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Introduction

Copper (Cu) is a trace element which is essential to life, especially in cellular biochemical reactions (including cellular respiration) and acts as a cofactor for many enzymes. However, at high concentrations, it may be toxic for living organisms. Increasing concentrations of Cu in the environment have been attributed to anthropogenic sources, especially in agriculture where it is used as an antifungal agent. The effects of Cu on energy metabolism in aquatic organisms are well documented. In fish, gills are the primary target of Cu,[1] where it causes ultra structural damage affecting respiratory, excretory or osmoregulatory functions of this organ. Furthermore, De Boeck *et al.*[2] showed a decrease in oxygen consumption in common carp exposed to Cu and suggested that Cu reduces ventilation and respiration rates. These results indicate a disturbance in aerobic metabolism, as observed by Rajotte and Couture,[3] who suggested that mitochondrial enzymes are a primary target for inhibition by this metal. Mitochondrial electron transport chain supplies over 95% of the total ATP requirement[4] in cells. Energetic status of organisms can then also be assessed through evaluation of chemical energy available at cellular level (adenylate loadings). The roach (*Rutilus rutilus*) is a cyprinid species found throughout Europe. This local and sedentary species is used in biomonitoring of aquatic environment,[5] thanks to its wide distribution in different types of environment. It represents a good bioindicator due to its robustness allowing it to develop in polluted environments.[6] The aim of this study was to determine the effect of Cu on energy metabolism in juvenile roach (to avoid reproductive factors) at different regulation levels (biochemical and molecular). The electron transport system (ETS) was then measured and the cytochrome c oxidase gene expression was followed to assess potential impact of Cu on respiratory chain. Finally, cellular energy was evaluated through ATP, ADP, AMP and IMP concentrations measurements.

Materials and Methods

Exposure conditions

Juvenile roaches were purchased from a commercial pond farm located in Champagne-Ardenne region (France). All fish were kept at the University of Reims first for two weeks in a 400 L aquarium before being transferred for one more week in 12 aquaria of 60 L (9 fish per aquarium). All along acclimatization and exposure, water temperature was maintained at 10°C, fish were fed *ad libitum* every two days with mud worms and photoperiod was kept constant (LD 12:12). Exposure began after three weeks of acclimatization; fish were then exposed at 10; 50 or 100 µg.L^{-1} of copper (using $CuSO_4^{2-}$) during seven days. Eight fish were sacrificed at the beginning of the experiment (T_0) to have reference measurements. During exposure, water was replaced every two days to keep constant Cu concentration. Cu concentrations were checked before and after each water replacement. No mortality was observed except for fish exposed to 100 µg.L^{-1} (50% after one day of exposure, 100% after 7 days). Fish (n=7 to 9) were sampled after 1 and 7 days. After a rapid dissection, white muscle was flash frozen in liquid nitrogen and kept at 80°C until biochemical and molecular analyses.

Analyses

Nucleotides of biochemical energy (ATP, ADP, AMP and IMP) were extracted with an acid solution of trichloroacetic acid following the technique used by Sébert *et al.*[7] The extracts were analyzed using a high performance liquid chromatography method with UV detection (254 nm) as previously described by Cann-Moisan *et al.*[8] The adenylate energy charge was computed as:

$$AEC = ([ATP] + 0.5\ [ADP])/([ATP] + [ADP] + [AMP])$$

Respiratory chain activity was measured following the activity of ETS according to De Coen and Janssen.[9] This method lies on the saturation of electron flux through mitochondrial membrane by adding high levels of natural substrates (nicotinamide adenine dinucleotide and Nicotinamide adenine dinucleotide phosphate). This activity was measured spectrophotometrically and following the substrates disappearance during 6 min at 490 nm.

Gene expression of cytochrome c oxidase subunit 1 (CCOX1) was measured by real-time quantitative polymerase chain reaction according to Livak and Schmittgen.[10] Specific primers were designed according to the coding sequence available in GenBank as HQ600768.1 (For: 5'-GGGTCACTTTTAGGCGATGA-3'; Rev: 5'-TTCGTGGGAATGCTATGTCA-3'). Actin and Ribosomal protein L8 genes were used as housekeeping genes (actinF: 5'-GCTGGAAGCAGCAGGTTATC-3'; actinR: 5'-CACACCATCCACACATCCAT-3'; RpL8F: 5'-ATCCCGAGACCAAGAAATCCAGAG-3'; RpL8R: 5'-CCAGCAACAACACCAACAACAG-3').

Statistical analysis

Statistical analysis was performed using Minitab 16 software. As all parameters were non-normally distributed (Kolmogorov-Smirnov test), non-parametric Kruskall-Wallis and Mann-Whitney U tests were used. Results are expressed as mean±S.E.M, excepted for molecular analysis where a box plot was used.

Results and Discussion

Relative gene expression of CCOX1 in fish exposed to Cu is shown (Figure 1). Expression of CCOX1 increased significantly ($P<0.05$) in all Cu concentrations except in fish exposed to 10 µg.L^{-1} and 50 µg.L^{-1} at T_1 compared to the T_0. Same results were observed comparing contaminated fish to non-exposed ones at their respective time of sampling (T_1 and T_7). CCOX is the last enzyme complex of the respiratory chain and so has a control on the oxidative phosphorylation process, and then is a key site for modulation of energy metabolism. Electron transport activity in white muscle of roach exposed to Cu is shown (Figure 2). No significant difference ($P>0.05$) was found in ETS activity, excepted at T_1 for roach exposed to 100 µg.L^{-1}. At T_1, electron transport activity tended to decrease, when Cu concentrations increased, with a significant decrease in fish exposed to 100 µg.L^{-1}. No difference was

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observed between times of exposure. ETS represents a valid alternative measure to whole animal respiration.[11] It's assumed that ETS activity is an overestimation of the maximal cellular respiration. Both molecular and biochemical approaches used here showed that aerobic metabolism seemed to be affected, but effects were opposite. Indeed, CCOX1 expression is stimulated while ETS activity decreased. We can hypothesize that electron flux was disturbed and that gene expression of CCOX1 was regulated to compensate this disturbance. Respiratory chain is subdivided in four complexes; Cu may affect one or more complexes, or also the membrane environment. This disturbance is early since an effect on ETS is observed from the first day of exposure and it depends on copper concentration. An increased expression of CCOX1 could take place at molecular level to compensate biochemical effect. This molecular response is also fast, since this increased expression is observed at the beginning of exposure and continues until the end of exposure. The hypothesis is supported by values of ETS activity at the seventh day, since no difference is observed at this time. It appears that maintaining electron transport activity at a significant level is essential since mitochondrial electron transport chain supplies over 95% of the total ATP requirement.[4] Then this response prevents a loss of cellular energy, especially ATP. A disturbance in aerobic metabolism has then been observed, especially on mitochondrial metabolism. This may be due to a decrease of oxygen availability in gills and then of ETS activity producing ATP, or could be due to a direct impact of Cu. Finally, results of the present study are in agreement with Pierron *et al.*[12] They observed an increase in gene expression of CCOX subunit 1 and a decrease of CCOX enzymatic activity in wild yellow perch (*Perca flavescens*) in response to Cu exposure in lakes. Moreover a decrease of citrate synthase enzymatic activity has been shown in wild yellow perch muscle.[3,13]

Focusing on energetic nucleotides, Figures 3 and 4 present the adenylate energy charge (AEC) and the concentration of ATP respectively. AEC is indicative of the metabolic energy available to the organism from the adenine nucleotide pool, mainly as ATP, at the time of sampling. AEC is an indicator of the metabolic energy state of cells and is defined as a ratio of

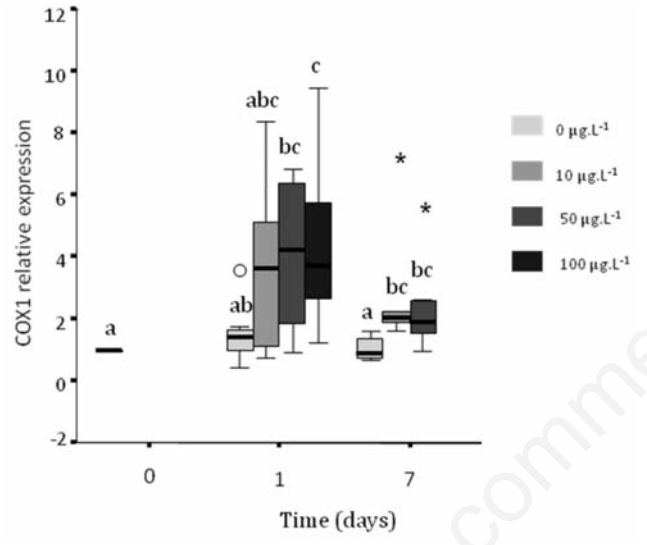

Figure 1. Relative gene expression of cytochrome c oxidase 1 during copper exposure in white muscle of roach. Different letters indicate significant differences (P<0.05). *Correspond to outliers.

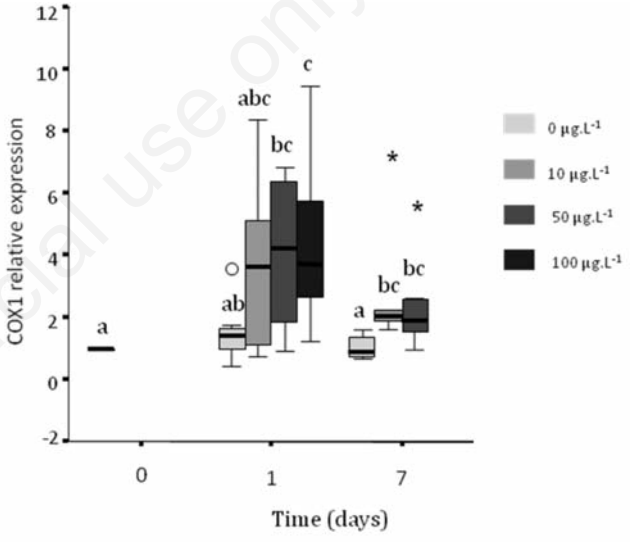

Figure 2. Electron transport system activity during copper exposure in white muscle of roach. Different letters indicate significant differences (P<0.05).

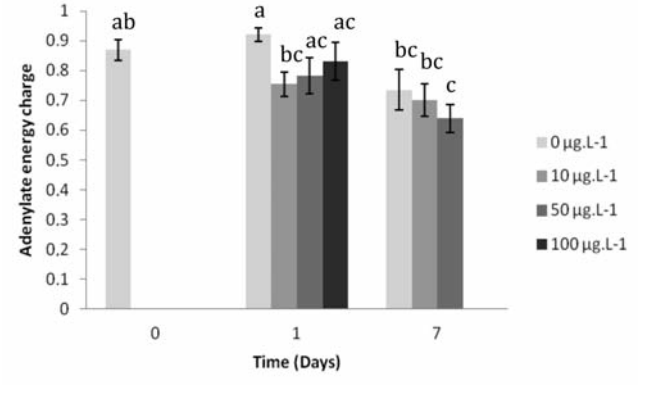

Figure 3. Adenylate energy charge in white muscle of roach exposed to copper. Different letters indicate significant differences (P<0.05).

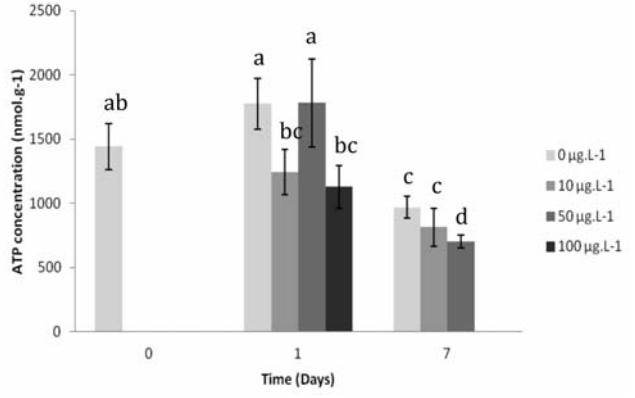

Figure 4. ATP concentration in white muscle of roach exposed to copper. Different letters indicate significant differences (P<0.05).

the different energetic nucleotides concentrations; it may vary between 0 and 1. AEC is equal to 1 when all energetic nucleotides are in the form of ATP, and equal to 0, when all energetic nucleotides are in the form of AMP.[14] If ATP concentration decreases, AMP deaminase[8] allows keeping a constant AEC by converting AMP into IMP, and then reducing the increase of AMP. In the present study, a significant decrease in ATP concentrations was observed in all concentrations tested after one and seven days of Cu exposure (excepted at 50 $\mu g.L^{-1}$ at T_1) compared to the control. This decrease was time and concentration-dependent. An upward trend in IMP level was also observed, but this increase was not significant. At T_1, AEC was lower in exposed fish compared to controls ($P<0.05$ at 10 $\mu g.L^{-1}$). Hence, the data suggest impaired cellular energy. In fact, ATP concentration decreased and IMP concentration increased in parallel, what indicates a cellular energy disturbance. There are several explanations for the changes in energetic nucleotides of exposed fish. First, a high metabolic demand as a direct effect of Cu[15] causes a decrease in ATP level in muscle, possibly for detoxification process. Second, impairment of oxygen transfer in gills may be observed during copper exposure, leading to hypoxia in different tissues of the organism, what may act on energetic nucleotides level leading to a decrease in ATP concentration.[1,16] Finally, Cu can inhibit key enzymes, such as ATPase,[17,18] hexokinase,[19] catalase,[20] etc.

In conclusion, this study revealed a disturbance on aerobic metabolism due to Cu exposure on juvenile roaches. During Cu exposure, two phases are observed: first, a compensatory phase at $T_{1;}$ and second, an exhaustion phase is observed at T_7. An increased expression of CCOX1 is observed, while ETS and AEC seem relatively constant during this period. In parallel, a decrease in ATP concentration is observed. However, organisms are able to cope with toxicant and keep a good energy balance ($AEC>0.7$). At T_7, AEC decreased depending on copper concentrations ($AEC<0.7$) showing a more important stress,[14] while the increase in expression of CCOX1 was lowered compared to T_1. Finally, after one week of experiment, organisms seem unable to compensate this stress.

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