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Effects of multigenerational exposure to elevated temperature on reproduction, oxidative stress, and Cu toxicity in *Daphnia magna*

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ABSTRACT

This study evaluated the effect of temperature (20 and 25 °C) on reproduction, oxidative stress, and copper (Cu) toxicity in *Daphnia magna* across three generations (F0, F1, and F2). Exposing *D. magna* to elevated temperature significantly decreased the number of offspring per female per day, the time to first brood, and body length compared to exposure to the optimal temperature ($p < 0.05$). In addition, elevated temperature induced a significantly higher production of reactive oxygen species and lipid peroxidation ($p < 0.05$). These findings suggest that *D. magna* likely responded to thermal stress by investing more energy into defense mechanisms, rather than growth and reproduction. In addition, oxidative stress at the elevated temperature gradually increased with each generation, possibly owing to the reduced fitness of the offspring. Exposing *D. magna* to 25 °C ($EC_{50}=34\pm3$ μg L^{-1}) substantially increased the median effective concentration of Cu in all generations compared to exposure to 20 °C ($EC_{50}=25\pm3$ μg L^{-1}), indicating a decrease in acute toxicity at elevated temperature. However, elevated temperature significantly increased the oxidative stress induced by a sublethal concentration of Cu (10 μg L^{-1}). The interaction between elevated temperature and Cu exposure appears to be synergistic; however, this needs to be confirmed using multiple generations in a long-term experiment.

1. Introduction

Temperature is the most important abiotic environmental factor that influences the behavior, physiology, phenology, and distribution of organisms, especially ectotherms (Chen and Stillman, 2012). For example, elevated temperature promoted accelerated molting and maturity in *Moina micrura* (Chen et al., 2015), and other changes in reproduction, such as total number of offspring and time to first brood (Engert et al., 2013). In addition, elevated water temperature may accentuate oxidative stress by increasing metabolic rate (Abele et al., 2002; Speakman, 2005; Williams et al., 2012). The formation of reactive oxygen species (ROS) and catalase (CAT) was accelerated in *D. magna* at elevated temperature (Becker et al., 2011). Increased lipid peroxidation and antioxidant enzyme activity, such as CAT and glutathione *S*-transferase, were also found in fish after exposure to elevated temperature (Madeira et al., 2013; Vinagre et al., 2012). Therefore, understanding the impact of thermal stress in aquatic systems is important as aquatic animals are subject to seasonal and daily temperature fluctuations (Sappal et al., 2014). As such, global warming is now regarded as a major threat for aquatic ecosystems (Williams et al., 2012).

Many studies have investigated the effect of temperature on metal toxicity in ectotherms (Heugens et al., 2006; Martínez-Jerónimo et al., 2006; Yang and Chen, 1996). Generally, elevated temperature may directly affect the toxicity of metal by increasing metabolic rate, including uptake and accumulation rates (Heugens et al., 2003; Muyssen et al., 2010; Wang et al., 2014). Toxic metals are well-known to generate ROS and cause oxidative damage to biomolecules (Lushchak, 2011; Vergauwen et al., 2013b), raising the possibility of synergistic effects between elevated temperature and metal exposure on oxidative stress (Lushchak, 2011; Muyssen et al., 2010). For instance, oxidative damage to lipids and activities of antioxidant enzymes was increased in *Perna viridis* exposed to mercury at an elevated temperature (Verlecar et al., 2007). However, a negative relationship between metal toxicity and water temperature has also been reported (Mubiana and Blust, 2007; Perschbacher, 2005). For instance, lower acute toxicity and internal concentration of copper (Cu) with increasing water temperature was observed in *Enchytraeus crypticus* (Cedergreen et al., 2013). Thus, the overall effect of temperature on metal toxicity remains equivocal.

Recently, multigenerational exposure to elevated temperature has gained much attention owing to the predicted impacts of global warming (Loureiro et al., 2015; Walsh et al., 2014). Changes in the maternal environment can lead to a reduction in the performance or quality of offspring (Marshall and Uller, 2007). For

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instance, *Caenorhabditis remanei* that were exposed to heat stress produced offspring that were more vulnerable to the stress of elevated temperature (Sikkink et al., 2014). Since ectothermic animals may encounter the additional stress of chemical exposure, the interactive effects with elevated temperature should be investigated across multiple generations (Noyes et al., 2009). Therefore, the aims of this study were: (1) to evaluate multigenerational effects of temperature (20 and 25 °C) on reproduction and oxidative stress in *Daphnia magna* across three generations (F0, F1, and F2); (2) to evaluate interactive effects of temperature on Cu toxicity toward *D. magna*. We used a freshwater filter-feeding crustacean, *D. magna*, as a model organism, as it is a recognized test organism for ecotoxicology studies, and serves as a vital link between producers (algae) and secondary consumers (fish) in aquatic food chains (Lampert, 2006). In addition, we used Cu as a model metal as it is a well-known toxicant for *D. magna* and a widely found contaminant in water bodies in Korea (Kim et al., 2012b; Yoo et al., 2013).

2. Materials and methods

2.1. Measurements of reproduction and body length

D. magna were obtained from the National Institute of Environmental Research, Korea and had been cultured in the laboratory since 2010. Daphnids were grown in Elendt M4 medium (pH = 7.8 ± 0.1 and hardness = 250 ± 25 mg L^{-1} $CaCO_3$) under a 16:8-h light: dark photoperiod at 20 °C. The culture medium was renewed twice weekly and organisms were fed daily with approximately 5×10^6 cells mL^{-1} of *Chlorella* spp.

For multigenerational testing, *D. magna* were exposed to two temperature treatments (20 and 25 °C) for three generations. Ten neonates (< 24-h-old) from the initial culture were used as the parental generation (F0) and cultured in glass beakers under the same conditions as the initial culture. Third brood neonates from the parental generation were used to culture offspring generations F1 and F2. Neonates from the second to sixth broods from each generation were used for further analyses.

Reproduction was assessed according to OECD Test Guideline 211 (OECD, 1996). For each generation, time to first brood, total number of offspring per female, and number of broods was determined over 21 d. No male and dead animals were found in either the control or treatment groups (data not shown).

In addition, body length was measured using adult *D. magna* (5-d-old) with 10 replicates. Neonates (< 24-h-old) from each generation were exposed to two temperatures, 20 and 25 °C, in Elendt M4 medium (pH = 7.8 ± 0.1 and hardness = 250 ± 25 mg L^{-1} $CaCO_3$) under a 16:8-h light: dark photoperiod for 5 d. Body length from the top of the head to the base of the spine was determined using an inverted fluorescence microscope (Axio Observer D1, Carl-Zeiss, Oberkochen, Germany).

2.2. Measurements of acute toxicity

Acute toxicity testing was performed according to OECD guideline 202 (OECD, 2004), with four replicates. Second brood neonates from each generation were exposed to one of five Cu ($CuCl_2 \cdot 2H_2O$, Sigma Aldrich, USA) concentrations ranging from 10 to 50 μg L^{-1} or a control. Concentrations were selected based on the median effective concentration (EC_{50}) for *D. magna* (22 μg L^{-1}) (Yoo et al., 2013). Each test well contained 10 mL of test solution and five neonates. The ISO medium (pH = 7.8 ± 0.1 and hardness = 250 ± 25 mg L^{-1} $CaCO_3$) used for dilution and control water was as described by the International Organization for Standardization (ISO, 2012).

Toxicity tests were conducted at two temperatures, 20 and 25 °C, with a 16:8-h light: dark photoperiod of 48 h; no food was given and the test medium was not renewed during this time. Animals not responding to gentle agitation for 15 s were counted as immobilized and the EC_{50} with 95% confidence limits was calculated using probit analysis. Standard reference toxicity tests were conducted between the acute toxicity tests using $K_2Cr_2O_7$ and compared with a control chart.

Concentrations of Cu in the test medium were analyzed using an inductively-coupled plasma-optical emission spectrophotometer (ICP-OES; 730 Series, Agilent Technologies, CA, USA) before and after acute toxicity testing. Calibration curves ($r^2 > 0.995$) were obtained daily using freshly prepared standard solutions, and the detection limit of the ICP-OES was 1 μg L^{-1}.

2.3. Measurements of oxidative stress

All measures of oxidative stress were assessed using adult *D. magna* (5-d-old) with three replicates, except for ROS measurements that used 20 replicates. Neonates (< 24-h-old) from each generation were exposed to two temperatures, 20 and 25 °C, in Elendt M4 medium (pH = 7.8 ± 0.1 and hardness = 250 ± 25 mg L^{-1} $CaCO_3$) under a 16:8-h light: dark photoperiod for 5 d.

The amount of ROS produced in *D. magna* was determined using 2′7-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma Aldrich, USA) following LeBel et al. (1992). After exposure to one of two temperatures (20 and 25 °C) with or without Cu (10 μg L^{-1} in ISO medium) for 48 h, each living daphnid was transferred to a 96-well plate containing 200 μL of 10 μM H_2DCFDA. A 25 mM H_2DCFDA solution was prepared with methanol and diluted with the ISO medium to a final concentration of 10 μM. The well plate was then incubated for 4 h in the dark at 20 and 25 °C. Fluorescence was measured using a fluorescence plate reader (Model Victor3, Perkin Elmer, NY, USA) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence intensity was divided by the dry weight of daphnids and normalized to the ISO control.

Levels of lipid peroxidation and glutathione peroxidase were analyzed following Barata et al. (2005). After exposure to one of two temperatures (20 and 25 °C) with or without Cu (10 μg L^{-1} in ISO medium) for 48 h, 30 (lipid peroxidation) and 20 (glutathione peroxidase) living daphnids were homogenized in phosphate buffer (100 mM) at pH 7.4. The homogenates were then centrifuged at 10,000 × *g* for 10 min and the supernatants collected for further analysis. Enzymatic measurements were conducted with a microplate spectrophotometer (BioTek Inc., Winooski, VT, USA) using a malondialdehyde (MDA) assay kit (NWK-MDA01, Northwest Life Science Specialties, Vancouver, WA, USA) and a GPx kit (K762–100, BioVision Inc., San Diego, CA, USA). All tests were carried out following the manufacturer instructions and enzyme activity was reported in units per mg protein (U mg^{-1}). Lipid peroxidation was evaluated by measuring the production of MDA reacting with thiobarbituric acid at 60 °C for 1 h. One unit of GPx activity was described as the amount of sample required to oxidize 1 μmol of NADPH to NADP at 25 °C for 1 min. Total protein concentration was measured following Bradford (1976) using bovine serum albumin (Bio-Rad Laboratories, Hercules, CA, USA) as an assay standard.

2.4. Statistical analysis

All statistical analyses were conducted in SAS program version 9.4 (SAS Institute Inc., Cary, USA). One-way analysis of variance (ANOVA) was used to analyze the statistical difference in body length among both generation and treatment groups. *T*-tests were used to analyze differences in body length among temperature