**Neuroendocrinology Letters** Volume 37 Suppl. 1 2016
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
*Web of Knowledge / Web of Science:* Neuroendocrinol Lett
*Pub Med / Medline:* Neuro Endocrinol Lett

# The effects of cypermethrin on oxidative stress and antioxidant biomarkers in marbled crayfish (*Procambarus fallax* f. *virginalis*)

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*Submitted: 2016-06-24 Accepted: 2016-10-30 Published online: 2016-12-18*

*Key words:* **crayfish; acute toxicity test; oxidative stress; antioxidants; pyrethroids**

Neuroendocrinol Lett 2016; **37**(Suppl. 1):53–59 **PMID:** 28263531 NEL370916A07 

## Abstract

**OBJECTIVES:** Pyrethroid insecticides are known to be highly toxic to non-target aquatic organisms. Toxic effects of different types of pyrethroids to fish has been well described but there is a dearth of data on their effects to aquatic crustaceans. The aim of this study was to assess the effects of a commercial product Cyperkill 25 EC (25% of cypermethrin as an active substance) on oxidative stress and antioxidants activity in marbled crayfish.

**DESIGN:** The juvenile crayfish were exposed to two concentrations of Cyperkill 25 EC: 0.02 μg.L$^{-1}$ (10% 96hLC50) and 0.05 μg.L$^{-1}$ (30% 96hLC50) for 72 hours. Activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-S-transpherase (GST) and reduced glutathione (GSH), as well as levels of thiobarbituric acid reactive substances (TBARS) were assessed in the whole-body homogenate.

**RESULTS:** Cyperkill 25 EC in both tested concentrations caused significant ($p$<0.01) decreased in levels of TBARS. Changes in the antioxidants activity were observed in every examined parameters except for the catalase. Cyperkill 25 EC in concentration 0.05 μg.L$^{-1}$ caused significant increase ($p$<0.05) of SOD activity and significant decrease of GST ($p$<0.01) and GSH ($p$<0.05) activity. The activity of GR was significantly ($p$<0.01) decreased in both treated groups.

**CONCLUSIONS:** This study suggests that Cyperkill 25 EC causes oxidative stress and also disruption of antioxidant systems in crayfish. Expanding to our previous knowledge, obtained results describe effects of cypermethrin also to non-target aquatic invertebrates and suggest a need of deeper understanding of mechanisms involved in this process.

ORIGINAL ARTICLE

**Abbreviations:**

| | |
|---|---|
| 96hLC50 | - concentration of test substance in water that kills 50% of tested organisms within a 96 h exposure |
| $ANC_{4.5}$ | - acid neutralization capacity |
| ANOVA | - analysis of variance |
| BSA | - bovine serum albumine |
| CAT | - catalase |
| CODMn | - chemical oxygen demand |
| DTNB | - 5,5´-dithiobis-2-nitrobenzoic acid |
| EDTA | - ethylenediaminetetraacetic acid |
| GABA | - gamma-aminobutyric acid |
| GC/ECD | - gas chromatography/electron capture detector |
| GR | - glutathione reductase |
| GSH | - reduced glutathione |
| GSSG | - oxidised glutathione |
| GST | - glutathion-S-transpherase |
| LPO | - lipid peroxidation |
| MDA | - malondialdehid |
| NADPH | - nicotinamide adenine dinucleotide phosphate |
| NBT | - nitroblue tetrazolium |
| PBS | - phosphate buffer saline |
| PP | - phosphate buffer |
| ROS | - reactive oxygen species |
| SOD | - superoxide dismutase |
| TBARS | - thiobarbituric acid reactive substances |

## INTRODUCTION

Contamination of the environment by various types of chemicals is one of the key worldwide problems. One of the most commonly detected substances in agricultural soils, surface and ground waters are pesticides (Scholz *et al.* 2012). Commercial sector, with the most massive pesticide use, is undoubtedly agriculture (Velisek *et al.* 2015). These substances significantly increase production of food for permanently increasing human population (Tilman *et al.* 2011). Water ecosystems are primarily endangered by the surface runoff from agricultural areas largely due to irresponsible and unprofessional application of pesticides. These substances may affect non-target organisms which play many important functional roles in the ecosystem (Schulz 2001).

Pyrethroids constitute the majority in the market with the synthetic insecticides and they are an active compound of many commercial products. Pyrethroids represent 17% of the word insecticides market in 2013 (Sparks 2013). These highly effective substances are commonly used in agriculture, households, human as well as veterinary medicine (Palmquist *et al.* 2012). Pyrethroids are synthetic substitutes for natural pyrethrins having increased photostability, while retaining the insecticidal activity and relatively low acute mammalian toxicity (Sparks 2013). Pyrethroids belong to the group of non-systematic insecticides. These insecticides kill insect by the touch or digestion of treated plants (Soderlund *et al.* 2002). They are divided into two types, but both impact the central nervous system. The first type provides a prolong opening of sodium channels in axonal membranes of neurons. The other type influences the GABA receptors, blocking chloride ion transport into the neurons (Werner & Moran 2008).

Cypermethrin [(alphaRS)-3-phenoxy-alpha-cyanbenzil (1RS,3RS,1RS,3SR)-3-(2,2-dichlorovinyl) -2,2-dimethylcyklopropan-karboxylat] is one of the most commonly used pyrethroid belonging to the second type including an alpha-cyano-3-phenoxybenzyl group. This type of pyrethroids has been shown as more toxic to fish (Breckenridge *et al.* 2009) while fish are extremely sensitive to the neurotoxic effects of this kind of pesticides. Cypermethrin is classified as highly toxic to fish with 96hLC50 usually less than 10 $\mu g.L^{-1}$ (Bradbury & Coast 1989). This substance is also used as an antiparasiticum in veterinary medicine, primarily as a protection of domestic animals against ectoparasites. In fish culture, cypermethrin is applied to enclosed salmon sea cages to treat salmonids for the infestations of sea lice *Lepeophtheirus salmonis* and *Caligus elongatus* (Treasurer & Wadsworth 2004).

The aim of the present study was to assess the effects of two sublethal concentrations of cypermethrin based insecticide Cyperkill 25 EC on oxidative stress and the antioxidant defence in juvenile marbled crayfish.

## MATERIAL AND METHODS

### *Chemicals*

The pyrethroids insecticide Cyperkill 25 EC (Agriphar S.A.,Rue de Renory, 26/1, B-4102 Ougreé, Belgium) with 25% of cypermethrin as an active compound.

### *Experimental animals*

Seventy two juvenile marbled crayfish (body weight: range of 266.8 mg – 357.4 mg, carapace length: 11.19 – 11.47 mm) were obtained from a laboratory culture at the Research Institute of Fish Culture and Hydrobiology in Vodnany, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Czech Republic.

### *Experimental protocol*

After acclimation, marbled crayfish were randomly divided and placed into nine plastic boxes, each containing eight individual chambers (inner dimensions AxBxC mm). System of individual keeping is often used during growth (Kozak *et al.* 2009) as well as toxicity tests in crayfish (Kouba *et al.* 2012) as it prevents cannibalism and aggression among crayfish. Boxes with crayfish were placed in aquariums with 10 L of the tested solution or water (in case of control group) with continuous aeration for 72 hours. These aquariums were located in laboratory with the light-dark cycle 16:8 h.

Two concentrations of Cyperkill 25 EC and control were used for the test, each group included 24 crayfish. Each group was tested in triplicates. The tested concentrations were: 0.02 $\mu g.L^{-1}$ (10% 96hLC50) for the first experimental group (1CYP) and 0.05 $\mu g.L^{-1}$ (30% 96hLC50) for the second experimental group (2CYP). These concentrations were chosen on base the preliminary acute toxicity test on marbled crayfish. In pre-

liminary acute toxicity test, 96hLC50 was 0.10 μg.$L^{-1}$ of Cyperkill 25 EC.

The solution was changed every 12 hours during the test. The crayfish were transferred into the other aquarium with the new solution every 12 hours. This provision ensured that concentration of the tested substance did not drop below 80% of the nominal concentration. The ensure agreement between nominal and actual cypermethrin concentrations, water was monitored by GC/ECD (gas chromatography/electron capture detector) (Kocourek & Hajslova 1989). Oxygen saturation, pH and temperature were measured every day. Water temperature during the test ranged from 21.8 to 23.2 °C, oxygen saturation >80% and pH ranged from 7.8 to 8.2. Other water parameters were as follows: $ANC_{4.5}$ 1.10 mmol.$L^{-1}$, $COD_{Mn}$ 1.1 mg.$L^{-1}$, total ammonia 0.03 mg.$L^{-1}$, $NO_2^-$ 0.02 mg.$L^{-1}$, $NO_3^-$ 5.92 mg.$L^{-1}$, sum of $Ca^{2+}$ + $Mg^{2+}$ 8.91 mg.$L^{-1}$.

*Preparation of samples*

After 72 hours, fourteen crayfish from each group were randomly selected. These selected crayfish were weighted and carapace length was measured. Samples were subsequently stored at –80 °C until following analysis.

Samples were weighed again and homogenized (1:10, w/v) with the ball homogenizer TissueLyser II (QIAGEN, Germany). The first half of samples from each group was homogenized with 50 mM PP buffer ($KH_2PO_4$) with 1 mM EDTA (pH 7.4). This whole-body homogenate was centrifuged (30 min at 15 000 rpm at 4 °C) and used for analysis of SOD and CAT. The second half of samples was homogenized in PBS buffer (0.8% NaCl, 0.02% KCl, 0.29% $NA_2HPO_4$*$12H_2O$, 0.02% $KH_2PO_4$ in deionised water, pH 7.2). Homogenate was centrifuged (Micro 200R, Hettich Zentrifugen, Germany) 15 min at 10 000 rpm at 4 °C and used for analysis of GR, GST, and GSH. Homogenate, including PBS buffer, was also used for analysis of TBARS, without centrifugation.

*Oxidative stress*

Oxidative stress was determined by thiobarbituric acid (TBARS) method, which evaluates level of lipid peroxidation product malondialdehid (MDA) following the methrod of Lushchak *et al.* (2005).

*Antioxidant parameters*

Total superoxide dismutase (SOD) activity was determined according to the method of Marklund & Marklund (1974) based on inhibition of NBT (nitro blue tetrazolium). For assessment of catalase (CAT) activity the method given by Beers & Sizer (1952) evaluating decrease of $H_2O_2$ was applied. Activity of glutathione reductase (GR) was determined by measuring NADPH oxidation following the method of Carlberg & Mannervik (1975). Total glutathione-S-transpherase (GST) activity measuring the reaction of 1-chloro-2,4-dinitrobenzene with GSH was determined according to the method of Habig *et al.* (1974). The level of reduced glutathione (GSH) was determined according to the method Ellmann *et al.* (1959) based on using DTNB (5,5´-dithiobis-2-nitrobenzoic acid) as a substrate.

*Total protein estimation*

Level of proteins was determined spectrophotometrically following the method of Bradford (1976) using BSA (bovine serum albumin) as a standard. All parameters were measured spectrophotometrically.

*Statistical analysis*

Data are expressed as mean ± SD. STATISTICA version 12.0 for Windows (StatSoft, Inc.) was used to perform the statistical analysis. Kolmogorov-Smirnov and Bartlett's tests were applied to assess data for normality and homoscedasticity, respectively. For data with normal distribution, one-way ANOVA was used. In case of non-normal distribution, a non-parametric Kruskal-Wallis test was performed. The significance levels for tests were $p<0.05$ and $p<0.01$.

## RESULTS

*Mortality*

Mortality in experimental groups during test is summarized in Table 1. Mortality was observed only in cypermethrin-treated groups, being 16.7 and 41.7% in 1CYP and 2CYP, respectively. No mortality was observed in control group.

*Oxidative stress*

The values on oxidative stress are depicted in Figure 1. Levels of lipid peroxidation in whole-body homogenate (expressed as TBARS) significantly decreased ($p<0.01$) in both cypermethrin-treated groups compared to the control.

*Antioxidant response*

Changes in SOD activity are shown in Figure 2A. Activity of this enzyme was significantly increased ($p<0.05$)

**Tab. 1.** Mortality in experimental groups during the test of Cyperkill 25 EC in marbled crayfish (*Procambarus fallax* f. *virginalis*).

| Group | Concentration (μg.$L^{-1}$) | Mortality | | | | Total (group) |
|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 72 h | Total | |
| 1CYP-A | 0.02 | 0 | 1 | 1 | 2 | 4 |
| 1CYP-B | 0.02 | 0 | 1 | 0 | 1 | |
| 1CYP-C | 0.02 | 0 | 0 | 1 | 1 | |
| 2CYP-A | 0.05 | 1 | 0 | 2 | 3 | 10 |
| 2CYP-B | 0.05 | 2 | 0 | 3 | 5 | |
| 2CYP-C | 0.05 | 0 | 0 | 2 | 2 | |

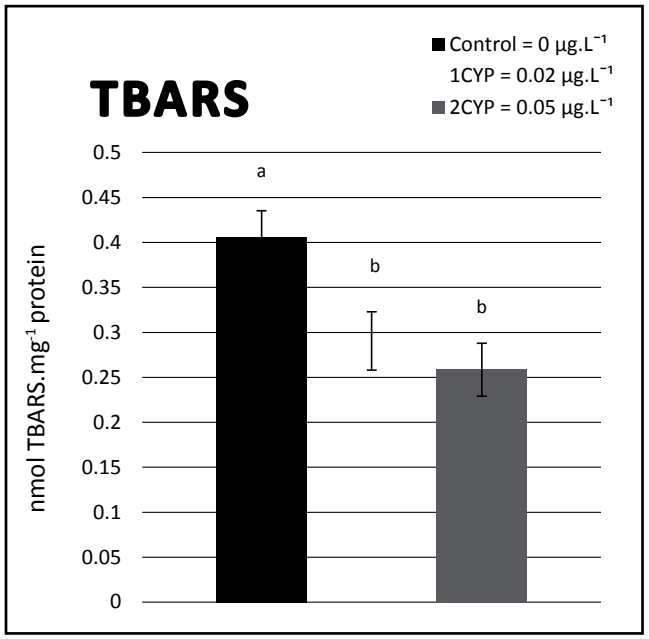


**Fig. 1.** The effect of acute exposure to Cyperkill 25 EC on thiobarbituric acid reactive substances (TBARS) in whole-body homogenate of marbled crayfish *Procambarus fallax* f. *virginalis*. Data are means ± SD. Significance levels observed are $p<0.01$ compared to the control group.

in 2CYP after exposure to Cyperkill 25 EC compared to other groups. No significant differences were found in activity of CAT (Figure 2B) in whole homogenate of crayfish among experimental groups. Activity of GST (Figure 3A) was significantly lower ($p<0.01$) in 2CYP group compared to the control. Significant declines ($p<0.01$) were observed in GR activity between control and cypermethrin-treated groups as well as between both treated groups after exposure (Figure 3B). The level of GSH was significantly ($p<0.05$) lower in the 2CYP group compared to the control (Figure 4).

## DISCUSSION

*Mortality*

Mortality was observed in both cypermethrin-treated groups, confirming that this synthetic pyrethroid is highly toxic to the crayfish. High acute toxicity of cypermethrin and other pyrethroids to a range of fish species has been recently extensively described, but there are a few information about toxicity of these substances further aquatic taxa, e.g. crayfish as a model invertebrates. Acute toxicity of three pyrethroids (cypermethrin, deltamethrin and cyfluthrin), a group of the most frequently used pyrethroids, to adult red swamp crayfish (*Procambarus clarkii*) was described in Morolli *et al.* (2006). Toxicity expressed as a 24hLC50 was 0.14 µg.L$^{-1}$ for cypermethrin, 0.17 µg.L$^{-1}$ for cyfluthrin and 0.22 µg.L$^{-1}$ for deltamethrin. Wu *et al.* (2012) reported 96hLC50 value 0.86 µg.L$^{-1}$ of deltamethrin for the same species. Difference between acute toxicity of deltamethrin for signal crayfish (*Pacifastacus leniusculus*) adults and larvae was reported by O'Reilly (2015). Observed 48hLC50 values were 0.86 µg.L$^{-1}$ for adults and 0.024 µg.L$^{-1}$ for larvae. These results suggest that pyrethroids are probably more toxic to early life stages compared to the adults.

*Oxidative stress*

Normal situation for cells is if production and degradation of ROS (reactive oxygen species) is in balance. Oxidative stress occurs if the production of ROS

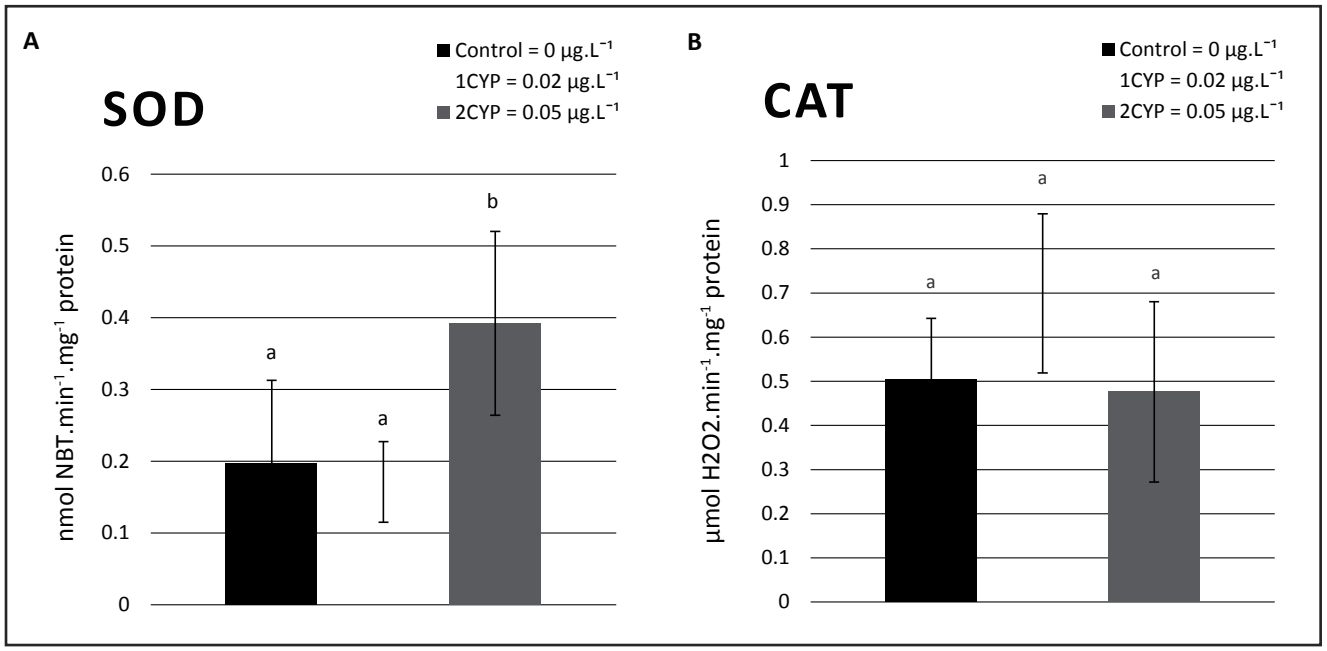


**Fig. 2A and 2B:** The effects of acute exposure to Cyperkill 25 EC on activity of SOD and CAT in whole-body homogenate of marbled crayfish *Procambarus fallax* f. *virginalis*. Data are means ± SD. Significance levels observed are $p<0.05$ compared to the control group.

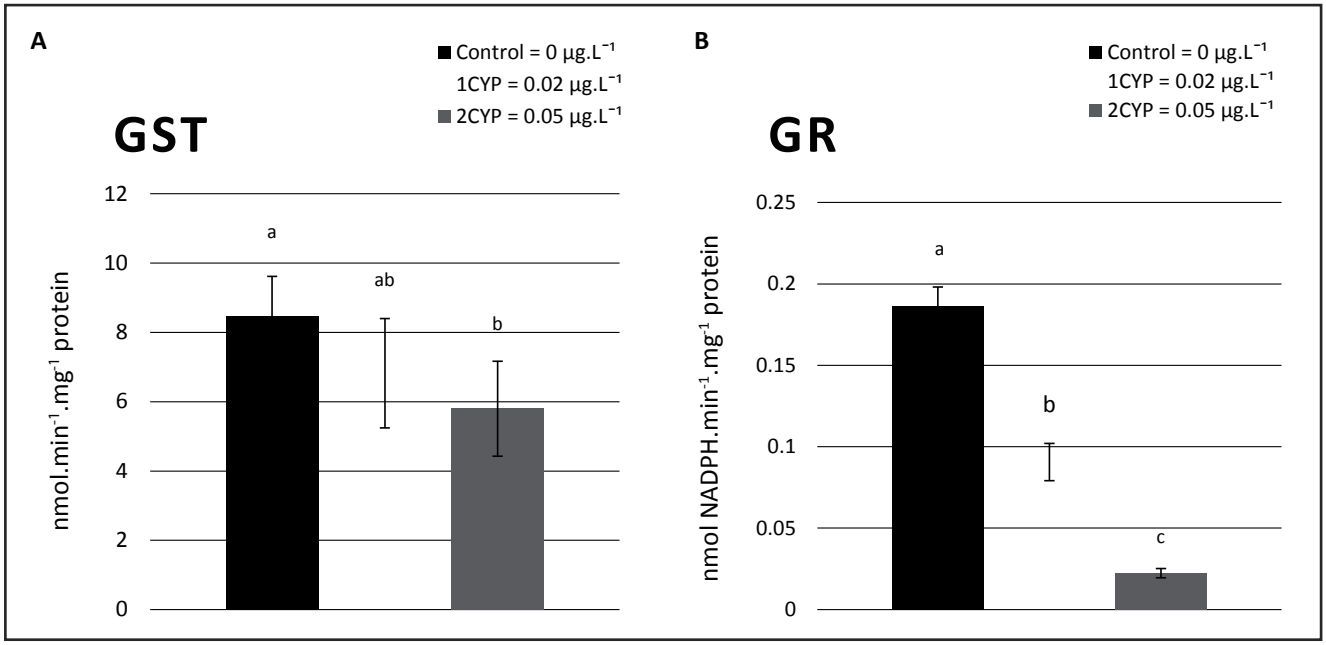


**Fig. 3A,B:** The effects of acute exposure to Cyperkill 25 EC on activity of GST and GR in whole-body homogenate of marbled crayfish *Procambarus fallax* f. *virginalis*. Data are means ± SD. Significance levels observed are $p<0.01$ compared to the control group.

is higher than the defence abilities of antioxidant systems (Lushchak 2011). Measurement of lipid peroxidation is one of the most usually utilized oxidative stress biomarkers. Lipid peroxidation is oxidation of lipids, primarily unsaturated fatty acids, resulting in a production of toxic aldehydes and hydroperoxides (van der Oost *et al.* 2003). In our test, the level of LPO significantly decreased in both cypermethrin-treated groups. These results suggest that antioxidant systems capacity didn't probably exhausted after exposure of 72 hours. On the contrary, significant increase of LPO was observed in hepatopancreas (Wei & Yang 2015a) and gills (Wei & Yang 2015b) of red swamp crayfish after 96 hours beta-cypermethrin exposure at concentrations ranging from 0.005 to 0.04 µg.L$^{-1}$. Cypermethrin also caused increase of LPO level in liver and kidneys of common carp (*Cyprinus carpio*) and in liver of Nile mouthbreeder, *Oreochromis niloticus* (Uner *et al.* 2001), in larvae homogenate of zebrafish, *Branchydanio reiro* (Shi *et al.* 2011) and rainbow trout, *Oncorhynchus mikkis* (Sakin *et al.* 2011) as well as in liver of rohu, *Labeo rorita* (Marigoudar *et al.* 2013). Significant increase of lipid peroxidation was observed in liver, brain and gills of common carp after acute deltamethrin exposure at 6.56 µg.L$^{-1}$ and 65.6 µg.L$^{-1}$, but after 96 h recovery time, the level of LPO returned to the physiological range (Stara *et al.* 2015). No changes in LPO level were observed by Richterova *et al.* (2015) in early life stages of common carp after 35 days cypermethrin exposure and by Stara *et al.* (2013) after exposure of juvenile common carp to zeta-cypermethrin.

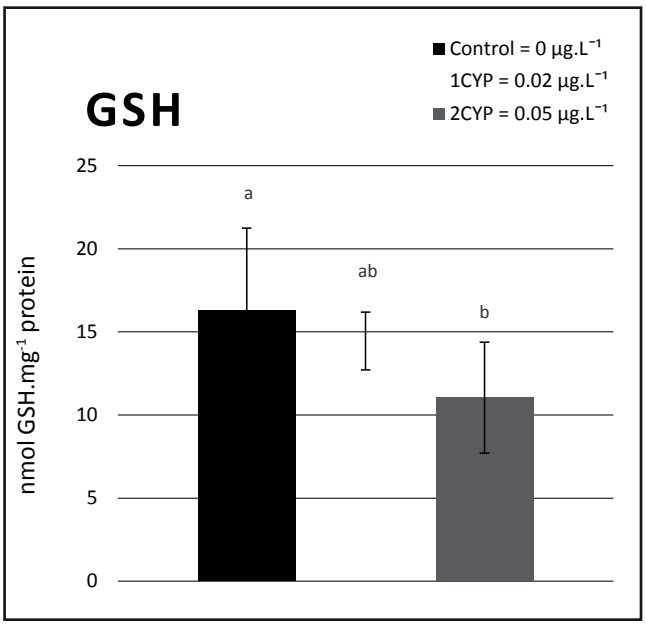


**Fig. 4:** The effect of acute exposure to Cyperkill 25 EC on level of GSH in whole-body homogenate of marbled crayfish *Procambarus fallax* f. *virginalis*. Data are means ± SD. Significance levels observed are $p<0.05$ compared to the control group.

*Antioxidant biomarkers*

Superoxide dismuases belong to the class of metaloenzymes which transform superoxide anions to molecular oxygen and hydrogen peroxide. SODs are one of the most important primary enzymatic antioxidants in cell protection (van der Oost *et al.* 2003). In our study,

activity of SOD significantly increased in 2CYP group, suggesting that decreased level of LPO was probably caused by increasing SOD activity. Significant increase of SOD activity was also observed after 48 hours with a following decline of this enzyme after 96 hours in hepatopancreas of the red swamp crayfish after exposure to beta-cypermethrin (Wei & Yang 2015a). In gills of identical species, SOD activity was higher after 24 h and lower after 48, 72 and 96 h exposure to beta-cypermethrin, respectively (Wei & Yang 2015b). Catalase changes hydrogen peroxide to water and oxygen (van der Oost *et al.* 2003). In our test no significant differences in CAT activity were observed among groups. Significant decrease in CAT activity was described in hepatopancreas (Wei & Yang 2015a) and gills (Wei & Yang 2015b) of red swamp crayfish after 96 hours exposure to beta-cypermethrin.

Glutathion reductase belongs to the secondary antioxidant enzymes. GR is a catalyst which converts oxidised glutathione (GSSG) to the reduced glutathione (GSH) (van der Oost *et al.* 2003). Our experiment showed reduction of GR activity between both cypermethrin-treated groups and control. Gltuathione-S-transpherases are multi-purpose antioxidant enzymes catalyzing reaction between glutathione and electrophilic xenobiotics. Reduced glutathione is one of the most important non-enzymatic antioxidants in cell protection against oxidative stress damage. The main functions of GSH are biotransphormation of electrophilic compounds and involvement in detoxification of peroxides (Valavanidis *et al.* 2006). Our experiment demonstrated significant decrease of GST and GSH in 2CYP group compared to the control. Activity of GR, GST and GSH after cypermethrin exposure has not been described in crayfish previously. Information about the activity of antioxidants are extensively described in fish. Rychterova *et al.* (2015) also observed significant decrease of GST and GR activity in early life stages of common carp after 35 days exposure to 7.2 $\mu g.L^{-1}$ of Cyperkill 25 EC. Exposure to 3 $\mu g.L^{-1}$ of cypermethrin caused significant increase of SOD and CAT in liver of Nile mouthbreeder and in liver and kidneys of common carp (Uner *et al.* 2001). Stara *et al.* (2013) reported effect of zeta-cypermethrin to juveniles of the same species, showing increasing activity of SOD and GR and decrease of CAT. Reductions of CAT and GSH activity were observed in rainbow trout larvae (Sakin *et al.* 2011), while increase of CAT activity was described by Marigoudar *et al.* (2013) in liver of rohu exposed at 0.4 $\mu g.L^{-1}$ and 4 $\mu g.L^{-1}$ of cypermethrin for 96 h. Acute exposure to pyrethroid deltamethrin causes significant changes in enzymatic activity of SOD, CAT and GR in liver, gills, brain, muscle and kidney of common carp at concentrations 6.65 $\mu g.L^{-1}$ and 65.6 $\mu g.L^{-1}$. After 96 h recovery time, higher GR activity in gills and higher CAT activity in intestine were observed at the highest concentration applied. Remaining parameters returned to the level of control group (Stara *et al.* 2015).

## CONCLUSIONS

Investigation of pyrethroids in connection with environment and their effects is of key importance, especially with focus on their previously overlooked effects on non-target organisms. Acute exposure to Cyperkill 25 EC has shown to alter the oxidative stress and antioxidant systems of marbled crayfish. The crayfish has been proved as a highly sensitive and appropriate organism for toxicological studies with pyrethroids. Measurement of oxidative stress and antioxidant biomarkers provides useful information for evaluating the toxic effects of cypermethrin on the non-target aquatic organisms (e.g. crayfish). However, more detailed studies must be conducted before application of such an approach for monitoring of the aquatic environment.

## ACKNOWLEDGEMENTS

The present study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects „CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program) and by the GAJU No. 012/2016/Z.

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