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Age effect on the antioxidant activity of *Daphnia magna* (Anomopoda: Daphniidae): Does younger mean more sensitivity?

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Abstract

It has been accepted that for most species newborns and senescent organisms are more sensitive than other ages to environmental stressors. Nevertheless, it must be considered that there are several biochemical and physiological compensatory processes which are not expressed with the same magnitude during the whole life cycle. With this aim, *Daphnia magna* individuals of different age were exposed to hexavalent chromium, Cr (VI), at two different sublethal concentrations (0.032 and 0.0064 mg l^{-1}), and the activity of some antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) were evaluated during most of the life cycle of this cladoceran. The antioxidant enzymatic activity showed an inverse relationship with respect to age. The activity of CAT, GR and GPx were increased in the two treated groups, at all life stages tested. On the other hand, the activity of SOD decreased in the same groups. Both, increase and decrease in the antioxidant enzymatic activities, showed significant differences with respect to the control group, being higher for the 0.032 mg l^{-1} group. The Cr (VI) LC_{50} was also estimated for these age groups, finding statistical differences among them. Even though adults exhibited higher responses, these enzymatic activity changes should not be interpreted as higher sensitivity, since the daphnids acute chromium toxicity followed a different pattern, with increasing LC_{50} values according to age.

Key words

Redox environment, antioxidant enzymes, ageing process, Cladocera, Cr (VI)

Introduction

Aquatic organisms can be exposed to several toxicants at different life stages. It has been examined the influence of organisms' age on the toxicity of pollutants and the general assumption, which has been established as a general rule in toxicology, is that young organisms are more sensitive than the older (Hoang and Klaine, 2007). At the present, some standardized toxicity test procedures recommend the use of individuals less than 24 h old, when daphnids are used as test organisms, as can be read in the USEPA (USEPA, 2002) and the OECD (2004) guidelines.

The effect of age has been tested in *Daphnia magna* neonates and juveniles. Hoang and Klaine (2007) exposed some daphnids to a single 12-h pulse of copper, zinc, selenium or arsenic, at different ages ranging from 3-h to 10-d old. The highest sensitivity they reported is comprised between 2- and 4-day old organisms for the assayed metals. Muyssen and Janssen (2007) used two age groups (0 and 7 day old organisms) which were exposed to copper and zinc, in both acute and chronic toxicity bioassays. The highest sensitivity recorded in the acute toxicity bioassays was obtained with the younger organisms for both metals. Nevertheless, they did not

find significant differences in the susceptibility during the chronic toxicity assays and concluded that acute and chronic toxicity data obtained from juvenile *D. magna* are more sensitive or equally sensitive than obtained from 7-d old organisms.

Chen *et al.* (1999) reported that juvenile and adult *Daphnia pulex* had similar sensitivity to arsenic. In the study carried out by Klein (2000) he found that the sensitivity of *D. magna* to chromium increased as a function of age between 2 and 26 h. The youngest organisms used in most studies on age effect were classified as 24-h old. This was due to the assumption of standardized bioassays methods that organisms 24-h old have a similar sensitivity (Lewis *et al.*, 1994).

Based on the assumption that juvenile stages are the most sensitive, the use of toxicity data derived with these juveniles will eventually result in the most protective water quality criteria. However, results on the inter-life-stage sensitivity for daphnids are often not consistent (Muyssen and Janssen, 2007). The studies of Johnston *et al.* (1987) and Bodar *et al.* (1989) showed that early life stages are more sensitive than older organisms. Sarma *et al.* (2007) also demonstrated that juveniles possess higher sensitivity in comparison to older organisms; when they exposed seven cladoceran species to mercury and methyl parathion, they found that the neonates of all the tested species exhibited the major effects due to toxicant exposure.

It has been accepted that younger organisms are the most susceptible to environmental changes and to the effect of xenobiotics, but there are differences among age groups physiology, such as the reduction of their antioxidant activity because of the ageing process, which conducts to the accumulation of oxidative species that can produce damage to cells and tissues; this is associated with a progressive increase in morbidity and mortality (Beckman and Ames, 1998). During lifetime, antioxidant mechanisms counteract the deleterious action of free radicals and reactive oxygen species (ROS) on macromolecules (Junqueira *et al.*, 2004). As all the organisms, *D. magna* is not an exception and also suffers the rise of oxidant species, as has been described by Barata *et al.* (2005). But, during these metabolic processes, a small proportion (2-3%) of free radicals may escape from the protective shield of antioxidant mechanisms, causing oxidative damage to cellular components (Ames *et al.*, 1993). Biological systems have developed during their evolution adequate enzymatic and nonenzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. The imbalance between the generation and the neutralization of ROS by antioxidant mechanisms within an organism is called oxidative stress (Davies, 1995), which has become an important subject for terrestrial and aquatic toxicity assessment (Livingstone, 2001; Valavanidis *et al.*, 2006).

Molecular biomarkers are used to test oxidative damage in biomolecules and various aspects of oxidative stress by free radicals in experimental animals. In addition to using primary and secondary products of free radical damage, biomarkers can monitor the status of various antioxidant defense mechanisms against free radicals. The antioxidant defense system of living organisms can be subdivided into enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), and nonenzymatic antioxidants, such as glutathione, vitamin E, ascorbate, β-carotene, and urate (de Zwart *et al.*, 1999). Since the discovery of the importance of free radical damage in the mechanisms of toxicity of many environmental pollutants (xenobiotics) there has been an increased application of biomarkers of oxidative stress in living organisms, especially in mammals (Kehrer, 1993; Nordberg and Arner, 2001). Molecular biomarkers of oxidative stress found widespread applications in the understanding of the mechanisms of response of aquatic organisms to chemical pollutants (Livingstone, 2001). Oxidative stress biomarkers comply with basic requirements for an endpoint, such as: responsiveness, low cost and simple procedures for determination, applicability under varied testing conditions; and sensitivity to a high number of environmental contaminants. Several authors used oxidative stress parameters as non-specific endpoints to assess the effects of single chemicals and complex mixtures of aquatic environmental contaminants (Nunes *et al.*, 2008).

The aim of this study was to evaluate how different life stages of the cladoceran *Daphnia magna* could be affected by the exposure to two different concentrations of the reference toxicant chromium (VI), since it has been described that younger organisms are more susceptible to xenobiotics than older life stages.

Materials and Methods

Daphnia magna Straus strain was provided by the Laboratory of Experimental Hydrobiology of the Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional (ENCB-IPN), and has been maintained and reproduced successfully in this laboratory for more than 20 years in reconstituted hard water (hardness: 160–180 as mg $CaCO_3$ l-1; pH: 7.8–8.0. U. S. Environmental Protection Agency, 2002). Despite it is an exotic species in Mexico, it is the test organism in current Mexican legislation for the assessment of acute toxic effects in freshwater environments, according to the guideline NMX-AA-087-1995-SCFI (SECOFI 1995).

Daphnia magna ages selected for this study were: neonates (<24 hr), 3, 5, 7, 14, 21 and 28 day old. These were selected considering that the age 7-d is related with the beginning of reproduction, so we could have information about effects in pre-reproductive period when ovary maturation is in process, and preparing the organisms for the first brood (Zafagnini, 1987); and reproductive stages, some of which have been suggested as endpoint in diverse protocols (Gersich and Milazzo, 1990; Gilhermino *et al.*1999). Organisms older than 28 d were not considered because mortality could be observed (Gómez-Díaz and Martínez-Jerónimo, 2009), and only healthy and vigorous organisms were considered in the experimental design.

Test organisms were obtained from controlled cultures carried out in 500 ml containers containing 400 ml of reconstituted hard

water (RHW) (USEPA, 2002) and 10 parthenogenetic females ion each container, fed on the green microalgae *Ankistrodesmus falcatus* at a concentration of $4X10^5$ cells ml^{-1} (Martínez-Jerónimo *et al.*, 1994); these parthenogenetic female batches were maintained at 25 ± 1°C, with a 16:8 photoperiod (light:darkness). The same procedure was used to obtain test organisms for all the assays, in order to avoid variations due to differences in culture conditions.

Acute toxicity bioassays with *Daphnia magna* Straus, 1820: Bioassays were performed according to the Protocol 202 of the Organization for the Economical Cooperation and Development (OECD, 2004), which is compatible with the procedure proposed by the USEPA (2002).

The bioassays were performed in quadruplicate with 5 organisms of every instar per replicate. Test containers were placed in an environmental chamber at a controlled temperature of 25°C and at 16:8 photoperiod during 48 hr. The assessed response was immobility or death of cladocerans. The criterion for test acceptance was a survival higher or equal to 90% in the control group. Finally, mortality data recorded at the end of the toxicity tests (48 hr) were used to determine the Median Lethal Concentration (LC_{50}) trough Probit analyses (Stephan, 1977).

Sublethal toxicity bioassays with *Daphnia magna* Straus, 1820: The selected age organisms were exposed to 0.032 mg l^{-1} and 0.0064 mg l^{-1} of Cr (VI) as potassium dichromate ($K_2Cr_2O_7$; 99.98% purity) (nominal concentrations, which did not differ from the real concentrations during test conditions, according to Martínez-Jerónimo and Martínez-Jerónimo, 2007), for 24 hr at 25°C, with photoperiod of 16:8 hr (light:darkness). For each treatment we have 5 replicates, with 20 organisms per replicate in the case of neonates and 3-d old organisms, and 10 organisms for replicate for all the others age groups. These two chromium sublethal concentrations were applied in order to evaluate how oxidative stress biomarkers could be altered in two different situations: the first one, with a relatively high amount of Cr (VI) (0.032 mg l^{-1}, equivalent to 1/5 LC_{50}), in which some mortality could be expected; and the second one, a low Cr (VI) concentration (0.0064 mg l^{-1}, equivalent to 1/25 LC_{50}), in which survivorship of all exposed organisms was expected. LC_{50} value for *D. magna* was previously determined by Martínez-Jerónimo *et al.* (2006), for the same cladoceran strain used in present study. After the exposure period, the organisms were frozen at -70°C until they were used for biomarker analysis.

For the determination of the antioxidant activity, organisms were homogenated in 2 ml of 100 mM phosphates buffer at pH 7.4. Then, the activity of SOD, CAT, GPx and GR was measured.

GR activity: This was determined by the method described by Calberg and Mannervik (1975), by measuring the consumption of NADPH at 340 nm, when it is used by the GR to reduce the glutathione from its oxidized form GSSG to its reduce form GSH. The substrate included NADPH 0.2 mM, GSSG 2 mM in Tris-HCl 10 mM buffer (pH 7). Absorbance was measured at time 0 and 10 minutes. Results are expressed as µmol of consumed NADPH min^{-1} protein mg^{-1}.

GPx activity: GPx activity was determined by a method based on the enzymatic use of reduced glutathione (Hafeman *et al.*, 1974; modified by Cano-Europa *et al.*, 2008) as follows: 100 µl of the homogenized organisms was added to 2 ml of 100 mM of phosphate buffer containing 0.4 mM EDTA, 10 mM sodium azide (NaN_3), and 2 mM GSH. This mixture was incubated for 5 min at 37°C, and then 1 mL of 1.25 mM H_2O_2 was added. Two samples were taken at 5 and 10 min and they were added to 0.6 ml of 1.6% metaphosphoric acid, and then diluted 1:2 with 400 mM phosphate buffer. Finally 250 µl of 5.5-dinitro-bis-2-nitrobenzoic acid were added and the absorbance was measured at 412 nm. Results are expressed as µg of GSH min^{-1} protein mg^{-1}.

Catalase activity: Catalase activity was measured by monitoring the enzyme-catalyzed decomposition of H_2O_2 (Aebi, 1984). Briefly, 100 µl of the organisms' homogenate was added to 3 ml of 100 mM phosphate buffer, pH 7.4, containing 30 mM of H_2O_2, and incubated 10 min at 37°C; absorbance was recorded at 240 nm. The decomposition of H_2O_2 by the catalase present in the samples follows first-order kinetics according to the equation $k=2.3t \log (Ao/A)$, where k is the first-order reaction rate constant, t is the time over which the decrease of H_2O_2 caused by catalase activity will be measured (3 min) and Ao/A is the ratio of optical densities at times 0 and 3 min. Catalase activity is expressed as k protein mg^{-1}.

Total SOD activity: The SOD activity was spectrophotometrically measured using 1.5 ml of a solution containing 10 µM NaN_3, 10 µM reduced cytochrome *c*, and 1 mM EDTA dissolved in 20 mM sodium bicarbonate and 0.02% triton X-100, pH 10.2. The enzymatic assay started by addition of 50 µl of xanthine oxidase (3.4 mg ml^{-1} in 0.1mM EDTA). The change in absorbance was monitored every 30 seconds at 550 nm. One unit of SOD activity is defined as that amount of enzyme that decreased the reduction rate of cytochrome *c* by 50%, as described by Crapo *et al.* (1978) and Cano-Europa *et al.* (2008).

With the enzymatic results, the rate change was calculated dividing the exposed group activities by the activity of the control group, considering the later as the 100%. These results were expressed as the percentage of the control group activity.

Total protein: Total protein was measured by the Bradford Method (Bradford, 1976) using bovine albumin as standard. The absorbance was measured at 595 nm.

Statistical analysis: All data sets from the antioxidant activity of *D. magna* were analyzed by two-way ANOVA and the Tukey's pairwise multiple comparison test; significant differences were established when $p<0.05$. These comparisons were carried out within age groups and within concentrations groups since the statistical interaction of the two factors tested, age and toxicant concentration (age X concentration interaction) showed to be significant ($P<0.05$).

Tukey's pairwise multiple comparison test and Fisher's LSD multiple comparison method were applied for the LC_{50} values among age groups and, as above; significant differences were identified when $P < 0.05$.

Results and Discussion

The acute toxicity bioassays with different life stages showed a pattern of increasing LC_{50} values as a function of organisms' age; in other words, juvenile organisms were more sensitive than mature life stages as it was referred by the correspondent statistical analyses, which showed significant differences among the groups tested ($P<0.05$). The LC_{50} for all age groups were situated in the range of 0.08-0.19 mg Cr (VI) l^{-1} (Fig. 1).

The antioxidant enzymatic activity in the control group and chromium exposed organisms decreased with respect to age, finding the highest activity in neonates and juveniles for all the enzymes and groups tested; therefore, the lowest enzymatic activity was achieved in the older groups. Statistical differences ($P<0.05$) were found among the groups with ages before the beginning of reproduction (<7-day old), and those which were into the reproductive period (> 7-day old). An exception to this pattern was the SOD activity, which increased until the fifth day and then its level got reduced with non statistical difference during the reproductive period (7-28 days old) ($F=0.275$; $P=0.76$). Chromium exposure reduced the SOD activity in both exposed groups. Nevertheless, the 0.032 mg l^{-1} was the most affected group since it presented the lowest SOD activity with respect to the control and the lower chromium concentration exposed groups (Fig. 2).

The GPx activity in the control group decreased as the organisms became adults and did not show significant changes during the period comprised between the 7th and the 28th days. The activity of this peroxidase in both groups, which were exposed to Cr (VI), showed two different patterns. First one comprised a slight decreased in the GPx activity in both groups until the 5th day, and after that, its activity was raised compared with the control (Fig. 2). Statistical differences were found mainly for the higher chromium concentration exposed organisms ($F=243.0$; $p<0.001$).

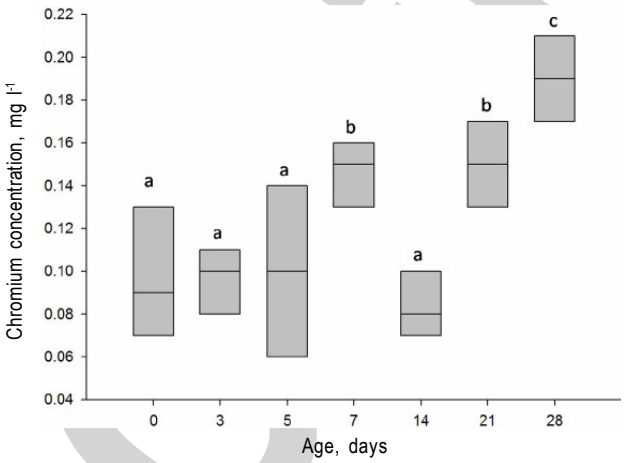

Fig. 1: Median Lethal Concentration (LC_{50}) at 48 hr (25°C) for the different life stages of *D. magna*. Bars indicate the median and the 95% confidence interval. Different letters above bars indicate significant differences *(P*<0.05)

CAT activity was also increased as result of exposure to chromium (Fig. 2), following the same pattern than the other two enzymes aforementioned, decreasing inversely to age. The increase in the CAT activity seems to be dependent on the metal concentration in both exposed groups, which were statistically different to the non-exposed daphnids, and between each other in all the tested age groups ($F=243.05$; $P<0.001$).

Glutathione reductase activity was not different for the control group and the 0.0064 mg Cr (VI) l^{-1}; this similitude was found within all the ages' groups. On the other hand, the reductase activity recorded for the 0.032 mg Cr(VI) l^{-1} showed to be statistically different from the other two groups, besides its activity was several times higher ($F=724.338$; *P*<0.001) (Fig. 3).

In general, the three groups used in this study followed a similar pattern in which the highest enzymatic activity is shown in younger organisms and it got reduced for the adult stages. As aforementioned, the alterations on the antioxidant activity of *D. magna* were dependent to the chromium concentration and the age of the organisms.

The ratio of change for all the enzymatic activities showed that the changes in the antioxidant activity of the cladoceran are related to the age and the toxic compound concentration. The highest changes were registered for the adults, not for the neonates and juveniles as it could be expected.

As it was expected, the antioxidant activity of *D. magna* decreased as a result of the ageing process as it was previously reported by Barata *et al.* (2005). This decrement was observed in the control group and also in those which were exposed to hexavalent chromium, showing the same pattern unless significant differences where found within age groups.

The antioxidant activity of peroxidases CAT and GPx, which play an important role as the first defense line against oxidative damage (Ames *et al.*, 1993), was increased in the chromium exposed groups. This could be due to the production of peroxides such as the hydrogen peroxide, which resulted from the chemical reduction of Cr (VI) to less toxic forms such as Cr V, IV or III. Some important natural antioxidants, such as the reduced form of glutathione (GSH), take part in the chromium detoxification (Standeven *et al.*, 1992; Liu and Shi, 2004). The increment activity of the peroxidases system of *D. magna* could be explained by the production of these peroxides that participate as enzymes substrates, for their consequent decomposition to water and hydroxyl radicals. Thus, the damage that could be caused by peroxides is avoided by these two enzymes at the expense of the GSH concentration, which decreased and formed the GSSG (its oxidized form) (Valko *et al.*, 2005).

In the aerobic organisms the superoxide anion is produced as a result of the chemical respiration. This high reactive oxygen species is neutralized by the SOD to produce hydrogen peroxide (Matés *et al.*, 1999), which is the final product of SOD activity. As it was observed in this study, the SOD activity in the chromium exposed

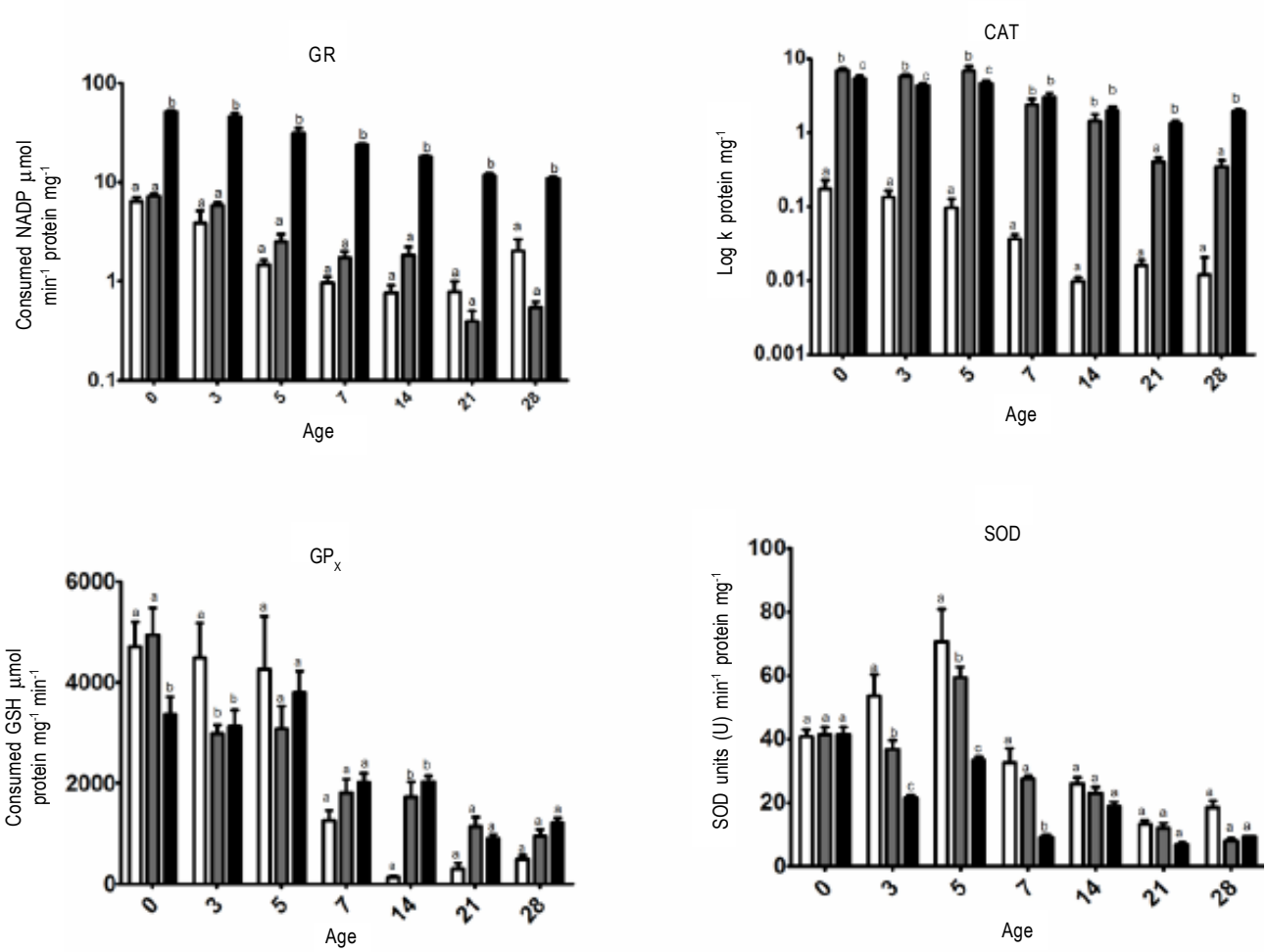

Fig. 2: Antioxidant enzymatic activity in *Daphnia magna* exposed to chromium (VI). Bars represent the mean ± standard error. Different letters indicate significant difference ($P<0.05$) between treatments within an age group.

groups followed a different pattern with respect to the other three enzymes, by decreasing its activity. This enzyme could be regulated by final product inhibition, since the SOD activity was reduced in these organisms (both for the effects of age and Cr (VI) concentration). In other words, when peroxides concentration rose, the SOD activity could be inhibited by them, since peroxides could participate as the SOD final product, or the SOD structure could have been damaged by the free radicals activity.

As aforementioned, the hexavalent chromium is detoxified by several routes which imply the participation of the glutathione (Valko *et al.*, 2005), in a direct way or by the enzymatic activity. The increment of oxidized glutathione could be compensated by the GR activity, turning this oxidized dipeptide into its reduced form to maintain the redox environment in a stable status by the redox pair [2GSH/GSSG]. In *D. magna*, the highest GR activity was obtained within the 0.032 mg l^{-1} exposed groups due to the intense consumption of glutathione in order to reduce the Cr (VI) to less toxic forms by enzymatic and non enzymatic pathways (Aiyar *et al.*, 1991).

Comparing the effects on the antioxidant enzymatic activity it is possible to consider that neonates and juveniles are more susceptible than the adults, but it has been considered the possible use of the ratio of change of the enzymatic activity. This ratio of change showed that the highest changes were obtained during the adult stages. The question is if they are more sensitive than the younger organisms. Neonates and juveniles possess higher antioxidant defenses than the adults but they have smaller sizes. As a result of this, their area/volume ratio becomes bigger; this means that their diffusion rate is faster and higher than the one of older organisms that are bigger size (Preuss *et al.*, 2008). A higher diffusion rate implies that younger organisms are more exposed to the medium components or toxicants and maybe this is the reason to have greater antioxidants defenses. This is the way to avoid oxidative damages in juvenile cladocerans, which plasticity may be increased due to their intrinsic capability to tolerate the surrounding medium influence. But the largest changes in the antioxidant activity were recorded for the adult stages, with organisms of bigger size but with lower diffusion rates and less antioxidant enzymatic activity. Furthermore,

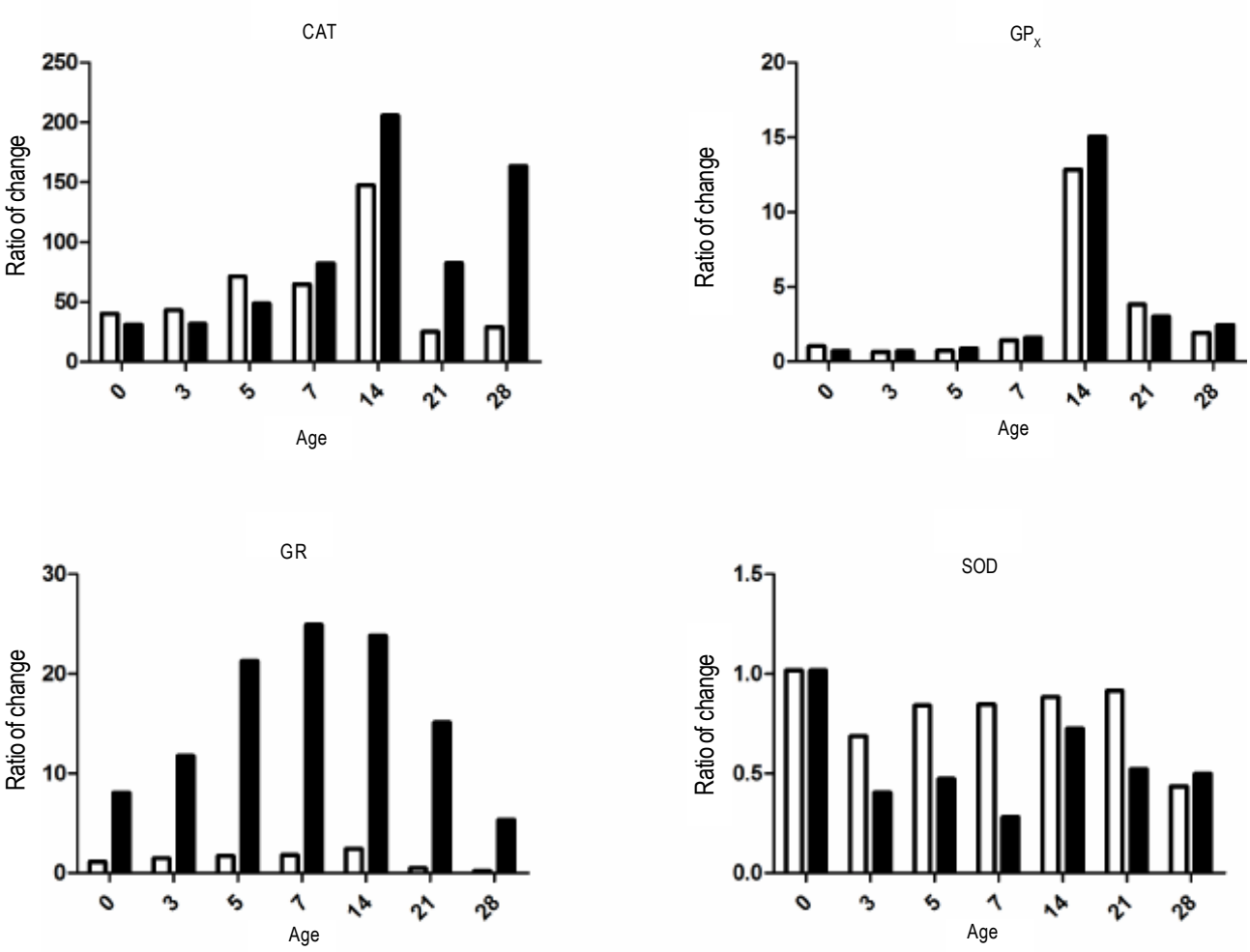

Fig. 3: Ratio of change in the antioxidant enzymatic activity of *D. magna* exposed to chromium (VI). White bars indicate the proportion of the 0.0064 mg l^{-1} exposed group enzymatic activity compared with the control group. Black bars indicate the ratio between the 0.032 mg l^{-1} exposed groups and the control one

this increment could prevent daphnids of oxidative damages for themselves and also on the embryos that they carry inside the incubator chamber. In nature, all of this could be a compensatory mechanism among stages; younger organisms which are more exposed to several medium substances but they are more capable to avoid damages because of their higher antioxidant defenses; and older organisms that are less exposed to the surrounding medium components and might require less antioxidant defenses but they are able to increased these activities to prevent oxidative damages.

There are several studies that have demonstrated that younger life stages are more sensitive than older organisms (Hoang and Klaine, 2007; Muyssen and Janssen, 2007) as the results obtained in this work, where once more has been proved that neonates and juveniles are the most sensitive organisms for acute toxicity bioassays although this could not correspond with the results of the oxidative stress biomarkers.

The antioxidant activity in *D. magna* is affected by age, reducing the activity of several important enzymes such as the SOD, CAT; GPx and GR, which could increase the sensitivity of mature life stages only at suborganism level, since they were not as sensitive as juveniles in the acute toxicity bioassays (organism and population level). For this reason, age is a key factor to be considered for the experimental design of toxicity bioassays with *D. magna*.

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