of GST are quite contradictory. McLoughlin and coworkers (2000) suggested low sensitivity of GST to organophosate (OP) insecticides in annelids and crustaceans. In addition, Steevens and Benson (1999), found that GST was not affected by 48h CPF exposure but is inhibited after 96h in *Hyalella azteca*. On the contrary, other studies have shown the induction of GST in *Hydropsyche pellucidula* (Berra et al., 2006) and *Chironomus riparius* exposed to OPs (Callaghan et al., 2002; Choi et al., 2000). In mollusks, an induction of GST occurred in *Corbicula fluminea* after exposure to fenitrothion

(Oneto et al., 2005). The same findings were highlighted in the recent study of Basopo and Ngabaza (2015). In fact, these authors found that the GST activity was significantly increased in snail *H. duryi*. Finally, Song and coworker (2017) in a study of *D. magna* showed that the GST was activated at low concentration and inhibited at high concentration of CPF reaching a maximum when the concentration was 360 ng/L and after 24h of exposure. The same authors reported an inhibition of GST activity after 48h exposure to increasing CPF concentrations (the lowest inhibition was obtained at the concentration of 360 ng/L). Our results partially confirmed these findings. In fact, we highlighted an increase of the GST activity following the increase of CPF concentrations even if at a higher time of exposure (96h) compared to previous studies. A possible explanation could be related to the tested concentrations. In our study, we were always below the concentration of 360 ng/L which was the peak of GST activity at 24h and the lowest level of inhibition at 48h. We hypothesize that the highest concentration of 250 ng/L tested in our study was not sufficiently high to induce the inhibition of GST activity even with an exposure of 96h.

- *AChE activity*

Acetylcholinesterase activity is one of the most important biomarker in the evaluation of the exposure to OPs and carbamate pesticides, and several studies, in which AChE has been used as a biomarker for anticholinesterase insecticides, are present in literature (e.g. Fulton and Key, 2001; Printes and Callaghan, 2004, Xuereb et al., 2009). For crustaceans, several studies report a concentration-dependent inhibition of AChE with OPs pesticides (Domingues et al., 2009 and references therein). These observations are in accordance with the expectations based on the mechanism of action of OPs pesticides. In our study we measured significant difference of AChE activity in the individuals exposed to both the selected concentrations compared to CTRL and DMSO, without a reliance of the enzyme inhibition in function of the concentration of CPF. No significant difference was found between 50 ng/L and 250 ng/L (diff = 0.04, 95% CI: (-0.07) – (-0.15), p = 0.37). A possible explanation of our results could be related to the tested concentrations which were not sufficiently high to induce a drastic change in the AChE activity. Indeed, in a previous work, Barata et al. (2004) measured the response of AChE to single dose exposures of OPs and carbamates insecticides. These authors described the AChE inhibition by means of an allosteric decay model with a period of no or low response at the low concentrations followed by an accelerated negative response as concentration increased.

3.2 Behavioral biomarkers (individual level) and potential link with molecular biomarkers (sub-individual level)

In this study, three swimming parameters (percent of active time, distance moved and swimming velocity) in *Daphnia magna* individuals were measured for each exposure condition. Behavioural responses were investigated in all the alive individuals at the end of the 96h exposure. Since the active velocity was significantly different in individuals treated with DMSO compared to the CTRL group ($F = 29.25$, $p < 0.0001$), the effects induced by CPF were compared to DMSO.

In Figure 2 the increasing or decreasing effects on *D. magna* swimming behaviour induced by different concentrations of CPF are normalized to DMSO.

An overall different profile is observed when the two concentrations of exposure are compared. In fact, at the lowest tested concentration (50 ng/L) a slight reduction in the % of active time of individuals can be observed (<5%), whereas a consistent decrease of both total distance moved and velocity (-24% and -25% respectively) is recorded. On the contrary, at CPF concentration of 250 ng/L the % of active time of individuals decreased notably (<17%), whereas a slight reduction in distance moved and an increase of velocity (-6% and 13% respectively) was noticed. The percent of activity time (i.e., how much of the time have the animal been in an active and inactive state) is calculated by considering, frame to frame, if the animal is moving a distance longer than a minimum threshold value (in pixels). Based on our results, a concentration-dependent reduction of the percent active time for individuals is demonstrated, meaning that as higher is the concentration of exposure as higher will be the period of inactivity or immobility.

The inhibition of AChE has been historically related to the mode of action of OP insecticides such as CPF. Indeed, in previous studies several authors have tried to link the inhibition of AChE activity with adverse effects at the organism level, including growth, reproduction and mortality or immobilization (Depledge and Fossi, 1994; Jemec et al., 2010) with contrasting results. For instance, Ludke et al. (1974) suggested the 50% inhibition of AChE as a threshold limit of a life-threatening situation. This limit was somewhat confirmed by Barata et al. (2004) in a study on *D. magna*. On the other hand, Phillips et al. (2002) linked acute exposures to CPF at levels causing mortality, to enzyme inhibition of >71% and >90% in juvenile and larval walleye (*Stizostedion vitreum*) respectively. In addition, no immobility of *D. magna* exposed to 100 μM of the OP acephate was observed, although the 70% inhibition of the enzyme activity was reached (Printes and Callaghan, 2004). These authors also found that different cholinesterase-inhibiting pesticides had different inhibition level associated with immobilisation of the exposed daphnids. These studies indicated that although AChE activity has been associated with mortality/immobilization, the association is species- and chemical-specific.

Given the key role of AChE in nervous system, it seems reasonable to relate swimming behaviour and the inhibition of this enzyme. Recently, Ren et al. (2017) investigated the role of AChE in swimming behaviour of *D. magna*. The authors concluded that 50% of AChE inhibition may cause changes in swimming behaviour in treated specimens. On the other hand, they also highlighted that there is no clear evidence for the role of AChE in the behaviour homeostasis. Similarly, in another study Xuereb and coworkers (2009) highlighted locomotor alterations in *Gammarus fossarum* exposed to CPF and the carbamate insecticide methomyl. The authors observed significant behaviour alterations for AChE inhibitions higher than 50% for both insecticides.

In our study we measured an inhibition of the AChE of about 22% (Fig. 1) without significant differences between the two tested concentrations. which is quite far from the threshold limits reported above. Therefore, we cannot establish a relationship between the AChE levels of inhibition and the percent of the reduced activity time.

Chevalier and coworkers (2015) highlighted a variability in behavioral changes during time in D. magna exposed to different concentrations of several pollutants with different mechanism of action (including an AChE inhibitor). In our study, the temporal variability of metabolic changes and swimming behavior was not taken into consideration. Consequently, our results should be regarded as a snapshot after 96h of exposure to CPF and this could have limited a more appropriate evaluation of the link between AChE inhibition and behavioural changes.

The same concentration-dependent trend obtained for the time of activity cannot be observed for the other two considered parameters (distance moved and active velocity). Indeed, as previously described, the decreases in the distance moved is significantly higher at 50 ng/L than at 250 ng/L (diff= -0.11, 95% CI: (-0.15) – (-0.06), $p < 0.0001$). Moreover, when speed is considered, a contrasting result is obtained with a significant decrease at the lowest tested concentration and even an increase at the highest one (diff= -0.17, 95% CI: (-0.20) – (-0.15), $p < 0.0001$). Probably, the Stepwise Stress Model (SSM) (Gerhardt, 1999, 2001; Gerhardt et al., 2005) can be a useful starting point to explain our findings. According to SSM, a cascade of regulatory behavioural stress responses is performed by the organisms either by increasing the toxicant concentration or the exposure time.

We hypothesize that the concentration of 50 ng/L of CPF after 96h of exposure was too low to activate regulatory or compensatory mechanisms at sub-individual levels such as the activation of the detoxifying enzymes (Fig.1) useful to maintain the homeostatic conditions. This situation has led to a significant reduction of both parameters indicating a condition of behavioural stress which can be associated to a mechanism of protective reaction due to a loss of coordination (Ferrando and Andreu, 1993; Wolf et al., 1998). On the contrary, the concentration of 250 ng/L of CPF stimulated the activation of the detoxifying enzymes (Fig. 1). The activation of these regulatory mechanisms allowed

393 the organisms to recover the movement capability (in terms of distance moved) and to activate another
394 behavioural response, that is the avoidance. In fact, the increased velocity of swimming can be
395 associated to the attempt of the organism to "escape" from the polluted aquatic environment and this
396 has been recognized as one of the first behaviour modulation in *Daphnia magna* (Ren et al., 2007).
397 On the other hand, detoxification process and antioxidant protection as well as the avoidance
398 behaviour require energy and this could help to explain also the reduction in the % of activity time in
399 individuals exposed at the highest tested concentration.
400

401 ## 4. CONCLUSIONS

402 This study was aimed at investigating potential links in the stress transition from the sub-individualto
403 the supra-individual levels in aquatic organisms. Our goal was achieved by measuring changes in
404 molecular and behavioral biomarkers in *Daphnia magna* exposed to sub-lethal concentrations of CPF.
405 The results have shown that daphnids were in a condition of stress in both conditions of exposure,
406 however, with a contrasting pathway. In fact, at the lowest tested CPF concentration we measured a
407 partial inhibition of the AChE and a significant decrease of some parameters related to swimming
408 behavior (distance moved and velocity), whereas the activity of antioxidant enzymes and GST
409 (molecular biomarkers) were not different from the control. In addition, the percent of activity time
410 (behavioral biomarkers**)** was slightly modulated in treated specimens in comparison with control. At
411 the highest tested concentration, we did not measure further inhibition of AChE suggesting that this
412 concentration was not sufficiently high to induce drastic changes in the activity of this enzyme. On
413 the other hand, we measured significant changes in antioxidant activity and GST suggesting that at
414 this concentration the organisms used a strategy of adaptation by synthesizing the antioxidant and
415 detoxification enzymes. At supra-individual levels, organisms showed the tendency to recover the
416 movement capability (distance moved) and also activated a mechanism of avoidance (increased
417 swimming velocity). However, a reduction in the percent of active time was noticed, and this was
418 attributed to the energy spent by organisms to activate the enzymes and the mechanism of avoidance.
419 Overall, our results suggest the existence of a link from sub- and supra-individual levels as the
420 activation or non-activation in the antioxidant and detoxifying enzymes activities can lead to different
421 modifications of the swimming behaviour in *D. magna*. Finally, although sub-lethal concentrations
422 of CPF elicited enzymatic and behavioural changes in *D. magna*, these cannot be directly related to
423 effects on their fitness or at higher ecological hierarchical level in a quantitative way. Therefore, they
424 cannot be considered into an environmental risk assessment procedure at this time and more effort
425 should be done in this direction.

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FIGURE CAPTIONS

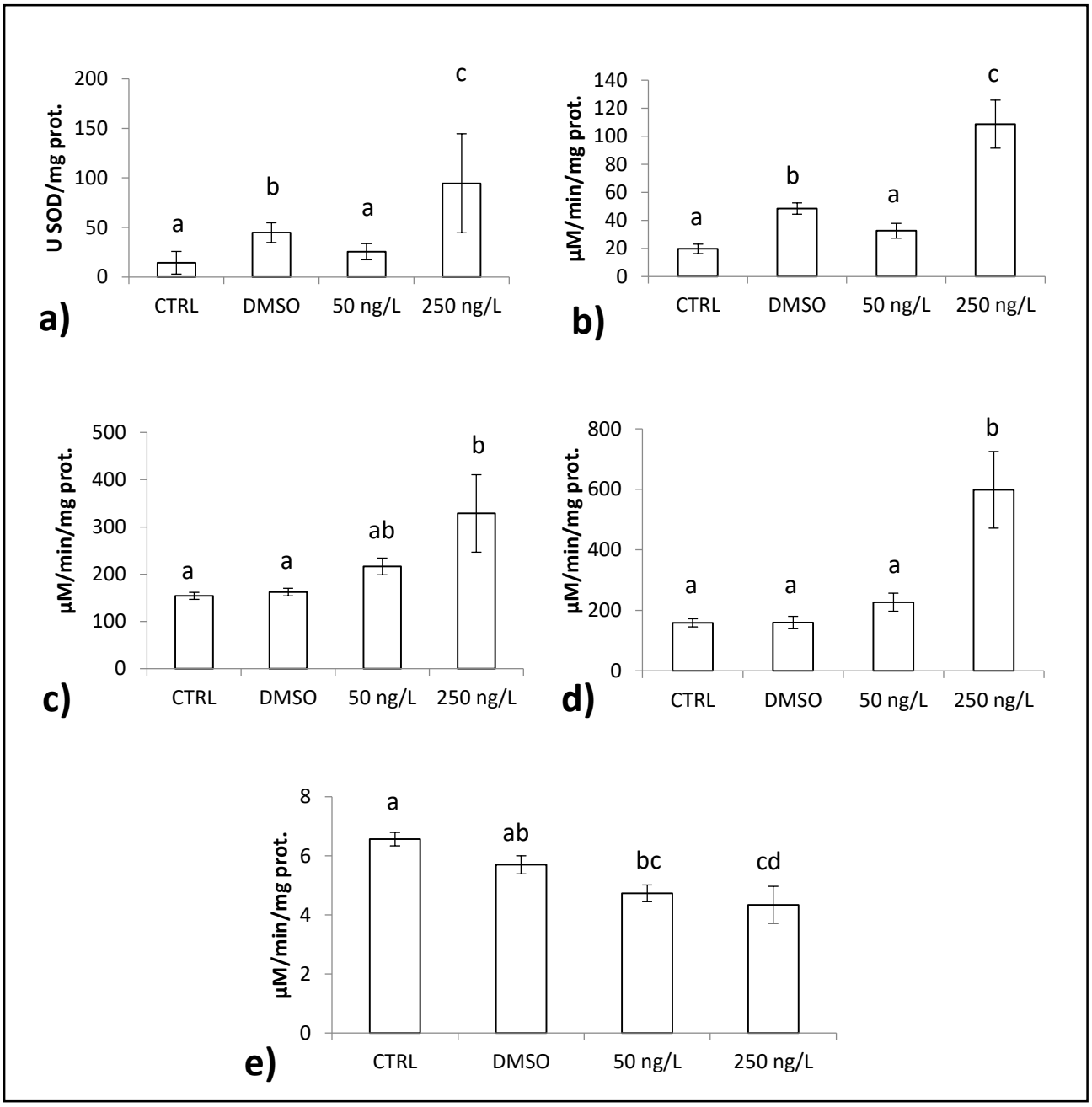

Figure 1: mean activity (±SD) of SOD (a), CAT (b), GPx (c), GST (d) and AChE (e) measured in 8d old individuals after 96h of CPF exposure (50 ng/L and 250 ng/L). Different letters indicate significant difference among groups.

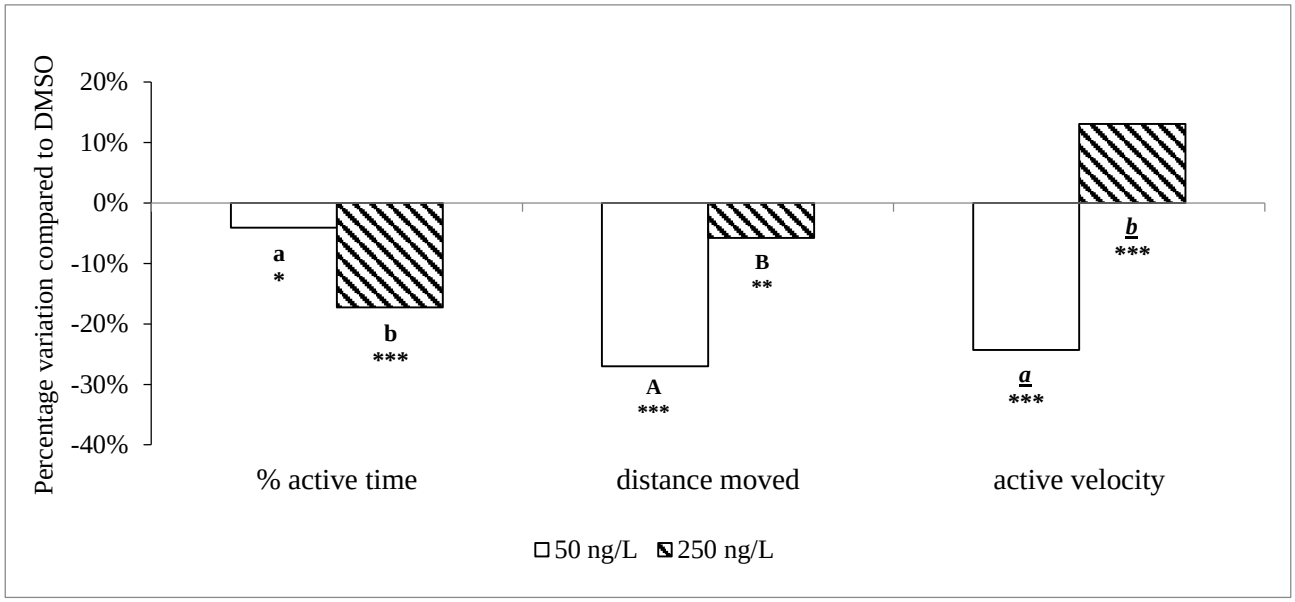

Figure 2: Histograms of increasing/decreasing effects (% active time, distance moved and active velocity) on swimming behaviour for *D. magna* individuals exposed to different concentrations of CPF (50 ng/L and 250 ng/L). Data are normalized to DMSO. Different letters indicate significant difference between the tested concentrations ($p < 0.05$). Asterisks indicate significant difference with DMSO (Significance codes: 0 ≤ '*' , 0.001≤ '**' , 0.01≤ '*' < 0.05).**