Combined cadmium and temperature acclimation in *Daphnia magna*: physiological and subcellular effects

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Combined cadmium and temperature acclimation in *Daphnia magna*: physiological and subcellular effects

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Abstract

Effects of temperature and Cd acclimation (≥ 6 generations) on life history and tolerance responses to stress in three clones of *Daphnia magna* was examined using a 2×2 design (20 and 24°C, 0 and 5 µg L^{-1} Cd). Endpoints include acute Cd and heat tolerance, individual traits such as ingestion rates, growth and reproduction responses and physiological attributes such as acute Cd and heat tolerance, energy reserves, electron transport system activity, haemoglobin and oxidative stress enzymes. Cd (20°C+Cd) did reduce reproduction, but acclimation to 24°C+Cd did not decrease reproductive output additionally. For energy reserves, on which Cd and temperature acted similarly, no synergistic effect could be demonstrated. Generally, the effect of 24°C+Cd was comparable to that of the 24°C acclimation. Cd acclimation at 20°C resulted in organisms which were more tolerant to acute Cd and heat shock challenge, while the contrary was observed at 24°C. A relationship between tolerance to Cd and heat shock and superoxide dismutase (SOD) activity was observed. Significant interclonal variation and genotype×environmental interactions in the measured traits evidenced that clones responded differently. As natural populations are invariably exposed to multiple stressors and genetic variability may change accordingly it is essential to improve our knowledge on the effects of such scenarios in order to allow a correct incorporation in ecological risk assessment methodologies.

Keywords: temperature, cadmium toxicity, interaction, water flea, energy, metabolism, oxidative stress

1. Introduction

Organisms like the zooplankter *Daphnia magna* that inhabit shallow or small water bodies may experience rapid diurnal or slow seasonal changes in temperature. On a larger time scale, global warming is considered as one of the new and important threats to freshwater habitats (Brönmark and Hansson, 2002). Additionally, heavy metal contamination remains an environmental concern in many of these habitats (Brönmark and Hansson, 2002). Therefore, an understanding of the interactive effects and mechanisms of multiple stressors (such as metal pollution and temperature) is critical for predicting tolerance limits and productivity of ectotherm populations.

The effects of temperature on metal toxicity, uptake and accumulation has been the subject of several studies (Cairns et al., 1978; Heugens et al., 2001, 2003; Sokolova and Lannig, 2008). Generally, the temperature-toxicity relationship for metals demonstrates that elevated temperatures tend to enhance toxic effects of metals on organisms which may be (partially) explained by the higher uptake rate of metals and a higher intrinsic sensitivity of the organisms.

The number of physiological studies on the combined effects of pollutants and temperature is far more limited. Energy metabolism can be regarded as a key target for the stress effects of temperature and toxic metals, due to the key role of energy balance in stress adaptation and tolerance (Sibly and Calow, 1989). According to a conceptual model of Sokolova and Lannig (2008) and references herein, metal exposure interferes with cellular processes such as ion homeostasis, protein stability and mitochondrial capacity and efficiency, leading to elevated costs for maintenance and detoxification and an impaired oxygen supply. These effects will be balanced as long as energy supply from food is sufficient and systemic functions (ventilation and circulation) and cellular machinery can provide enough ATP to sustain elevated basal metabolism. With rising temperature, the synergistic effects of

temperature and metal exposure on energy demand override the aerobic metabolic machinery, resulting in progressive hypoxemia. This mismatch between elevated energy demand and limited energy supply during these combined exposures will lead to elevated mortality and whole-organism physiological stress. The above-described model can also explain the interference of metal exposure with thermal tolerance resulting in a rapid onset of tissue hypoxemia and aerobic energy deficiency, and therefore decreasing temperature limits in metal-exposed animals.

Many studies, of which several used daphnids as a test species, contribute elements in favour of the model. However, it seems plausible that due to the fact that similar physiological processes (e.g. respiration, oxygen transport and oxidative stress) are involved in temperature and cadmium stress the outcome of their combined stress may be non-intuitive. Hallare et al. (2005), for instance, found pre-hatched zebrafish embryos to be less sensitive to Cd at higher temperatures due to the higher expression of heat shock proteins (hsp70). After hatching, however, larvae showed an increased sensitivity to Cd at higher temperature. Also it is not clear if detoxification and elimination rates of metals increase at higher temperatures (Heugens et al., 2001). Especially, for long-term exposures it may be hypothesized that physiological acclimation to one stressor may reduce the effects of the other.

In the present study *D. magna* was acclimated for ≥ five generations to two temperatures (20 and 24°C) and two Cd concentrations (0 and 5 μg L^{-1}). Endpoints measured at the end of the acclimation period were reproduction, acute Cd and heat shock tolerance, growth and ingestion rate, energy reserves and oxygen consumption, haemoglobin content and catalase (CAT) and superoxide dismutase (SOD) activities. To account for possible interclonal variation, three clones originating from the same pond were tested. By using a combination of long-term acclimation, interclonal variation and moderate changes in temperature and Cd we aimed at a more realistic assessment of the (future) effects of

temperature and Cd exposure on the fitness of *D. magna* in contrast to most standardized laboratory tests.

2. Materials and methods

2.1 Acclimation conditions

The experiments were performed with three clones (F, H and T) of *D. magna* obtained from ephippia that were isolated from sediment collected in the Blankaart pond (Belgium). Upon hatching organisms of each clone (F1 generation) were cultured individually in polyethylene cups at 20°C in 40 ml of modified M4 medium (Elendt and Bias, 1990). The original composition of this medium was adjusted by replacing Na_2EDTA and $FeSO_4$ solutions by natural DOC (4 mg C L^{-1}). The DOC was sampled in Bihain (Ruisseau de St. Martin, Belgium) using a portable Reverse Osmosis (RO) system (PROS/2). The amount of trace elements introduced to the test medium due to the addition of DOC was measured and was negligible. Individuals were fed daily with a mixture of the green algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio based on cell counts. Food was added at 250, 500 and 750 µg dry weight of algae per individual per day during week one, two and three, respectively. New acclimation generations, each time started with 3rd to 6th brood neonates, consisted of ten replicates. The offspring of the second generation cultured at 20°C was divided and used to initiate a third generation at 20°C and a first generation at 24°C. A schematic overview of the acclimation treatments is presented in Figure 1. After two subsequent generations at the respective temperatures, the offspring of each temperature treatment was divided and then used to initiate the control and 5 µg L^{-1} Cd treatments (added as $CdCl_2$ to the test medium). This lead to our final four acclimation series. Daily, the age-specific survival and reproduction was recorded.

When the control culture (20°C) had reached a duration of minimum 8 generations (i.e. 8 or 9 generations depending on the clone), 100 individuals from the fifth brood of each treatment were collected and transferred to polystyrene aquaria containing 2 litres of modified M4 medium and the respective temperature and Cd concentration (density of 1 *Daphnia* per 20 ml). The acclimation time of the 20°C+Cd, 24°C and 24°C+Cd treatments at this moment is presented in Figure 1. The medium was gently aerated and renewed two times a week. Organisms were fed 160 µg algae mix per *Daphnia* during the first week and 240 µg during the second week. Daily, the food concentration in the aquaria was measured and adjusted to these levels. The offspring of this generation was used in acute Cd toxicity tests and heat shock experiments and to start of a final generation of which organisms (14-days old) were used to measure length, ingestion rate, energy reserves, energy consumption, haemoglobin concentrations and antioxidant enzyme activities. In parallel, the individual culturing of the three clones at the different treatments continued. A final monitoring of these individual cultures started simultaneously for the three clones when acclimation had reached at least 9, 5, 8 and 6 generations at 20°C, 20°C+Cd, 24°C, and 24°C+Cd, respectively.

2.2 Acute toxicity test and heat shock experiment

Acute toxicity assays were performed following OECD guideline 202 (OECD, 1996). For each clone, three replicates of ten juveniles (< 24 h old) were exposed to at least five Cd concentrations and a control. Experiments were performed in the modified M4 medium (see section 2.1) and the respective temperature with a light:dark cycle of 16h:8h. Each test vessel contained 40 mL of test medium. After 48 h the number of immobilized organisms in each vessel was counted and 50% effective concentrations (48hEC50 values) with their 95% confidence limits were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). Reported 48hEC50s are based on measured (dissolved) Cd concentrations.

Heat shock tests were performed according to Kivivuori and Lahdes (1996). For each clone, 20 juveniles (< 24 h old) were exposed for 15 minutes to at least five temperatures (range 35 to 38.5°C). Animals were placed in 40 ml of culture medium in a warm water bath and the temperature in the test vessels was recorded with a precision of 0.1 °C. Following the heat shock, organisms were transferred to medium at room temperature for 30 minutes (recovery period) after which the number of dead organisms was determined and the lethal temperature for 50% of this test population (15minLT50) was calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977).

2.3 Length and ingestion rate

For each clone and acclimation treatment ten organisms were measured. The daphnids were placed on a marked (precision 0.1 mm) microscope slide and a digital picture was taken using a microscope equipped with a camera. The daphnids' length was determined from the top of the head to the base of the spine (UTHSCSA Image Tool for windows v 3.00).

For the determination of ingestion rate sixteen daphnids were removed from the aquaria and distributed over four replicate polyethylene cups containing 40 ml of medium identical to that in the test aquaria (water characteristics and food concentration). One additional replicate without organisms was used for calculating the correction factor (A). At the test initiation and after an 8-hour period, the algal concentration was measured and the filtration rate (F) and the ingestion rate (I) were calculated following Ferrando and Andreu (1993):

$$F = \frac{V}{n}\frac{(\ln C_0 - \ln C_t)}{t} - A$$

$$A = \frac{\ln C_0 - \ln C'_t}{t}$$

$$I = F\sqrt{C_0.C_t}$$

where C_0 and C_t are the initial and final food concentration (cells/mL), t is the time of exposure, n is the number of organisms per vessel, V is the test volume, and C'_t is the final cell concentration in the control cup (without daphnids added).

2.4 Energy reserves and electron transport system (ETS)activity

Energy reserves were measured as the sum of protein, carbohydrate and lipid content in the organisms. The activity of the ETS, which is a measure of the maximum aerobic capacity of an organism, was determined as an indication of energy consumption. All measurements were made following De Coen and Janssen (1997). For each energy fraction and ETS measurement three replicates of five 14-day old organisms were used. Daphnids were collected, shock-frozen in liquid nitrogen and stored at -80°C until analysis. The different energy contents (mg per organism) were measured spectrophotometrically and converted into mJ equivalents. Measurement of the ETS activity was based on the spectrophotometric determination of INT reduction (p-IodoNitro Tetrazolium Violet) and expressed as mol O_2 per organism per minute.

2.5 Haemoglobin, catalase (CAT) and superoxide dismutase (SOD)

Haemoglobin concentrations were measured using the protocol developed by Hildemann and Keighley (1955). For each clone three replicates of ten 14-day old organisms were collected and homogenised in 200 µl cold deionised water. An additional 1 ml cold deionised water was added and the sample was centrifuged for 5 minutes at 10 000 g. 1.2 ml of the homogenate was transferred to a cuvet to which 800 µl deionised water and 30 µl 0.1 % potassium cyanide (KCN) was added. The absorbance of the final preparation was measured spectrophotometrically at 415 nm, compared with a reagent blank and standardised against powdered bovine haemoglobin (Sigma H-4632) over a range of 0.015 to 0.15 g L^{-1}.

To measure CAT activity, three replicates of ten 14-day old organisms were collected from each clone and homogenised in 50 mM phosphate buffer (pH 7.0). Samples were centrifuged for 5 minutes at 10 000 g. CAT activity was measured as the decrease in absorbance at 240 nm due to H_2O_2 consumption (Aebi, 1984). 100 µl of the homogenate was transferred to a cuvet and 400 µl phosphate buffer and 500 µl 20 mM H_2O_2 (Merck) were added. The absorbance was measured every 6 seconds during 42 seconds. One unit of CAT is defined as the amount of sample that will decompose 1 µmol of H_2O_2 to oxygen and water per minute.

SOD activity was determined using an indirect method involving the inhibition of cytochrome *c* reduction. The reduction of cytochrome *c* by $O_2^{\cdot-}$ is monitored by the absorbance increase at 550 nm (McCord and Fridovich 1969). The same homogenate as that prepared for the CAT measurements was used. 15 µl of the homogenate was transferred to a cuvet together with 400 µl xanthine oxidase (10 units/ml, Sigma X-1875) and 2.6 ml reaction cocktail (pH 7.8) with a final concentration in the cuvet of 50 mM phosphate buffer, 0.1 mM EDTA, 0.1 mM cytochrome *c* (Sigma C-7752) and 0.05 mM xanthine (Sigma X-0626). The absorbance was measured at 550 nm every 10 seconds during 1 minute. One unit of SOD is defined as the amount of sample causing 50% inhibition of cytochrome *c* reduction.

2.6 Physico-chemical measurements

During the acclimation period, oxygen concentrations and pH of the test medium were monitored on a weekly basis. Cd samples were 0.45 µm filtered and acidified with 1% 14 N HNO_3 prior to analysis. Cd concentrations in the acclimation cultures and in acute toxicity tests were measured using ICP-MS (Perkin-Elmer Elan DRC-e) and flame atomic absorption spectrophotometry (SpectrAA100-Varian), respectively. DOC concentrations were measured with a TOC analyzer (TOC5000, Shimadzu, Duisburg, Germany).

2.7 Statistical analyses

48hEC50s and 15minLT50s and their 95% confidence limits were compared pair-wise using the method described by Wheeler et al. (2006). The significance ($p<0.05$) of the effects of temperature, Cd, clone and their interactions on the additional endpoints was investigated using three-way analysis of variance (ANOVA). If ANOVA assumptions (i.e. homogeneity of variances and normality) were not fulfilled data were rank transformed prior to analysis. Post-hoc Duncan's test was used to demonstrate significant differences between treatments. Superscript letters indicate significant differences between treatments within one clone: values sharing the same letter are not significantly different.

3. Results

Physico-chemical measurements of the test medium showed that oxygen levels ranged from 8.6 to 9.0 mg L^{-1} and pH from 7.31 to 7.73. The Cd concentration in the control medium was < 0.01 µg L^{-1}. The nominal 5 µg L^{-1} Cd treatment of freshly prepared medium corresponded to measured values of 4.30 ± 0.23 and 4.26 ± 0.17 µg L^{-1} Cd at 20 and 24°C, respectively. At the time of renewal of the test medium (i.e., 'old' medium) Cd concentrations had decreased to 2.86 ± 0.58 and 2.85 ± 0.74 µg Cd L^{-1}, respectively. Average DOC concentrations ranged from 3.70 to 4.10 mg L^{-1} in fresh medium and from 5.03 to 5.74 mg L^{-1} in old medium.

Reproduction of the surviving females, time to first brood and the number of broods produced during 21 days are presented in Table 1. Survival was ≥ 80% in all treatments and was considered not affected. The significance of the effects of the different treatments on reproduction and additional endpoints is summarized in Table 2. Generally, total reproduction

was highest in the 24°C treatment. This higher reproductive output was linked to a shorter time to first brood and a larger number of broods during the 21-day period. Cd significantly decreased total reproduction, but did not increase time to first brood or decrease the total number of broods produced. The combined effect of temperature and Cd (24°C+Cd) did not result in a reduction of the total reproductive output of the organisms or the time to first brood compared to the Cd acclimated organisms at 20°C.

The results of the acute Cd toxicity tests are presented in Figure 2A. Cd acclimation did significantly increase the Cd tolerance only in clone H, while acclimation to 24°C significantly decreased the 48hEC50 values of all three clones. The average acute Cd tolerance of the three clones decreased from to 112 ± 14 $\mu g\, L^{-1}$ at 20°C to 64 ± 12 $\mu g\, L^{-1}$ at 24°C. The 48hEC50 of organisms acclimated to the 24°C+Cd was similar to that of organisms acclimated to 24°C. Interclonal variation was significant only at 24°C (control and in combination with Cd).

The results of the heat shock experiment are presented in Figure 2B. For the three clones acclimation to 20°C+Cd increased the 15minLT50 significantly. In clone F and H, acclimation to 24°C and 24°C+Cd resulted in organisms with similar heat tolerance as the control (20°C). In clone T, heat tolerance at 24°C was significantly lower compared to 20°C. Interclonal variation was significant in all acclimation treatments except at 20°C (control).

The effect of temperature and Cd acclimation on the size of the organisms is shown in Figure 3A. Length ranged from 3.13 ± 0.33 to 3.43 ± 0.15 mm. Although variation was limited and no clear trend as a function of Cd or temperature acclimation was obvious, three-way ANOVA analysis demonstrated a significant effect of temperature and a significant clone×Cd and clone×temperature×Cd interaction. Ingestion rates (Figure 3B) ranged from 2.02×10^5 to 2.40×10^5 cells $org^{-1}\, h^{-1}$. They were shown to be significantly affected by temperature. Interclonal variation as well as the interaction clone×Cd were significant.

Total energy reserves (sum of proteins, carbohydrates and lipids) are presented in Figure 3C. The response of clone H and F was similar: at 20°C Cd reduced energy reserves significantly by 20 and 38%, respectively. At 24°C energy reserves decreased by 46 and 47%, respectively. There were no significant differences between 24°C and 24°C+Cd acclimated organisms. In clone T, energy reserves of control organisms (20°C) were lower than in the other two clones and Cd acclimation slightly increased the energy reserves of the organisms. Three-way ANOVA demonstrated a significant effect of clone, Cd, temperature and their interactions. On average the proteins, carbohydrates and lipids accounted for 11, 21 and 68% of the total energy reserves, respectively. Proteins and lipids were similarly affected by the acclimation treatments and were therefore both responsible for the observed changes in total energy reserves of the organisms. The energy transport system (ETS) activity, which is a measure of the energy consumption in an organism is presented in Figure 3D. Three-way ANOVA analysis only indicated a significant interaction of temperature×Cd. Values ranged from 0.364 ± 0.032 and 0.613 ± 0.397 nmol O_2 per organism per minute.

In the three clones, Cd induced a significant and almost 50% increase in the haemoglobin content of the daphnids, while temperature did not have a significant effect (Figure 4A). Acclimation to 24°C+Cd resulted in the same haemoglobin content as measured in the 20°C+Cd treatment. CAT activity is presented in Figure 4B. Two-way ANOVA within each clone did not demonstrate significant differences in CAT activity among treatments but according to three-way ANOVA the enzyme was significantly affected by temperature. In clone F, H and T acclimation to 24 °C decreased the CAT activity by 13, 38 and 39%, respectively. Interclonal variation was significant. Generally, superoxide dismutase (SOD) activity was significantly increased by acclimation to Cd (up to 43% at 20°C) and significantly decreased (up to 16%) by acclimation to 24°C (Figure 4C). The combined 24°C+Cd treatment resulted in an SOD activity similar to that of the 24°C control.

4. Discussion

According to conceptual models described in Heugens et al. (2001) and Sokolova and Lannig (2008) the interactive effects of elevated temperature and trace metal exposure have two consequences in aquatic ectotherms: (1) the sensitization of an organism to metal toxicity and (2) a decrease in thermal tolerance limits in metal-exposed animals. At higher temperatures, due to the increased metabolic activity, metals are mostly found to be more toxic to aquatic invertebrates including *Daphnia* sp. (e.g. Cairns et al., 1978; Heugens et al., 2003). The results of our present study indeed confirm the proposed temperature-induced increase in (acute) metal sensitivity: comparing the 48hEC50s of organisms acclimated to 20°C (112 ± 14 µg L^{-1}) and 24°C (63 ± 12 µg L^{-1}) it was observed that an elevated temperature significantly decreased the acute Cd tolerance of the daphnids. The rather limited increase in acute Cd tolerance due to Cd acclimation (20°C+Cd compared to 20°C), with a maximum increase in 48hEC50 by a factor of 1.3, is comparable to other multi-generation acclimation studies with *Daphnia* (LeBlanc, 1982; Muyssen and Janssen, 2004).

According to the concept of oxygen-limited thermal tolerance in aquatic ectotherms (Pörtner, 2001), it was expected that metal stress combined with elevated temperature would shift critical temperatures to the lower values (Sokolova and Lannig, 2008 and references herein). However, in the present study this decrease in thermal tolerance limits in metal-exposed animals was not found. To the contrary, organisms acclimated to Cd (20°C+Cd) performed significantly better in heat shock tests compared to the controls (20°C). As will be discussed further in this section, the increase in thermal tolerance may be related to the fact that Cd acclimated organisms had a higher activity of the antioxidant enzyme SOD, which may have protected the organisms during heat challenge as well as acute Cd exposure. Studies demonstrating a positive effect of elevated temperature on Cd sensitivity or vice versa are rare.

Nevertheless, as similar physiological pathways such as oxidative stress are involved in exposure to high temperature and Cd it seems plausible that physiological responses induced to counteract one stressor will also reduce the effects of the other. Hallare et al. (2005), for instance, found pre-hatched zebrafish embryos to be less sensitive to Cd at higher temperatures due to the higher expression of heat shock proteins (HSP70). However, after hatching the larvae were more sensitive to Cd at elevated temperature.

The physiological basis for the observed shifts in metal and temperature tolerance is often poorly documented. In the present study we tried to link endpoints at different levels of biological organisation. In addition, to enhance ecological relevance, we used organisms acclimated for several generations to sub-lethal combinations of temperature and Cd exposure. According to Sokolova and Lannig (2008) long-term acclimation is an important understudied aspect of temperature-pollution interaction.

Energy metabolism can be regarded as a key target for the stress effects of temperature and toxic metals, due to the key role of energy balance in stress adaptation and tolerance (Sibly and Calow, 1989; Pörtner, 2002). Both elevated temperature and toxic metals may result in an increased basal metabolic demand. In the present study, we indeed found both temperature and Cd to significantly reduce energy reserves (sum of proteins, carbohydrates and lipids) in two of our studied clones. In 20°C+Cd exposures, and similar to the results of Soetaert et al. (2007), the lipid fraction was affected most. In 24°C exposures, proteins and lipids were affected most. However, a synergistic effect of temperature and Cd on energy reserves was not present, i.e. the energy reserves of organisms acclimated to 24°C+Cd were not lower than these of organisms acclimated to 24°C.

The fact that reduced energy reserves were observed means that the energy demand due to temperature and Cd exposure must have exceeded the energy supply. Temperature is known to determine the rates of all physiological and biochemical reactions in aquatic

ectotherms and an elevated temperature will consequently result in an increase in energy demand for basal metabolism. Metal exposures typically lead to an increase in the aspects of cell metabolism responsible for cellular detoxification (e.g. HSPs, metallothioneins (MTs) and antioxidants) and repair. In the present study the activity of the antioxidant enzymes CAT and SOD were determined as a measure of cellular detoxification. It was observed that at 20°C Cd significantly increased the activity of SOD (up to 43%), while CAT activity was not affected. Similarly, Barata et al. (2005) did demonstrate an increase in the activity of SOD, total glutathione peroxidase (GPX) and glutathione S-transferase (GST), but not CAT, in *D. magna* following exposure to 2-5 μg Cd L^{-1}. SOD (and not CAT) appeared in a list of annotated genes of *D. magna* responding to Cd (6 μg L^{-1}) from a microarray analysis by Connon et al. (2007). The reason for the discrepancy between SOD and CAT response may be related to the fact that CAT (together with GPX) is only acting as a second line defence against reactive oxygen species (ROS), detoxifying H_2O_2 produced by SOD. Surprisingly, an elevated temperature significantly decreased the activity of both enzymes in our study. It is likely that the temperature stress posed on the daphnids in the present study was not sufficient to induce antioxidant activity and that SOD and CAT enzymes therefore followed the general decrease in proteins observed at 24°C (cfr. energy reserves). The present study demonstrates that acclimation to 24°C+Cd resulted in a response similar to that of 24°C, thus a decrease in CAT and SOD activity. It is hypothesized that the reduction in antioxidant enzyme activity in the 24°C and 24°C+Cd treatments is related to the observed decrease in heat shock tolerance and acute Cd tolerance. Similarly the increase in SOD activity following Cd acclimation may be linked to the increase in heat shock tolerance and Cd tolerance of the Cd exposed organisms at 20°C. HSPs and MTs were not measured in the present study, but most likely will have contributed to the stress response of our daphnids as they both play an important role in cellular protection against toxic metals and temperature stress. Information on the effects of a

combined temperature and metal stress on MT and HSP expression in aquatic ectotherms is rare. Ivanina et al. (2009) concluded that the absence of heat-induced HT upregulation in Cd-exposed oysters could reflect a capacity limitation of the transcriptional response or the sufficient level of protection rendered by elevated MT levels. The authors also observed that Cd exposure in these oysters affected the ability to mount adequate HSP response to acute warming, which might be explained by cellular energy deficiency that can limit the amount of ATP available for HSP synthesis and/or function.

One possible mechanism to balance an additional demand for energy is to increase the energy supply from food. In the present study additional energy was not acquired by ingesting more food. Similarly, Burns (1969) did not find an increase in ingestion rate of *D. magna* in the 20-25°C temperature range. This may be partially attributed to the fact that food levels in our experiments were well above threshold levels resulting in ingestion rates that were already maximal and therefore masking (minor) changes in food intake due to temperature or Cd exposure. Besides food intake, up-regulation of digestive enzymes (which was not measured in the present study) can also be considered a compensatory mechanism to cope with reduced energy reserves. This was demonstrated in *D. magna* following Cd exposure (Soetaert et al., 2007).

An alternative mechanism to ensure that enough ATP is available for basal metabolism is to adjust systemic functions (ventilation and circulation) and cellular metabolic machinery, e.g. mitochondrial function (Sokolova and Lannig, 2008). In the present study the activity of the ETS – a measure for oxygen consumption and representing maximal metabolic activity - was measured as well as the concentration of haemoglobin, the central element of circulatory oxygen transport. Trace metals may strongly affect mitochrondrial function, e.g. by reducing activity of the ETS and ATP production (De Coen and Janssen, 2003; Cherkasov et al., 2006). A combination with elevated temperature would further exaggerate hypoxemia

due to the elevated oxygen demand on the one hand, and lower O_2 solubility at high temperatures on the other (Cherkasov et al., 2006; Lannig et al., 2008). In the present study ETS activity was not affected by temperature or Cd. Reduced ETS might have occurred at higher Cd concentrations as observed by De Coen and Janssen (2003). Moreover, the response of ETS activity is not necessarily identical to the effective respiration rates in daphnids (Simčič and Brancelj, 1997). Physiological changes related to respiration such as an increase in ventilation rate and heart rate might have preceded changes at the molecular level (Pörtner, 2002; Paul et al., 2004).

Haemoglobin concentrations – as a measure of oxygen transport capacity - were not increased by elevated temperature, although this observation has been made by other authors for *D. magna* (Lamkemeyer et al., 2003; Seidl et al., 2005). This might be an additional indication of the lack of (oxidative) stress in animals acclimated to 24°C (cfr. SOD activity). Increases in haemoglobin concentration in the above mentioned studies were observed at 30°C (compared to 20°C). Other reasons for the lack of effects on haemoglobin observed in the present study include the fact that the method used did not allow to measure possible shifts in O_2 affinities of haemoglobin and subunit composition while these are also important in determining the oxygen transport capacity (Paul et al., 2004). Moreover, measured concentrations of haemoglobin in the 20°C acclimated organisms, i.e. 0.025 mg Hb org^{-1} (83 mg Hb g DW^{-1} assuming an average DW of 0.3 mg), were already quite high and comparable to those in animals described as haemoglobin-rich (Kobayashi and Tanaka, 1991). And finally, multi-generation acclimation might have decreased haemoglobin levels as observed by Seidl et al. (2005). Cd, however, significantly increased haemoglobin concentrations in *D. magna*, confirming the presence of oxidative stress as already concluded from SOD activity measurements. Other authors have found decreases as well as increases in haemoglobin content or expression in *D. magna* following Cd exposure but these seem dependent on the

Cd concentration and the subunit under consideration (Berglind, 1986; Soetaert et al., 2007; Connon et al., 2008). The 24°C+Cd acclimation resulted in haemoglobin concentrations similar to those of the 20°C+Cd acclimation thus increasing the oxygen carrying capacity in the organisms.

Proceeding to the organismal and population level, it can be concluded that energy reserves do not automatically translate into reproductive output. However, it should also be taken into consideration that reproduction data and energy reserves were obtained from different organisms which were not cultured under identical conditions (cultured individually and in aquaria, respectively). For temperature acclimation, despite the reduced energy reserves at 24°C, no reduction in total reproduction was observed. This was caused by the earlier start of reproduction and larger number of broods within the 21-day period (Sakwínska, 1998; Heugens et al., 2006). The higher number of offspring produced does not necessarily mean that more energy was invested in that offspring. Yampolsky and Scheiner (1996) did demonstrate a trade-off between daphnid offspring size and number as a function of temperature resulting in more but smaller offspring at higher temperatures. At 5 $\mu g\ L^{-1}$ Cd the reduction in energy reserves did reflect in the reproductive output of clone H and T. Cd decreased the time to first brood in clone F and H, but the total number of broods was not affected. Generally, reproduction in 24°C+ Cd was comparable to that of 20°C+Cd, with lower total reproductive output than in the control (20°C and 24°C) and a longer time to first brood than at 24°C. Thus temperature did not enhance the adverse effect of Cd on *D. magna* reproduction as observed by Heugens et al. (2006).

Finally, the interclonal variation demonstrated in the present study deserves some discussion. Although, the number of clones tested for all endpoints was limited to three for practical reasons, we also conducted a parallel study with identical acclimation treatments on 11 additional clones. Here, life-history traits (length, reproduction, r_m) were monitored and

estimates of quantitative genetic variation and genetic correlation between traits and between-environments were obtained (Messiaen M *et al*., in preparation). Interclonal variation in daphnids' performance and physiology has been well-studied in the absence of stressors (e.g. Brookfield 1984) as well as under single stress such as temperature and metal exposure (MacIsaac et al., 1985; Baird et al., 1990; Mitchell et al., 2004; Guan and Wang, 2006). In most cases variation between clones was shown to be (highly) significant although some studies have shown that the genotypic differences in (Cd) tolerance may converge from lethal to sublethal responses and that this convergence could be related to the specific mechanisms of detoxification and energy allocation processes (Baird et al., 1990; Barber et al., 1990). In the present study, although clonal variation were significant for several endpoints (Table 2), differences between clones in the single as well as in the combined treatments were relatively small never exceeding a factor of 1.7. This can be attributed to the long-term acclimation and convergence of responses as indicated by Barber et al. (1990) and Baird et al. (1990) or can also partly be due to the fact that the three clones originated from the same (pristine) environment. Nevertheless, our three clones did not respond similarly to temperature and Cd acclimation which can be derived from the significant genotype × environment interactions shown in Table 2 (T × C and Cd × C). Both interactions affected endpoints important for determining fitness of the organisms (e.g. reproduction and energy reserves). The combined interaction of temperature and Cd (T × Cd × C) was significant for length and energy reserves. Similarly, Barata et al. (2002) concluded that genotype and genotype × environmental factors governed population responses in *D. magna* exposed to Cd and zinc mixtures.

Conclusion

It was hypothesized that a combined exposure to elevated temperature and Cd would lead to the sensitization of an organism to metal toxicity and to a decrease in thermal

tolerance limits in metal-exposed animals. By measuring a series of physiological endpoints related to energy reserves and consumption, oxygen transport and oxidative stress we aimed at gaining insights in the underlying mechanisms of such combined exposures. The present results demonstrate that at 24°C *D. magna* was indeed more sensitive to acute Cd stress. However, the thermal tolerance limits were highest in Cd acclimated organisms. Changes in (acute) tolerance toward temperature and Cd corresponded to changing activities of SOD. Cd acclimation increased haemoglobin concentration similarly at 20 and 24°C, while temperature had no significant effect. Energy reserves were reduced by both Cd and temperature but the combined effect was not synergistic. Energy reserves were not reflected in reproductive output which was reduced by Cd but not by temperature (highest reproduction at 24°C). The combined effect (24°C+Cd) did not result in a reduction of the total reproductive output of the organisms or the time to first brood compared to the Cd acclimated organisms at 20°C. It can be concluded that the combined effect of temperature and Cd acclimation was not predictable. If this was caused by the (sub-lethal) Cd concentration and temperature under investigation or the long-term acclimation period cannot be discriminated by the current experimental design. Future research will aim at monitoring the acclimation process as a function of time, including a wider range of temperature and Cd concentrations.

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Table 1. Reproduction per surviving female (offspring per female) ± SD, time to first brood (days) ± SD and number of broods ± SD of three clones (F, H, T) of *D. magna* acclimated to different combinations of temperature and Cd.

Acclimation	Reproduction			Time to first brood			Number of broods		
	F	H	T	F	H	T	F	H	T
20°C	88 ± 15^{a}	96 ± 11^{B}	$89\pm7^{bc'}$	9.6 ± 0.7^{c}	8.8 ± 0.6^{B}	$7.3\pm0.5^{ab'}$	4.8 ± 0.8^{a}	5.1 ± 0.3^{A}	$4.9\pm0.4^{a'}$
20°C + 5 µg L^{-1} Cd	85 ± 5^{a}	84 ± 13^{A}	$70\pm25^{a'}$	7.3 ± 0.5^{b}	7.8 ± 0.4^{A}	$7.4\pm0.5^{ab'}$	5.2 ± 0.4^{a}	5.2 ± 0.4^{A}	$5.2\pm0.7^{b'}$
24°C	101 ± 10^{b}	96 ± 7^{B}	$99\pm9^{c'}$	6.6 ± 0.5^{a}	7.9 ± 0.6^{A}	$6.9\pm0.3^{a'}$	6.0 ± 0.0^{b}	5.8 ± 0.4^{B}	$6.0\pm0.0^{c'}$
24°C + 5 µg L^{-1} Cd	94 ± 8^{ab}	86 ± 8^{AB}	$75\pm12^{ab'}$	7.6 ± 0.7^{b}	7.2 ± 0.4^{A}	$7.8\pm1.1^{b'}$	6.0 ± 0.0^{b}	5.4 ± 0.5^{AB}	$6.0\pm0.5^{c'}$

Superscript letters indicate significant differences between treatments within one clone: values sharing the same superscript letter are not significantly different.

Table 2. *p* values for three-way ANOVA for effects of temperature (T), cadmium (Cd) and clone (C) and their interactions on the different endpoints measured in *Daphnia magna.*

Endpoint	T	Cd	C	T × Cd	T × C	Cd × C	T × Cd × C
Reproduction *	**< 0.01**	**< 0.01**	**0.04**	0.23	0.09	0.11	0.49
Length *	**0.01**	0.76	0.12	0.05	0.72	**0.01**	**< 0.01**
Ingestion rate *	**< 0.01**	0.63	**< 0.01**	0.26	0.79	**0.01**	0.66
Energy reserves	**< 0.01**	**0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
ETS activity *	0.96	0.89	0.73	**0.04**	0.30	0.21	0.82
Haemoglobin *	0.67	**< 0.01**	0.67	0.14	**< 0.01**	0.40	0.94
CAT	**<0.01**	0.92	**0.02**	0.51	0.06	0.98	0.74
SOD	**< 0.01**	**< 0.01**	0.14	**< 0.01**	0.32	0.31	0.27

*Data were rank transformed prior to analysis.

Figure captions

Figure 1. Schematic time line of the acclimation treatments. Numbers in boxes indicate acclimation generations.

Figure 2. (A) Acute cadmium tolerance (48hEC50) and (B) heat shock tolerance (15minLT50) of three clones of *Daphnia magna* acclimated to combinations of temperature (20 and 24 °C) and cadmium (control and 5 $\mu g\,L^{-1}$ Cd). Error bars represent 95% confidence intervals.

Figure 3. (A) Length (N=10), (B) ingestion rate (N=4), (C) energy reserves (N=3) and oxygen consumption (N=3) of three clones of 14-day old *Daphnia magna* acclimated to combinations of temperature (20 and 24 °C) and cadmium (control and 5 $\mu g\,L^{-1}$ Cd). Error bars represent standard deviation.

Figure 4. (A) Haemoglobin concentration, (B) catalase (CAT) activity and (C) superoxide dismutase (SOD) activity in three clones of 14-day old *Daphnia magna* acclimated to combinations of temperature (20 and 24 °C) and cadmium (control and 5 $\mu g\,L^{-1}$ Cd). Error bars represent standard deviation (N=3).

Figure 1

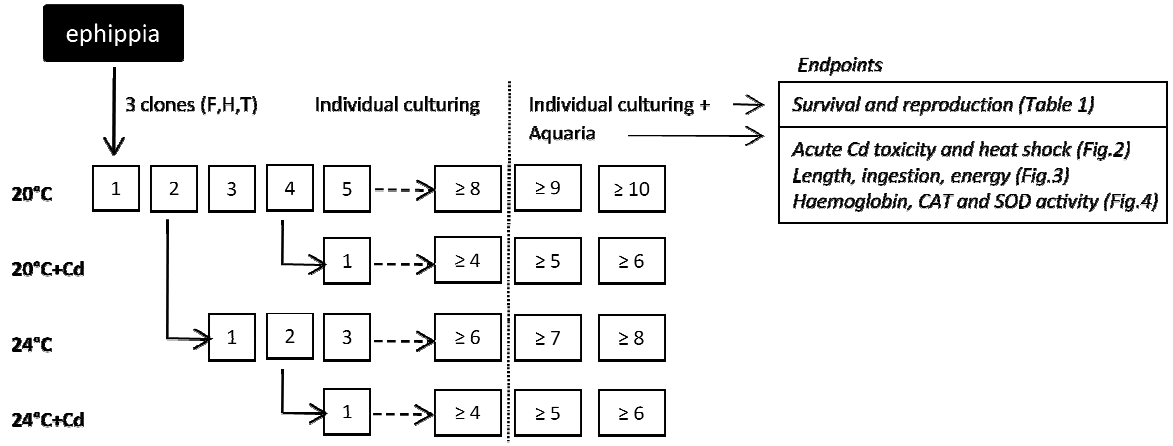
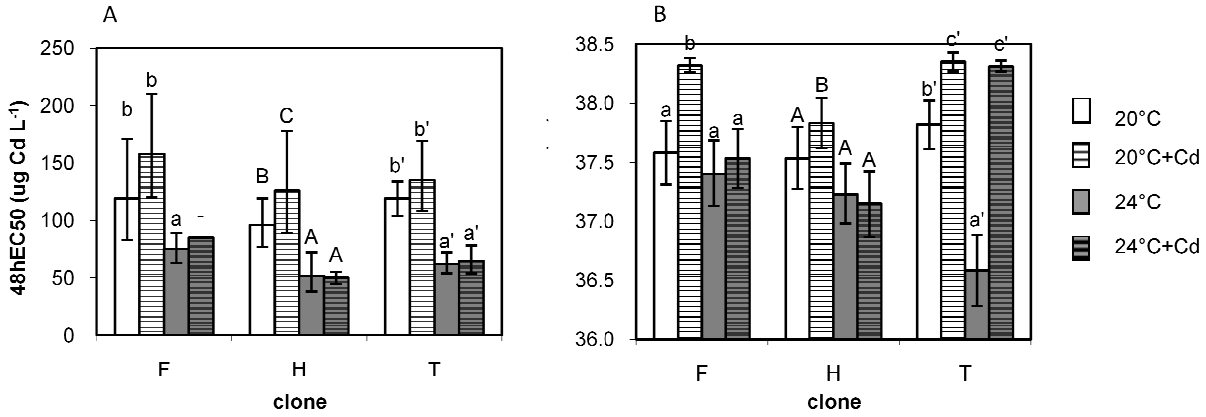
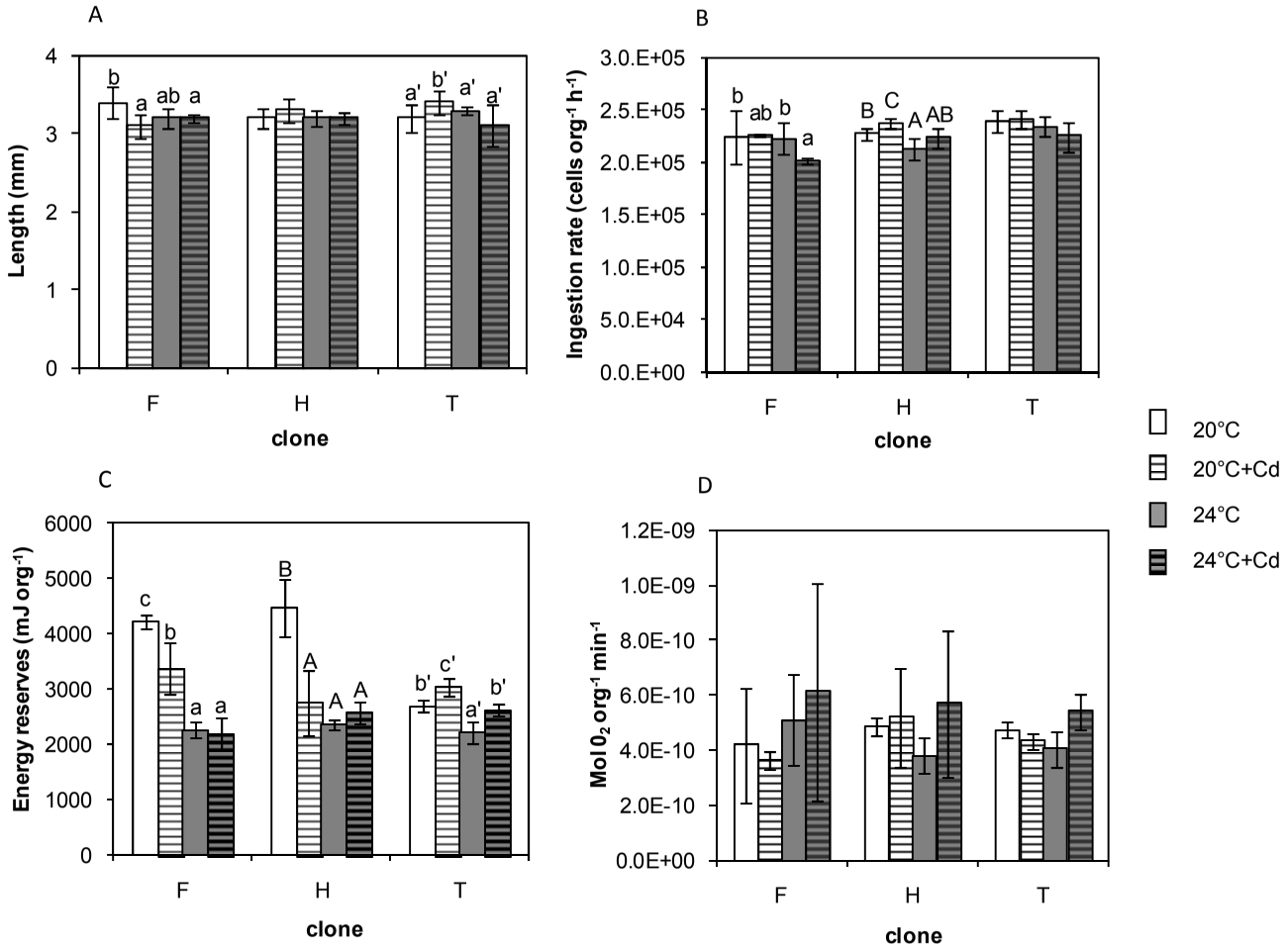

ephippia
3 clones (F,H,T)
Individual culturing
Individual culturing +
Aquaria
Endpoints
Survival and reproduction (Table 1)
Acute Cd toxicity and heat shock (Fig.2)
Length, ingestion, energy (Fig.3)
Haemoglobin, CAT and SOD activity (Fig.4)
20°C
20°C+Cd
24°C
24°C+Cd
1
2
3
4
5
≥ 8
≥ 9
≥ 10
1
≥ 4
≥ 5
≥ 6
1
2
3
≥ 6
≥ 7
≥ 8
1
≥ 4
≥ 5
≥ 6

Figure 2


A
B
48hEC50 (ug Cd L-1)
250
200
150
100
50
0
38.5
38.0
37.5
37.0
36.5
36.0
F
H
T
clone
20°C
20°C+Cd
24°C
24°C+Cd

1

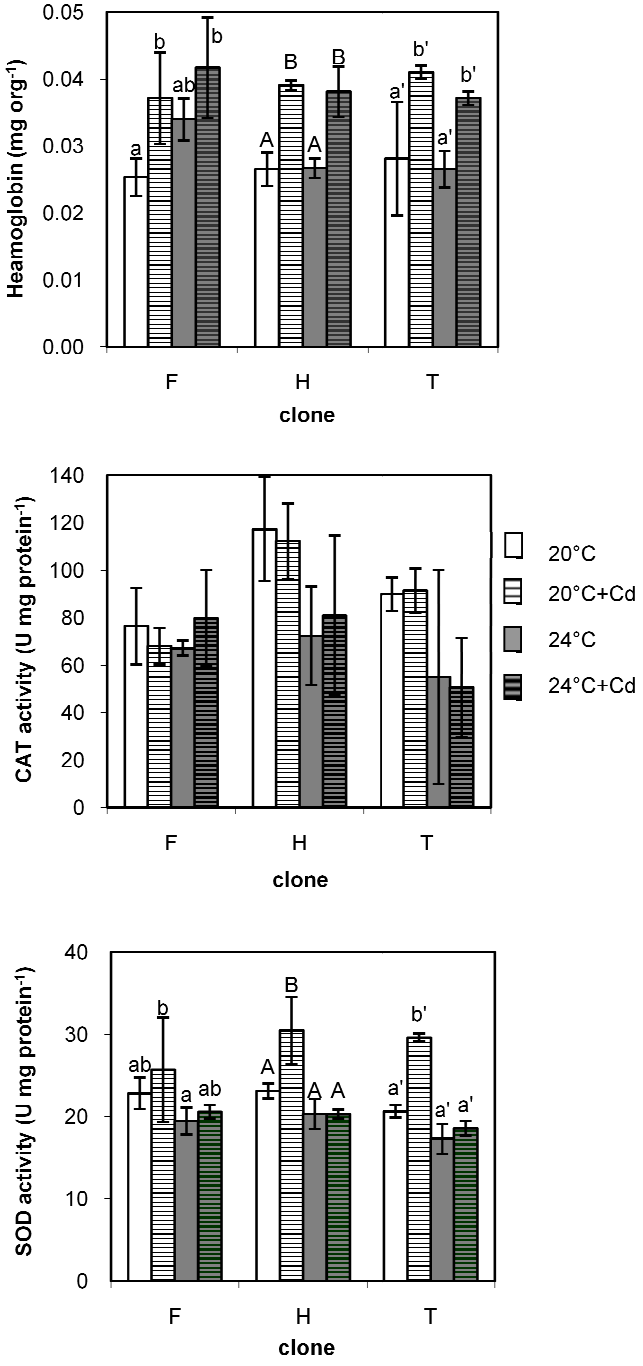
2 Figure 3


A
Length (mm)
clone
F
H
T
B
Ingestion rate (cells org-1 h-1)
C
Energy reserves (mJ org-1)
D
Mol O2 org-1 min-1
20°C
20°C+Cd
24°C
24°C+Cd

3

1

2 Figure 4



3

4