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Effects of Acclimation on Temperature Tolerance and Oxidative Damage in Daphnia magna

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Effects of Acclimation on Temperature Tolerance and Oxidative Damage in *Daphnia magna*

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ABSTRACT

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By

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Freshwater zooplankton crustacean *Daphnia* frequently face strong temperature fluctuations in its natural environment, which necessitates adaptive plastic responses. This study focuses on changes in lipid peroxidation and total oxidative capacity in *Daphnia* tissues in response to long-term and short-term temperature changes. Long-term acclimation to 28ºC helped *Daphnia* survive longer at lethally high temperatures. This difference, however, was not accompanied by changes in lipid peroxidation, indicating that it isn't a good measure of damage or predictor of temperature tolerance.

On the other hand, total oxidation capacity was lower 28ºC- than in 18ºC-acclimated *Daphnia*, suggesting that acclimation resulted in higher amounts of antioxidants in *Daphnia* tissues. Exposure to hypoxia, known to up-regulate antioxidant pathways in *Daphnia*, further elevated heat tolerance in 28ºC- acclimated individuals. Yet, manipulations of glutathione, an important antioxidant, while predictably affecting oxidative capacity, didn't influence heat tolerance in *Daphnia*, suggesting that other antioxidants may play a significant role in it.

DEDICATION

I would like to dedicate my thesis to my family and friends who have always believed in me, even on the days that I didn’t.

ACKNOWLEDGEMENTS

I would like to first acknowledge Dr. Lev Yampolsky. I will never be able to thank him enough for taking me under his wing and allowing me to work in his lab these past two years. Through him, I have gained valuable knowledge, research experience, and a deeper love for science that will help me in my future endeavors. He helped to push me everyday to become better myself and leave the program a better student and researcher than when I first arrived.

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I would also like to extend gratitude to Dr. Dhirendra Kumar for allowing me to come into his lab and use his platereader to complete my research and to Dr. Aruna Kilaru for helping to analyze data. Also, I would like to thank Bret Coggins, my fellow lab mate, for all the time and effort he has put into this project as well.

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CHAPTER 1

INTRODUCTION

Daphnia magna are planktonic crustaceans that play pivotal roles in freshwater zooplankton communities and are often used as a model organism for studies in ecology and ecological physiology. *Daphnia* are suitable model organisms for these studies, due to their reproduction mode, cyclic parthenogenesis, which makes it possible to maintain genetically identical genotypes throughout a variety of environments (Colbourne et al. 2011). An additional advantage is provided by a fully sequenced genome (Colbourne et al. 2011) that allows to approach the identification of genetic variation behind ecologically important traits. In their natural environment of small bodies of water, such as ponds, *Daphnia* are often exposed to broadly varying conditions, including significant temperature and oxygen content changes (Becker et al. 2011; Zeis et al. 2013). If current climate change trends continue for another 50-100 years, global average temperature is predicted to increase by as much as 4ºC and frequency of exposure to much higher temperatures is expected to increase as well (Geerts et al. 2015). Organisms facing these changes in the environment may experience any of the three possible outcomes: ability to respond to elevated temperatures through plastic response that allows them to adjust their physiology to survive new conditions, show an evolutionary response to novel selection pressures, or become extinct. Being able to understand the mechanisms of both plasticity and potential response to selection will help to begin to predict what the overall ecological response will be (Geerts et al. 2015), in particular when the organism in question plays a pivotal role in the ecosystem.

There are studies are being done to see if and how organisms are evolving along with the climate change resulting in local adaptation (Yampolsky et al. 2014) or quick response to temperature manipulations (Geerts et al. 2015). Such studies are interesting because they estimate *Daphnia*'s ability to evolved in response to, and thus to survive, the climate change. It is not known exactly where the adaptation is occurring, based on *Daphnia* responses to a temperature increase, that simulated the 4ºC future warm up, and resurrection study of the adaptive changes of *Daphnia* over

the span of 40 years, it was concluded that they can evolve to survive in the climate change and continue to do so (Geerts et al. 2015). The main goal of this study though, is to look at the lipid peroxidation and total antioxidant capacity response to the acclimation effect.

Harmful effects of stressful high temperatures on organisms include a wide range of damages such as: protein denaturation, cellular membrane excessive fluidity and increased oxidative stress. Accordingly, organisms employ a variety of physiological and biochemical responses to either ameliorate or to cope with such damages. For example, short-term responses to heat shock are dominated by overexpression of heat shock proteins (HSPs), molecular chaperones that enable correct folding of proteins and have the capacity to prevent or correct misfolding or mis-aggregation caused by high temperature (Richter et al. 2010; Mikulski et al. 2011). In *Daphnia* quick up-regulation of some HSPs are known to provide a rapid protect against high temperature and to modulate life span (Schumpert et al. 2014). On the other hand, in long-term acclimation studies, few HSPs show a consistent up-regulation (Williams et al. 2011) and it is not clear how strong their role is in providing increased temperature tolerance in heat-acclimated *Daphnia*. Several long-term acclimation studies pointed out that heat-acclimated *Daphnia* may be responding by up-regulating pathways responsible for protection against oxidative stress (Becker et al. 2011; Williams et al. 2011).

Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and the capacity of the antioxidant defense system to neutralize them. ROS are radical and non-radical oxidants that, if allowed to accumulate, can cause DNA and lipid damage, protein degradation, and enzyme inactivation (Monaghan et al. 2009). ROS are by-products of the normal oxygen metabolism, which means throughout the *Daphnia*'s lifetime, they will acquire oxidative damage as they age, slowly accumulating damages that don't typically put them at immediate risk of harm (Barata et al. 2005). But as the temperature increases, there is a higher respiration (and other catabolic reactions) rate within the *Daphnia*, which result in higher production of ROS. The increased amount of ROS synthesized leads to a higher rate of lipid peroxidation (Abele et al. 1998). Lipid peroxidation is a result of damage to

lipids, and targets mainly the membrane and its function, but also has the ability to create side reactions that cause damage to DNA and proteins as well (Monaghan et al. 2009; Lushchak 2011). The ROS production due to the rising temperature accumulates much quicker than that from the natural accumulation due to aging, making it harder for the antioxidant defense system to keep up with the neutralization of the ROS and prevent extensive damage. In aquatic organisms oxidative damage can be exacerbated by the disparity between metabolic demand for oxygen, which increases with temperature, and oxygen availability that decreases with temperature. This can lead to hypoxic conditions in tissues, resulting in inability of mitochondria to function properly, including reduced capacity to neutralize ROS (Becker et al. 2011).

In order to prevent this damage, organisms use a complex antioxidant defense system that encompasses different mechanisms of ROS neutralization. These include mechanisms that control the accumulation of ROS within the cell and those that remove or repair what has been damaged (Monaghan et al. 2009). Some antioxidants that are part of this defense system include enzymes that utilize ROS, such as superoxide dismutase (SOD) and catalase (CAT), and enzymes that prevent formation of lipid peroxides, such as glutathione peroxidase (Barata et al. 2005). Glutathione, the necessary component for glutathione peroxidase protective mechanism, is the most active antioxidant within the cells that works as a redox system to neutralize the ROS. Glutathione peroxidase works by breaking down compounds, such as hydrogen peroxide (H_2O_2). To do this, reduced glutathione (GSH) reacts with the H_2O_2 and produces a less harmful oxidized glutathione (GSSG) and creates less possibility for damage (Barata et al. 2005; Becker et al. 2011). This antioxidant system is not expressed constitutively, but rather it is up-regulated in response to stress the organism is experiencing (Barata et al. 2005).

As the temperature continues to rise, the oxygen demand of *Daphnia* increases, but the amount of oxygen available decreases. This creates a hypoxic environment, which is a decrease in the amount of dissolved oxygen available for use by an organism (Frappell and Wiggins 2002). To continue to survive in a hypoxic environment, *Daphnia* must be able to efficiently retain and use the small amount of oxygen available. A mechanism that the *Daphnia* employ in this situation is an

increase in the hemoglobin concentration (Zeis et al. 2013). When *Daphnia* are in hypoxic conditions the expression rate of different paralogous genes coding for different hemoglobin subunits change, resulting in production of hemoglobin molecules that have an increased oxygen affinity in fat tissues. This change in hemoglobin expression allows *Daphnia* tissues to increase oxygen supply and capacity (Zeis et al. 2009). This mechanism could be used to help *Daphnia* survive in the lethal temperature. Since the changes in hemoglobin expression increase its oxygen affinity in the tissues, which should help *Daphnia* to more efficiently store oxygen to be used.

Survival in ever-changing conditions of their environments requires *Daphnia* to display a high degree of phenotypic plasticity, i.e., the ability of identical genotypes to produce variable phenotypes in response to environmental changes (Hoffmann and Willi 2008). In this study, we considered the plastic response *Daphnia* had to stressful environments by manipulating their temperature and oxygen content. In particular, in response to temperature, *Daphnia* show a strong acclimation effect: after prolonged exposure to high, but not lethally high temperature it gains ability to better tolerate lethal temperatures (Yampolsky et al. 2014). Several studies have indicated that a similar response can be observed after a mild exposure to free radicals eliciting protective mechanisms resulting in higher tolerance of subsequent oxidative stress (Monaghan et al. 2009; Maulucci et al. 2016 Mar 21). Allowing *Daphnia* to become acclimated to a temperature that is close to the upper limit of their tolerance range (28ºC) is likely to give them time and opportunity to up-regulate antioxidant defenses against oxidative damage, which would help them to survive longer at the lethal temperature. If this is true, it would be predicted that there would be less oxidative damage in the acclimated *Daphnia* when compared to those not acclimated to high temperature. Alternatively, one may hypothesize that *Daphnia* up-regulate antioxidants just enough to maintain a constant, acceptable level of damage despite increased oxidative stress at higher temperature. In this case one should expect similar level of oxidative damage between acclimated and un-acclimated *Daphnia*, might expect changes in oxidative damage after a short-term transfer from high to low temperatures and vice versa. For example, one might expect lower levels

of oxidative damage in *Daphnia* acclimated to 28ºC but transferred, shortly before measurement, to 18ºC, where oxidative stress is lower. Also, one may expect the addition of glutathione, known to be up-regulated during these conditions (Becker et al. 2011) to decrease oxidative damage, while inhibition of its synthesis pathway should increase the amount of damage. If glutathione is indeed the key antioxidant that offers protection against high temperature-induced oxidative damage, such manipulations of glutathione availability should then have an effect on *Daphnia* thermal tolerance.

CHAPTER 2

METHODS

Daphnia magna

Daphnia clones were maintained in 200mL plastic vials filled with COMBO water medium (Kilham et al. 1998) (Table 1). The COMBO medium was changed in each vial every two days. During the changing of the medium, juveniles were removed and placed into new vials, so that each vial contained either 5 adult *Daphnia* or 10 juvenile *Daphnia*. They were fed every two days with *Scenedesmus obliquus* algal culture that was grown in the lab.

Table 1. Names and native locations of *Daphnia magna* clones used

Clone	Location
F1	Hybrid between clones from Tvärminne, Finland and Ismaning, Germany
GB-EL75-69	London, UK
IL-M1-8	Jerusalem, Israel
IR-GG1-7	Lake Guru-göl, Iran
RU-RM1-009	Moscow, Russia
RU-YAK1-1	Yakutia, Russia

Acclimation

Individuals from each of the 6 *Daphnia* clones were separated into two different temperature treatment groups, un-acclimated and acclimated, and placed into corresponding incubators. The un-acclimated *Daphnia* were kept at 18ºC, while the acclimated were kept at 28ºC. During the acclimation period, feedings for

Daphnia being acclimated to 28ºC were increased to everyday, while feedings for the un-acclimated in 18ºC were kept the same at every two days.

Thermal Tolerance

After the 2 generations, a long-term acclimation, individuals from each clone in the temperature treatments were place in separate 50mL plastic vials, containing COMBO water at their acclimation temperature. Half of the vials would be exposed to the lethal temperature in a water bath set 37ºC, while others would be unexposed (the control). The exposed *Daphnia* were placed into a water bath at staggering times to achieve an exposure time gradient, consisting of 0-minute, 30-minute, 60-minute, and 120-minute exposures to the lethal temperature. This gradient was set up by first placing the *Daphnia* that would be exposed for the full 120-minutes in the water bath, followed by the addition of the 60-minute exposed *Daphnia* 60-minutes later, and then the finally the 30-minute exposed *Daphnia* were added 30-minutes later. Once the *Daphnia* were in the water bath, the temperature within vials was monitored by placing a thermometer in a vial that was *Daphnia* free and only contained COMBO medium and checking the temperature every 2-minutes, until the water in the vials reached 37ºC which took approximately 10-minutes. Once the water in the vials reached 37ºC the temperature was maintained by continuing to watch the temperature in the *Daphnia* free vial. The vials containing *Daphnia* were then monitored by quickly removing them from the water bath to check for movement and the placing them back into the water bath. *Daphnia* were monitored until they became immobilized, which was considered to be when the *Daphnia* sunk to the bottom of the vial and were unable to lift themselves. Once they were immobilized, the time was marked and they were removed. Once removed they were examined under a microscope to see if they still had a heartbeat, which could be seen, as well as to measure their body size and size of their clutch.

Short-Term Switches

Thermal tolerance was also measured in *Daphnia* that had their temperature treatments switched, for a short-term acclimation period. This switch was accomplished by switching *Daphnia* from their original temperatures to the opposite. For example, *Daphnia* that had spent 2 generations at 18ºC were switched into the 28ºC incubators and vice versa. These switched *Daphnia* were given a 4-day acclimation period to their new temperature treatments and then their thermal tolerance was measured as described above.

Hypoxia

A mild hypoxic environment was created filling vials with boiled COMBO medium. The oxygen content was measure, after the water had cooled, and found be around 5mg/L. The boiled COMBO water was then used to completely fill 50mL plastic vials. These vials were made to maintain the hypoxic condition of the water as the test was ran. The lids of the vials had a hole drilled into them, in which a 1000-micron pipette tip was placed into to prevent the formation of any bubbles. Once the vials were filled, they were left to cool for a day before being used. After the cooling period, the vials were placed into the 18ºC and 28ºC incubators to help avoid any temperature shock when the *Daphnia* are added to the vials. The *Daphnia* were added to the vials by quickly removing the pipette tip from the lid and placing the *Daphnia* in the water. Along with the hypoxic vials, normoxic vials filled with 18ºC or 28ºC COMBO and used as a control. Before being placed in the water bath, the *Daphnia* were either given a 0-minute or 90-minute acclimation period to the hypoxic environment. After the respective hypoxia acclimation period, the *Daphnia* were placed in the 37ºC water bath and their thermal tolerance was measured.

Glutathione Manipulations

In order to augment the antioxidant effect, *Daphnia* were kept in water containing 0.1 mM, 0.05 mM or 0 mM (control) of glutathione (Sigma-Aldrich).

Adult *Daphnia*, 7-14 days old, were placed in the concentrations of glutathione for 4 days prior to temperature tolerance and oxidative damage measurements.

To disrupt glutathione synthesis juveniles <48 hours old were placed into 30 ml flasks containing 1, 0.5, 0.2, 0.1, or 0 mM of buthionine sulfoximine (BSO, Sigma-Aldrich), a potent inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis pathway (Anderson 1998).

Temperature tolerance and oxidative damage were measured after 5-days of exposure.

Lipid Peroxidation and Total Oxidation

The Image iT kit was used to measure the lipid peroxidation in the *Daphnia*, using BODIPY 581/591 as the fluorescent reporter. The oxidized lipids fluoresced at 528nm and reduced at 590nm. After the *Daphnia*'s thermal tolerance was measured, they were homogenized, centrifuged for 4 minutes at 8,000rpm and 50µL of the supernatant was plated onto a black flat bottom 96-well plate. Blank fluorescence were read on a BioTek platereader at 485nm excitation/528nm emission and 530nm excitation/590nm emission wavelength. After that 50µL of 20µM of ImageIT dye solution was added to each well and the plate was incubated for 30-minutes and measured again, which resulted in the estimate of lipid peroxidation. The plate was then incubated again for 24-hours and re-measured to obtain the amount of total oxidation that had occurred in the samples. Blank fluorescence was subtracted from the 30-minute and 24-hour readings and the 528/590 ratio (i.e. the ration between oxidized and reduced dye) was used a measure of oxidation occurring in the sample.

Statistical Analysis

A General Linear Model was used to analyze the data on the JMP Software with either temperature tolerance or the 528/590 ratios as responses and acclimation temperature, 37ºC exposure, clones and all the interactions as the sources of variance. Response variables were log-transformed for the sake of normality.

CHAPTER 3

RESULTS

Acclimation Effect: Long-Term vs. Short-Term

The acclimation temperature affected the time until immobilization (T_{imm}) of the *Daphnia* when subjected to the lethal temperature. *Daphnia* that were acclimated to 28ºC waters were able to tolerate and survive in the stressful environment significantly longer than those that were not (Figure 1). A short term (4 day; ~1 molting cycle at 18ºC) eliminated approximately half of this acclimation effect (Figure 1, hatched bars). Likewise, short-term exposure resulted in some increase of temperature tolerance, although it was not significant (Figure 1).

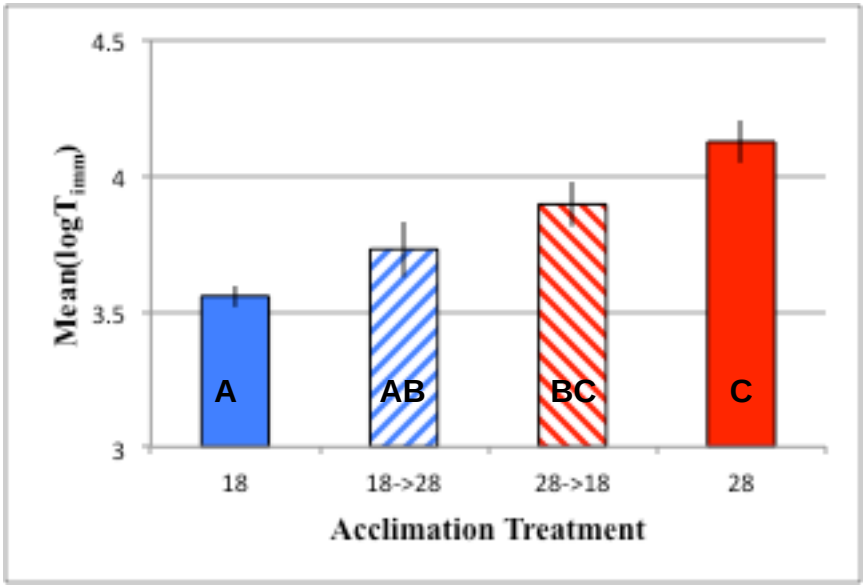

Figure 1. Mean log-transformed time until immobilization at 37ºC in *Daphnia* acclimated for 2 generations to either 18ºC (blue) or 28ºC (red). Solid bars: no switch; hatched bars: 4-day switch to the opposite temperature. 1-way ANOVA: df_num=3; df_den=47; F= 12.23; P<5E-6. Tukey test: Means not sharing a letter are different with at least 0.05 confidence level) Here and elsewhere, vertical error bars represent standard error.

Lipid Peroxidation and Total Oxidation

No difference in lipid peroxidation, measured as the ratio of oxidized to reduced Image IT dye, F528/F590 after 30 min incubation, was detected between *Daphnia* acclimated to 18ºC and 28ºC (Figure. 2A). However, after exposure to the lethal temperature, 28ºC- acclimated *Daphnia* show a higher level of lipid peroxidation than the 18ºC- acclimated ones (Figure 2A, Table 2). This difference is the largest (although not significant in Tukey test, P>0.05) after 60 minutes exposure, which is the duration of exposure at which there is the greatest difference in mortality/immobilization between the 28ºC- and 18ºC- acclimated *Daphnia*, indicating that higher temperature tolerance in the 28ºC- acclimated *Daphnia* is not achieved by lowering the amount of heat-generated lipid peroxidation.

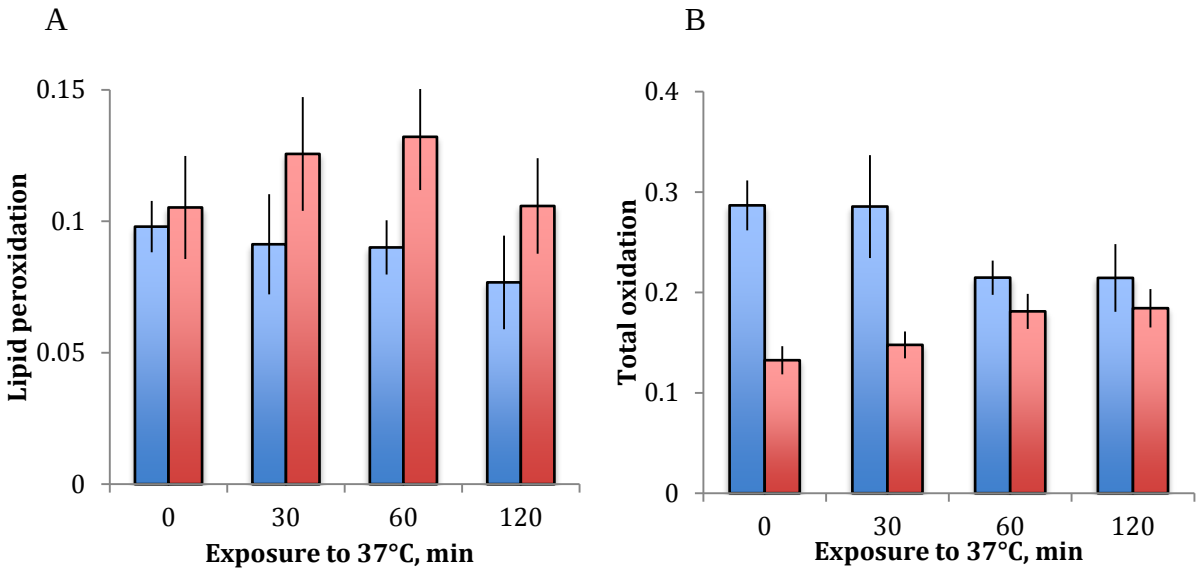

Figure 2. Lipid peroxidation (ratio of oxidized to reduced Image IT dye, F528/F590 after 30 min incubation, A) and total oxidative capacity (ratio of oxidized to reduced Image IT dye, F528/F590 after 24 hours incubation, B) in *Daphnia* acclimated to either 18ºC (blue) or 28ºC (red) and exposed to 37ºC for 0 – 120 min.

Table 2. 2-way ANOVA of the effects of acclimation temperature (18ºC vs. 28ºC), exposure to the lethal temperature (37ºC) and their interaction on lipid peroxidation and total oxidation.

Source	DF	Sum of Squares	F Ratio	Prob > F
Lipid peroxidation				
AccT	1	0.0391	5.51	0.02
Exposureto37,min	3	0.0113	0.53	0.66
Exposureto37,min*AccT	3	0.0108	0.51	0.68
Error	215	1.5268		
Total oxidation				
AccT	1	0.3901	24.29	<.0001
Exposureto37,min	3	0.011	0.23	0.88
Exposureto37,min*AccT	3	0.1809	3.76	0.01
Error	216	3.4689		

On the other hand, the 18ºC- acclimated *Daphnia* show higher total oxidative capacity (measured as the ratio of oxidized to reduced Image IT dye, F528/F590 after 24 hours incubation) than their 28ºC- acclimated counterparts (Figure 2B, Table 2). This difference, in contrast to lipid peroxidation, is the highest in *Daphnia* unexposed to lethal temperatures and disappears after such exposure (Figure 2B and the interaction term in Table 2). This indicates that high temperature-acclimated *Daphnia* contain higher amounts of antioxidants in their tissues, but this difference disappears as antioxidants are used during exposure to lethal temperature.

In the short-term acclimated *Daphnia*, there was a significant decrease in the amount of lipid peroxidation in *Daphnia* down switched (28ºC to 18ºC), indicating that once the *Daphnia* were in a less stressful environment they no longer need the protection they acquired by the long-term acclimation. When *Daphnia* were up shifted (18ºC to 28ºC) there was no significant change in the amount of lipid peroxidation. The lipid peroxidation in these switches was like that of the 28ºC-

acclimated *Daphnia*, indicating that when *Daphnia* were placed in a more stressful environment they quickly began trying to protect themselves (Figure 3A).

In the switches, lower total oxidation observed in 28ºC-acclimated *Daphnia* was quickly lost during the 4-day 28º->18º switch, but was not as quickly acquired in the reciprocal switch (Figure 3B). In fact, *Daphnia* in the 18º->28º treatment tended to show even higher total oxidation than non-switched 18ºC-acclimated ones (Figure 3B), although this difference was not statistically significant. Thus, short-term changes in total oxidation do not mimic temperature tolerance changes observed during the same temperature switch.

A. B.

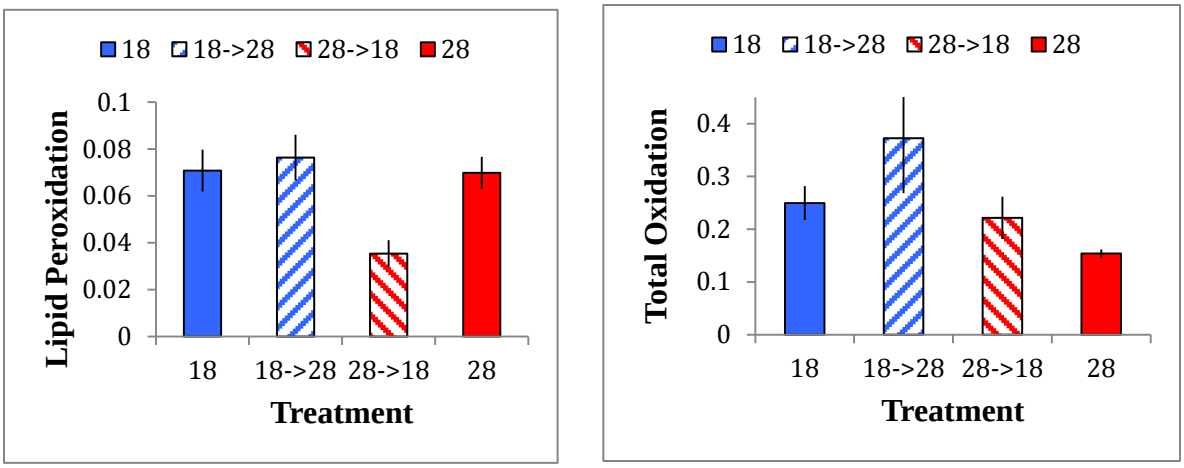

Figure 3. Lipid Peroxidation (Figure A) and Total Oxidation (Figure B) measurements in long-term acclimated (solid bars) and short-term, 4-day, acclimated (hatched bars) *Daphnia*

Hypoxia

The hypoxia tests showed that the hypoxic environment did act as a protection mechanism, allowing the *Daphnia* to survive longer in 37ºC water, but only when the *Daphnia* were given the 90-minute acclimation period (Figure 4). The *Daphnia* given that 90-minute acclimation had a significantly higher T_{imm} in the hypoxic

environment compared to the normoxic. The 90-minute acclimation affected both temperature treatments equally; meaning *Daphnia* from both the 18ºC and 28ºC increased their T_{imm} at equal rates when in the hypoxic environment.

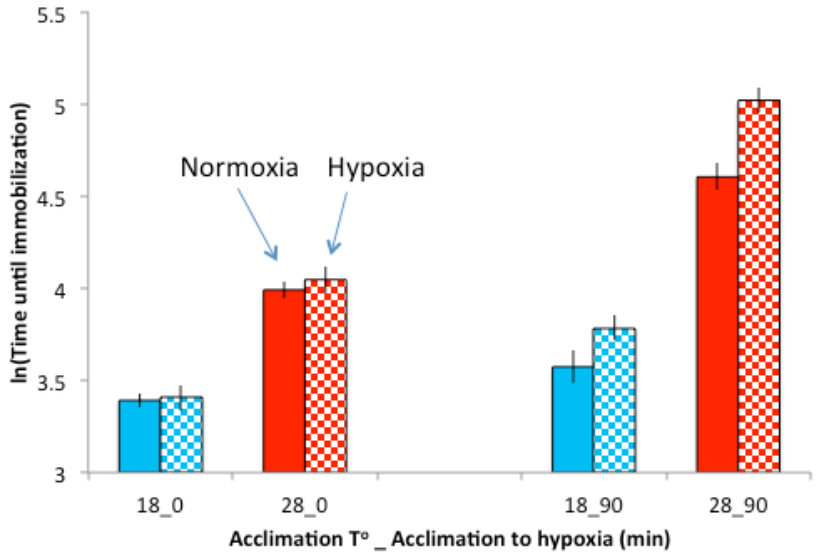

Figure 4. The effect of mild hypoxia (patterned bars) in both18ºC (blue bars) and 28ºC (red bars) acclimated *Daphnia* and after either 0-minute or 90-minute hypoxic acclimation

Glutathione Manipulation

The addition of the antioxidant glutathione and buthionine sulfoximine (BSO) had no effect on the T_{imm} of *Daphnia* in the lethal temperature. The high concentrations of glutathione did not increase the thermal tolerance of *Daphnia*, while the BSO did not cause *Daphnia* to lose the acclimation effect and decrease their thermal tolerance (Figure 5). The addition of glutathione or BSO also did not have an effect on lipid peroxidation between the control and exposed *Daphnia* (Figure 6A, Figure 7A). There was a decrease in total oxidation as the concentration of glutathione increased, but an increase in it as more BSO was added (Figure 6B, Figure 7B). This further indicates that total oxidation depends on the amount of antioxidants in the tissues.

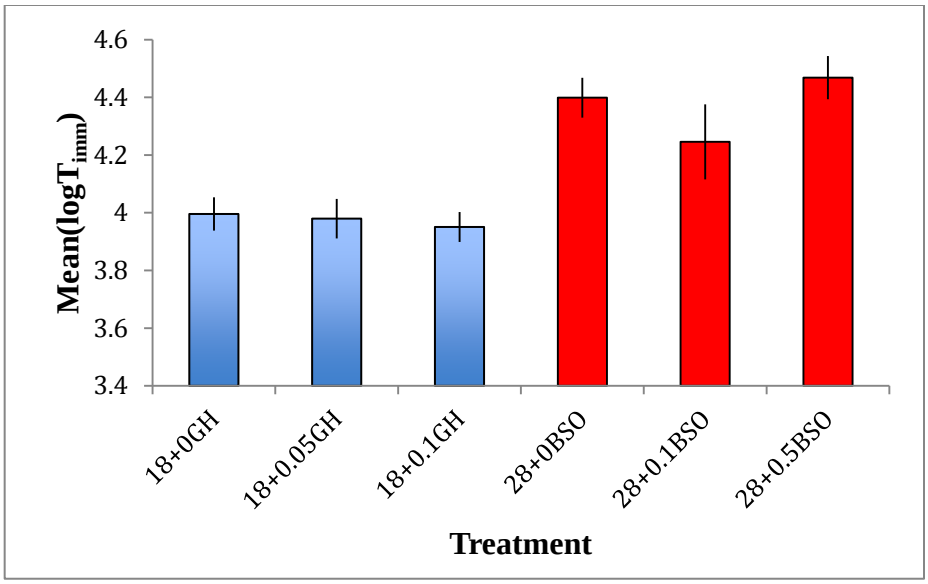

Figure 5. Effect of glutathione augmentation (GH, blue bars) and inhibition of glutathione synthesis pathway (BSO, red bars) on *Daphnia* thermal tolerance

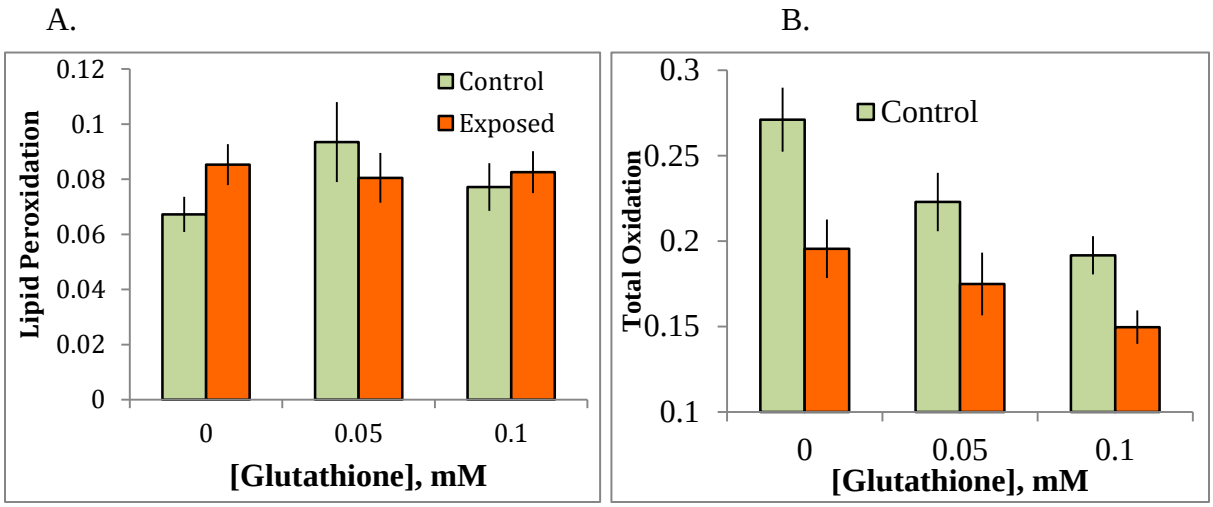

Figure 6. The effect of the augmentation of glutathione synthesis pathways on lipid peroxidation (Figure A) and total oxidation (Figure B) between the control, unexposed *Daphnia* (green bars) and *Daphnia* exposed to 37ºC (orange bars).

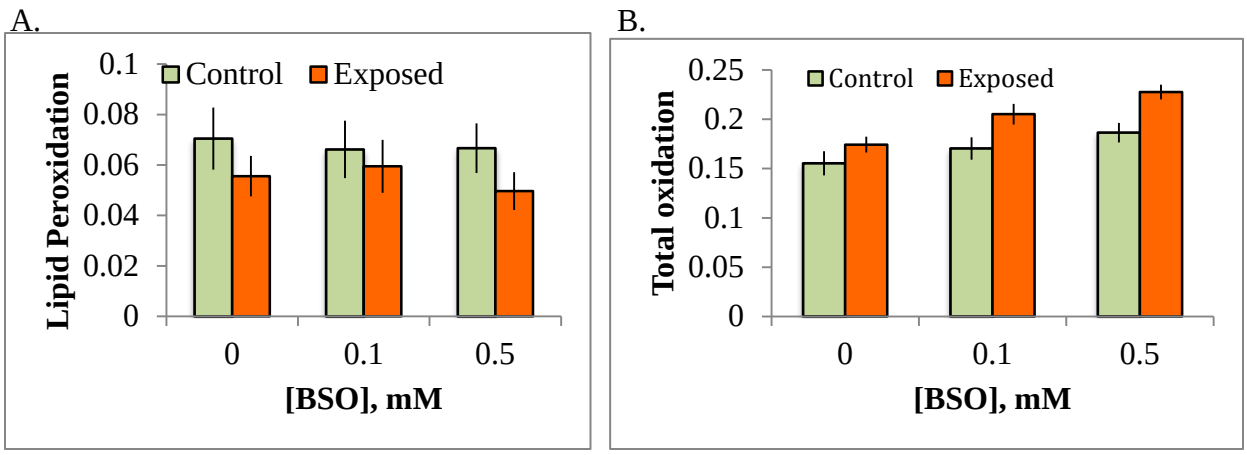

Figure 7. The effect of the inhibition of glutathione synthesis pathways using buthionine sulfoximine (BSO) on lipid peroxidation (Figure A) and total oxidation (Figure B) between the control, unexposed *Daphnia* (green bars) and *Daphnia* exposed to 37ºC (orange bars).

CHAPTER 4

DISCUSSION

With the continuously changing climate, *Daphnia* must have mechanisms that help to tolerate these changes to allow them to continue to survive. Our study found that long-term acclimation to a mild stressor, like the 28ºC, helped *Daphnia* to tolerate and survive longer in the lethal water temperatures, compared to un-acclimated *Daphnia*. This change was accompanied by a significantly higher total oxidation capacity than in un-acclimated *Daphnia* (Figure 2B; Table 2), suggestive of an effect of antioxidants on temperature tolerance. This is further corroborated by the increased temperature tolerance in *Daphnia* exposed to mild hypoxia (Figure 4), known to up-regulate antioxidant pathways including glutathione synthesis in *Daphnia* (Becker et al. 2011). This effect is observed only when *Daphnia* are acclimated to hypoxic conditions for 90 minutes and not immediately after the onset of hypoxia, indicating that some organismal response and not lower oxygen concentration *per se* is responsible for increased temperature tolerance. All these observations point towards a role antioxidant mechanisms play in *Daphnia* acclimation to higher temperature.

On the other hand, the hypothesis that the acclimation to higher temperature helped protect *Daphnia* exposed to lethal temperatures by reduction of lipid peroxidation (LPO) is not confirmed. Such reduction could be hypothesized for two reasons. Firstly, 28ºC-acclimated *Daphnia*, as indicated above may possess stronger antioxidant protections, thus reducing oxidative damage to lipids. Secondly, it may be expected that cellular membranes of 28ºC-acclimated *Daphnia* may contain lower amounts of unsaturated fatty acid side chains, thus presenting a small target for lipid peroxidation (Barata et al. 2005; Schlechtriem et al. 2006). Neither of these mechanisms appear to manifest in our data. To the contrary, we observed that LPO increased in long-term 28ºC acclimation those *Daphnia*, relative to their un-acclimated counterparts (Figure 2A; Table 2). The difference between sensitive un-acclimated and tolerant 28ºC-acclimated *Daphnia* increased with the exposure to the lethal temperature, becoming the highest after 60 min exposure (at which point the

difference in survival between the 28ºC- and 18ºC-acclimated individuals is also the highest). This difference is observed despite the expected lower target and possible higher antioxidant protection in the 28ºC-acclimated *Daphnia*. This unexpected result may indicate that LPO may not be a measure of damage to the organism, but rather a measure of protective action of unsaturated fatty acid side chains in *Daphnia* lipids, acting as scavengers of free radicals and thus ameliorating the effect of increasing temperatures.

This hypothesis is consistent with the differences in LPO among short-term switch treatments (Figure 3A). The only treatment different from the 3 others in LPO amount detected was the 28º->18º short-term switch, which showed lower LPO. It is suggestive of a protective role of LPO maintained at a constant level over different temperature, reflecting temperature-specific need for protection (and possibly reflecting some sort of highest acceptable level of oxidative damage to lipids). During a short-term 28º->18º switch *Daphnia* prepared to withstand lipid peroxidation rate typical for 28ºC, find themselves in a less oxidative environment and show lower LPO. Alternatively, this may indicate that the 28º->18º switch *Daphnia* still possess the level of unsaturation in membrane lipids acquired during long term acclimation to the higher temperature (Barata et al. 2005; Schlechtriem et al. 2006) and thus a smaller target for LPO, while experiencing a lower oxidative stress at their current, lower temperature.

While LPO may be a protection mechanism, the total oxidation appears to be a measure of antioxidants available. As the *Daphnia* are long-term acclimated to 28ºC they up-regulate the antioxidant defense system, which allows them to neutralize ROS quicker and create a better protection against the oxidative damage due to the exposure to the lethal temperature when compared to the un-acclimated *Daphnia*. With this ability to ward off oxidation for a longer amount of time, the acclimated *Daphnia* would have a lower amount of total oxidation. The total oxidation does eventually begin to even out between the acclimated and un-acclimated as exposure time increases because the acclimated *Daphnia* eventually use up their antioxidants and no longer have an advantage over the un-acclimated. These findings are consistent with previous indications of the role of antioxidants in *Daphnia* response to

heat and hypoxia (Zeis et al. 2009; Becker et al. 2011; Williams et al. 2011; Zeis et al. 2013).

During short-term switches total oxidation was found to be quickly lost but not quickly acquired. Since it takes longer acquire then it would indicate that total oxidation is an adaptive mechanism. This would make sense because looking back to the long-term acclimated *Daphnia*, when *Daphnia* had a longer-acclimation period to a mild temperature they were able to up-regulate more antioxidants to help them survive better but when they were given a short-acclimation they were unable to quickly build up antioxidants.

Although these protection mechanisms are very important to the survival of *Daphnia* in stressful environments, *Daphnia* don't seem to maintain them at all times. Within 4-days of being removed from the stressful environment and placed back into normal conditions, *Daphnia* were found to have lost the protection they had built up during the 28ºC long-term acclimation. Indicating that although the LPO and antioxidants protect *Daphnia* they are only activated once *Daphnia* have been exposed to stressors, and not maintained at all times to be prepared for stressful environments.

Yet, it proved difficult to pinpoint a specific antioxidant mechanism causative for the acclimation effect. As mentioned above, glutathione is one of the most active antioxidants within the body, so it would be thought that this would be the antioxidant that was responsible for much of this protection. While, as expected, altering total oxidation, supplementing glutathione and inhibiting glutathione-synthesis pathway (Figure 6, Figure 7), these manipulations do not have any discernible effect on temperature tolerance (Figure 5), indicating that other antioxidants must be playing a more significant role in acclimation, possibly hemoglobins (Williams et al. 2011; Zeis et al. 2013).

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