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Does Thermotolerance in *Daphnia* Depend on the Mitochondrial Function?

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

by

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August 2019

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Keywords: Mitochondrial function, *Daphnia*, Temperature tolerance, Mitotoxins, Acclimation,

Membrane potential

ABSTRACT

Does Thermotolerance in *Daphnia* Depend on The Mitochondrial Function?

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Rajib Hasan

Thermotolerance limit in aquatic organism is set by the ability to sustain aerobic scope to sudden temperature shifts. This study tested the genetic and plastic differences in thermotolerance of *Daphnia* that can be explained by the differences in the ability to retain mitochondrial integrity at high temperatures. Five genotypes with different biogeographic origins were acclimated to 18°C and 25°C. We developed a rhodamine 123 *in-vivo* assay to measure mitochondrial membrane potential and observed higher fluorescent in heat damaged tissues as the disruption of the mitochondrial membrane potential. Significant effects on temperature tolerance were observed with CCCP and DNP but not with NaN₃. Effects of toxins were significant in temperature sensitive genotype and high concentration of lactate was observed in 18°C acclimated genotype only. We conclude that genetic and physiological differences are intricately linked to the ability of sustaining aerobic respiration at high temperatures which sets limit to the thermotolerance.

ACKNOWLEDGMENTS

I would like to thank, Dr. Lev Yampolsky, who has guided me and supported me with directions and guidance.

To my committee members, Dr. Aruna Kilaru and Dr. Jones, I would like to thank you both for the advice and critiques that contributed to my research and kept it on track.

I also want to thank to Mark, Bret, Quinn, Cora and Taylor with whom I had great experiences to work with.

I am also very thankful to Dr. Arceo Gomez for letting me use the fluorescent microscopes and his whole lab for supporting me in their lab.

I am thankful to the Department of Biological Sciences at East Tennessee State University for giving me the platform to enhance my knowledge with practical experiences.

Thanks to my parents for supporting me all the way through!

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CHAPTER 1

INTRODUCTION

Poikilothermic Temperature Tolerance and Respiration

Every organism has certain range of temperature tolerance where they perform best and outside of this range the temperature becomes stressful for that organism. It has been a mystery that organisms vary greatly in their limits of temperature tolerance despite the thermodynamic and the chemical constraints imposed by temperatures (Sibly and Calow 1986; Schmidt-Nielsen 1997). These differences are clear not only in the interspecific level but also in the extended intraspecific level as well, and, the variability of thermotolerance in organism manifests both in heritable local adaptation and in phenotypic plasticity (Yampolsky et al. 2014). The adaptive plasticity is considered as the major playing role in the evolutionary mechanisms such as the evolution of novel plastic response or phenotypes as well as in speciation (Pfennig et al. 2010; Oomen and Hutchings 2015). Though the causative mechanism of adaptive response to the environmental stress is the vital goal in the field of biological research such as modern physiology and ecological genomics (Hoffmann and Willi 2008; Morris and Rogers 2014), little is known about the genetic and biochemical mechanism of such adaptive phenomena.

Aquatic organisms inhabit a wide range of the geographical areas and habitats that differ in temperature regimes and biogeographic distribution. The tolerance limit to stresses caused by the shift in ambient temperature largely determines this wide distribution. Temperature tolerance limit in aquatic water breathing organism is set by the ability to sustain respiration during hypoxia caused by the increased temperature (Pörtner 2002, 2010; Pörtner and Knust 2007). Critical temperature (T_c) at which the partial transition from aerobic to anaerobic state occurs, correlates with the environmental temperature and explains the biogeographic distribution of

aquatic ectotherms such as fishes, mollusks, crustaceans and annelids as well as the shifts in their tolerance limit due to the climate change (Pörtner 2002, 2010; Pörtner and Knust 2007; Pörtner and Farrell 2008). Studies on temperature tolerance in zooplanktons do point toward the role of mitochondria in setting up the limit to temperature tolerance (Yampolsky 2014; Coggins et al. 2017). Hence, the temperature induced biochemical and physiological adjustments appear to be the prior mechanism that affect mitochondrial functions in maintaining the aerobic scope which, in turn, defines the temperature limits before the adjustments of the molecular function take place (Pörtner 2001).

Thermotolerance in *Daphnia*

Daphnia is an ecologically important aquatic organism that is widely used as a model organism in many fields of biological studies due to its reproduction through parthenogenesis, a reproductive phenomenon that ensures the genetically identical individuals as replicates or across environments. It has short-term generation time as well as characterized ecology, particularly convenient for studying the phenotypic plasticity and local adaptation to heat-tolerance for its easy culture in the laboratory setting. Being capable of sustaining the life in wide range of temperature as well as in oxygen concentrations, this organisms can be acclimated to temperature between 10°C and 30°C (Lamkemeyer et al. 2003) and the PO₂ (oxygen partial pressure) between 2-74kPa (Zeis et al. 2003) where it also can tolerate anoxia condition induced at 5°C -34.5 °C for few hours or days as well (Paul et al. 1998; Zeis et al. 2004).

Since, temperature plays an important role in physiology, biochemistry, behavior and anatomy etc. of the poikilothermic organisms as their body temperature is directly affected by the water temperature, the clear understanding of the correlation of these reaction norms with the temperature shifts in *Daphnia* is crucial for unwinding the mechanisms of the tolerance.

Thermotolerance in *Daphnia* shows both phenotypic plasticity (through acclimation) and local adaptation (Paul et al. 2004; Yampolsky et al. 2014; Geerts et al. 2015). Though behavioral responses such as breathing and ventilation are the short-term adjustment to the acute temperature in *Daphnia* (Pinkhaus et al. 2007), the ability to tolerate high temperature differs mainly for phenotypic plasticity (through acclimation) and local adaptation (Paul et al. 2004; Yampolsky et al. 2014; Geerts et al. 2015) .

There are several lines of evidence that rapid evolution and local adaptation of the temperature tolerance in *Daphnia* (Yampolsky et al. 2014; Geerts et al. 2015) are correlated with the heritable changes in respiratory functions such as hemoglobin expression (Costantini 2014; Yampolsky et al. 2014) and the level of antioxidant defenses (Oexle et al. 2016). Likewise, plastic response to temperature often includes similar changes (Williams et al. 2012), implicating the connection to sustaining the mitochondrial function. Additionally, plastic response to temperature has been shown which includes changes in whole body lipid composition, particularly the abundance of polyunsaturated fatty acids (Coggins et al. 2017). The higher unsaturation of fatty acids in mitochondrial membrane leads to lipid peroxidation, which in turn results in high release of ROS or free radicals damaging membranes' integrity and causing eventual cell death (Pamplona et al. 2000; Al-Gubory 2012). Hence, the antioxidants have crucial role for maintaining the cellular integrity by sustaining the aerobic scope at high temperature to protect the cell from oxidative damage.

Hemoglobin, has important role in antioxidant capacity, sustains the aquatic life (Wittmann et al. 2008; Beers and Shidel 2011) including *Daphnia* (Pirow et al. 2001; Seidl et al. 2005; Paul et al. 2004) either in acclimated or at the local site during the exposure to high temperature and hypoxia. The acclimation to hypoxia (Fox et al. 2003, Kobayashi and Hoshi

1982) and temperature (Lamkemeyer et al. 2003) affect the Hb regulation to respond to the stress. The mechanism of decreasing the hypoxia in the acclimated *Daphnia* is done by the increasing the expression of of different Hb mRNA and hemolymph Hb which eventually and actively lessen the effects of oxygen imbalance due to sudden acute environmental changes (Becker 2011). The quantity and quality of hemoglobin is greatly influenced by the higher temperature and tissue hypoxia (Gerke et al. 2011). The increased expression of Hb protein which releases oxygen to the tissue at temperature changes, was observed in heat-tolerant genotypes or warmer climate genotypes or genotypes acclimated to higher temperature. This observed plasticity of hemoglobin which helps sustaining the respiration as well as the survival even at higher temperature (Yampolsky et al. 2014).

Mitochondrial Function and Oxidative Stress

In addition to the critical importance in meeting the high energetic demands at elevated temperatures (Somero 2002; Pörtner et al., 2007), mitochondria are the major sources of reactive oxygen species production (Turrens 2003). Increase of the concentration of ROS in the cell due to the reduced electron transport chain because of the shifts in the environmental temperature and the oxygen availability in the tissues or habitat (Bonawitz et al. 2007), has the detrimental effect for the cellular integrity. Disrupted operation of electron transport chain leads to production of superoxide (Jastroch et al. 2010), which can further react with the formation of other free radicals or cause lipid peroxidation. ROS also acts as the signaling molecules despite their toxicity effect (Matés et al. 2008), induces the antioxidants mechanism to minimize tissue damage. Several antioxidant defense mechanisms such as H₂O₂ scavenger CAT (catalase) (Storey 1996) which prevents the accumulation of ROS and glutathione (GSH and GSSG) that is involved in degradation and detoxification of ROS (Maher 2005). So, maintaining the

mitochondrial metabolism is very crucial to prevent the damage caused by ROS in the cell. On the other hand, failure to mitochondrial membrane-based functions can trigger other biochemical defense mechanisms or total collapse of the cellular functions.

Mitochondrial Toxins Effects on Mitochondrial Function

Mitochondrial toxins affect the mitochondria at different stages of electron transport chain complexes as well as at the membrane phosphorylation. Toxins like dinitrophenol (DNP) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) are protonophores, the uncouplers of the oxidative phosphorylation that move the protons across mitochondrial membrane and particularly affects the ATP synthase. These result in the reduced electrochemical gradient and blockage of the ATP synthesis (Heytler 1963; Cunarro and Weinder 1975; Woronowicz et al. 2015). On the other hand, sodium azide (NaN_3) inhibits the function of complex IV or cytochrome C oxidase in ETC. As the result, oxygen consumption decreases due to the inhibition of electron transfer to oxygen through the complexes of the ETC (Trumpower 1990; Trumpower 2002; Kake-Guena et al. 2017; Chen and Lesnefsky 2006). The inhibition of the cytochrome oxidase also increases the production of H_2O_2 or other ROS which further reduces the mitochondrial membrane potential (Rahn et al. 1991). Thus, the effects of different mitotoxins effects on thermotolerance may provide some mechanistic understanding of the role of mitochondrial function in temperature tolerance.

Rh-123 Probe for Measuring the Mitochondrial Membrane Potential and Membrane Fluidity

The rhodamine fluorescent probe rhodamine 123 (Rh-123) is a cationic dye that can be accumulated in the matrix of mitochondria (Nicholls et al 2000; Baracca et al. 2003)

proportionally to mitochondrial membrane potential. The loss of membrane potential is indicated as the loss of the dye or the fluorescent intensity (Chazotte 2011) inside mitochondria, whereas damage is measured by the increase of overall tissue fluorescence since the dyes diffuse back into the cytoplasm when the mitochondrial membrane is damaged. We therefore propose that *in vivo* staining with Rh-123 can provide valuable information about the level of mitochondrial membrane potential and its loss due to temperature or mitotoxins.

Hypothesis and Predictions

The findings described above clearly indicate the importance of mitochondrial function in dealing with the acute and gradual temperature changes in the environment. However, as of now, there is no direct assay to measure mitochondrial membrane potentials in *Daphnia* in the context of acclimation and adaptation to temperature. Since rhodamine 123 widely used in assays of mitochondrial membrane potential in isolated mitochondria and in cell culture systems (Emaus et al. 1986; Baracca et al. 2003), we propose it also can be implemented *in vivo* in *Daphnia* and should allow us the proximate measurement of mitochondrial membrane potential by fluorescent microscopy. This would allow to test the following predictions of the hypothesis that mitochondrial functions set the limit to the thermotolerance in *Daphnia*:

1. Loss of mobility during exposure to high temperature should be accompanied by the loss of mitochondrial membrane potential which is detectible as the overall increase of tissue fluorescence in rhodamine 123 stained *Daphnia*.

2. The disruption of mitochondrial membrane potential by mitotoxins at their nonlethal concentration should reduce heat tolerance and should mimic the effect of lethal temperature.

3. The high temperature and mitotoxin induced damage, which is detectable as the increase of tissue fluorescence, should correlate with genetically and physiologically determined temperature tolerance, i.e. with differences among genotypes and the acclimation regimes.

4. Loss of mobility during exposure to lethal temperature should be accompanied by accumulation of lactate in tissues due to collapse of aerobic scope.

CHAPTER 2

MATERIALS AND METHODS

Cultures of *Daphnia* Genotypes and Temperature Acclimation

Daphnia magna from different geographic locations (Table 1), including both temperature sensitive and temperature tolerant genotypes (Yampolsky et al. 2014) were maintained in 100 mL jars with COMBO water (Kilham et al. 1998) at both 25 °C and 18 °C with 5-6 individuals per jar. *Daphnia* were fed with *Scenedesmus obliquus* every other day to the concentration of 100,000 cells/ml and the water was changed every 4 days that ensures proper nutrition as well as the oxygen solubility. The acclimation was achieved by transferring adult *Daphnia* into either 25°C and 18°C incubators and maintaining the clones for two generation before the experiments were conducted. This two-generation acclimation ensures the elimination of the maternal effects, the possibility of the phenotypes in the offspring determined by its mother genotype and the environment it experiences.

Table 1. *Daphnia* Genotypes of different geographic location used in the study. The ID of genotypes with geographic coordinates, habitat and temperature sensitivity are showed. Among the clones, IL-MI-8 and FI-FSP1-16-2 have the highest variability of temperature tolerance whereas GB-EL75-69 has the least.

Clone ID	Type of Habitat	Latitude	Longitude	Acute Temperature Tolerance
IL-MI-8	Mediterranean pond	31° 42' 52.42"	35° 3' 3.38"	High
GB-EL75-69	Year-round pond	51°30'26"	-0°7'39"	Low
FI-FSP1-16-2	Summer rock pool	60 ° 10.062"	25° 47.677"	Low
FR-SA-1	Mediterranean pond	43° 27' 37.06"	4° 39' 09.83"	Low
HU-K-6	Lake	46° 47' 33.3"	19° 10' 53.84"	High

Measurement of Mitotoxins Effects on Heat Tolerance and Survival

Adult *Daphnia* (II-M1-8 clone) were exposed to varying concentrations of three mitotoxins, CCCP (Carbonyl cyanide m-chlorophenyl hydrazine), DNP (Dinitrophenol) and NaN₃ (Sodium azide) for 48 hours. Concentrations used were below LD50 estimated for this genotype (Table 2) where less than 10% mortality was recorded during the exposure. 0.5% DMSO (Dimethyl sulfoxide) was used as the vehicle to dissolve mitotoxins in water. DMSO did not affect T_{imm} (see below), the measurement of the survival and thermotolerance capability. At the end of the 48h exposure period, heat tolerance was measured as time until immobilization at 37°C (Williams at al 2012). Adult *Daphnia* were placed individually into 30 ml vials with COMBO water with mitotoxins or vehicle added at the same concentration during exposure, and then transferred to water bath with increasing the temperature up to 37 °C at 10-12 interval. Ability to sustain swimming was checked every 1-3 min and the time of immobilization (T_{imm}) recorded. The heartbeat was also monitored to ensure the elimination of false positive result. None of the toxins caused any additional mortality or immobilization in 2 hours trials at room temperature.

Table 2: *Different Concentrations of mitotoxins Used in Measuring the Survival Rate of the Daphnia.*

Mito-Toxins	The chemical name	Low Concentration	Medium Concentration	High Concentration (approx. 24 h LC50)
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine	0.01 µM	0.1 µM	1 µM
DNP	Dinitrophenol	0.1 µM	1 µM	10 µM
NaN3	Sodium Azide	0.05 µM	0.5 µM	5 µM

In order to assess the effect of genotype and acclimation temperature on mitotoxin toxicity juvenile *Daphnia* <48 h old were collected from mothers from 5 different clones (Table 1) acclimated to either 15°C, or 20°C, or 25 °C for two generation. These juveniles were placed into 1.5 mL Eppendorf, 10 in each, with COMBO water with either 0 or 1 µM of CCCP. Death of individuals was recorded every 3 hours for whole 24 hours.

Membrane Potential Measurement by Rh123 Assay

The measurement of mitochondrial membrane potential was measured by the rhodamine 123 (Rh-123) assay and the fluorescent microscopy. Neonate *Daphnia* were exposed to water solution of Rh123 (with 0.5% DMSO as vehicle) with concentrations between 0.5 to 10 µM for 24 hours in the dark at the same temperature to which they had been acclimated. After 24 h of exposure, *Daphnia* were rinsed with COMBO water and their head and heart regions were photographed with a 10x objective on a Leica fluorescence microscope with a broad path filter with excitation of 495 nm and emission >515 nm. Fluorescence intensity was analyzed using ImageJ software with the median of intensity within each region of interest used as the measure of tissue fluorescence. Microscopy was conducted either with or without the exposure to either heat (20-60 min at 37 °C) or to mitotoxins at the "high" concentration (Table 2) for 1-3 hours. For the saturation curves only, individuals that were still alive after the 60 min exposure to 37°C were included into the analysis to eliminate non-specific loss of mitochondrial membrane potential due to postmortem tissue degradation.

Lactate Accumulation Measurement

The measurement of lactate accumulation with or without exposure of high temperature were conducted by using the Promega lactate assay kits, Lactate-Glo™ Assay with luminescence was measured on a BioTek plate reader in a 96-well plate, with calibration to mM lactate conducted per manufacturer's instructions.

Statistical Analysis

Statistical analysis was conducted in JMP (SAS Institute 2012). The General Linear Model was used for statistical analysis on JMP software with analyzing the clones of two different acclimated temperature separately or on the clones pooled. The 3-way Analysis of Variance (ANOVA) was performed to determine the correlation between variables such as acclimation temperatures, concentration, survival and the time. Michaelis-Menten curves were fit to Rh123 saturation data using JMP. Non-linear fit platform was performed with the parameters F_{\max} , fluorescence at saturation (suggested proxy for total mitochondrial capacity) and K_m , the concentration of fluorophore at which $\frac{1}{2}$ of F_{\max} is achieved (suggested proxy for the inverse of mitochondrial membrane potential). Mortality of *Daphnia* in the CCCP toxicity experiment was analyzed by means of Parametric Survival Fit in JMP Survival platform.

CHAPTER 3

RESULTS

Mitotoxins Effect on the Thermotolerance

Time until immobilization in adult *Daphnia* exposed to various concentrations of mitotoxins is shown on Fig.1. The vehicle (0.5% DMSO) did not differ from untreated control ($P>0.2$). The highest concentration of two protonophore toxins (CCCP and DNP; 1 μM and 10 μM respectively) significantly reduced time range during which *Daphnia* failed to retain swimming ability during exposure to the lethally high temperature ($P<0.001$). The third toxin, an electron transport chain disruptor, sodium azide (NaN_3) did not have any effect on time until immobilization ($P>0.5$).

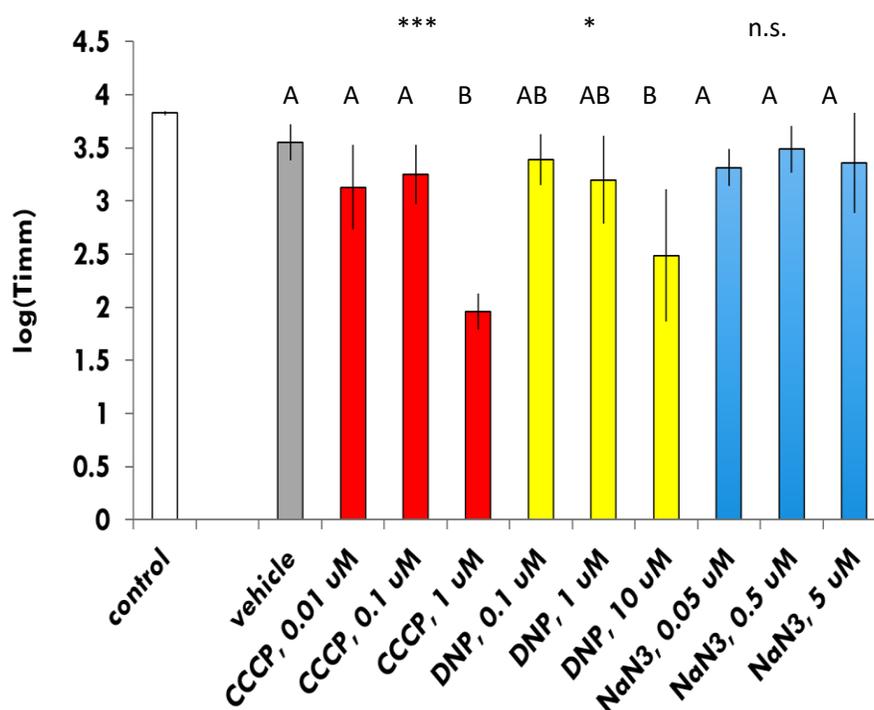


Figure 1. Mitotoxins Effect on the Time Until Immobilization (Timm, minutes, log-transformed) at 37 °C. The horizontal axis are the plots of the different concentration of the toxins and the vertical axis is the survival rate when exposed to these toxins with different concentration. Red bars represent CCCP effects on survival rate at different concentration, Yellow bars are the DNP effects and the Blue bars are the effects of NaN_3 . Grey bar represents

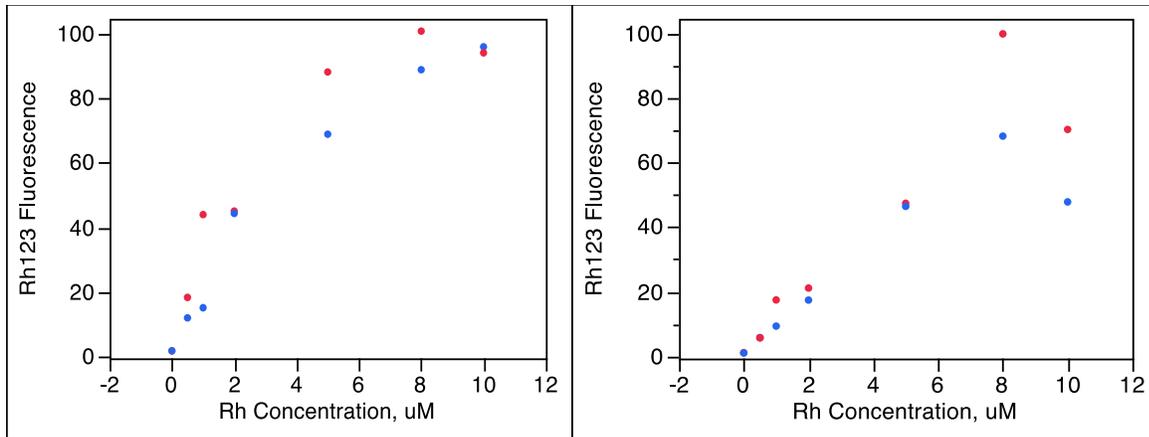


Figure 2. Rhodamine 123 in vivo assay saturation curves. Blue dots: 18 °C acclimated *Daphnia*; red dots: 25 °C acclimated *Daphnia*. A, B – control; C, D – 60 min exposure to lethal temperature. Regions of interest (ROI): A, C – head, B, D – heart.

Table 3. Rhodamine 123 saturation measurement. Fitted Parameters (and their standard errors) of Michaelis-Menten equation to Rh123 fluorescence in head and heart of *Daphnia* as a function of fluorophore concentration. F_{max} – fluorescence at saturation, K_m – concentration of fluorophore at which $\frac{1}{2}$ of F_{max} is achieved.

Treatment	ROI	Acclimation temperature	
		18 °C	25 °C
F_{max} (arbitrary units)			
Control	Head	66.9 (6.1)	86.0 (8.6)
60 min at 37 °C	Head	154.9 (16.8)	126.0 (12.4)
K_m (μ M)			
Control	Head	1.8 (0.5)	2.0 (0.6)
60 min at 37 °C	Head	6.1 (1.4)	2.6 (0.7)
Control	Heart	2.2 (0.9)	4.0 (1.1)
60 min at 37 °C	Heart	6.9 (5.7)	12.4 (15.2)

In control treatment, 25 °C-acclimated *Daphnia* showed a higher value of F_{max} in both

head and heart regions where standard errors do not overlap, suggest the higher total mitochondrial capacity (Table 3). However, the K_m parameter is only slightly higher in the 25°C-acclimated *Daphnia* ($P>0.1$), is indicating that the affinity of mitochondria to Rh123, and therefore membrane potential is not affected by the acclimation temperature. Standard errors of estimated parameters for the 37 °C-exposed *Daphnia* are much higher (Table 3). For the head region the K_m parameter is significantly lower in the 25°C acclimated *Daphnia*, is indicating, unexpectedly higher release of fluorophore at a given concentration due to mitochondrial membrane potential loss during the exposure to high temperature.

Mitotoxins Mimic the Effect of Heat on Mitochondrial Membrane Potential

Tissue fluorescence increased significantly and equally in both tested genotypes, the temperature sensitive GB-EL75-69 and the temperature tolerant IL-MI-8 clones (Fig. 3). As predicted, about 4 hours exposure to mitotoxins mimicked the effect of high temperature exposure on mitochondrial membrane potential (Fig. 4). However, one genotype out of two showed the effects of high temperature during the exposure to the three mitotoxins: the effects of mitotoxins was only observed in the heat-sensitive GB-EL75-69 genotypes, but not in the heat-tolerant IL-MI-8 genotypes (Fig. 4).

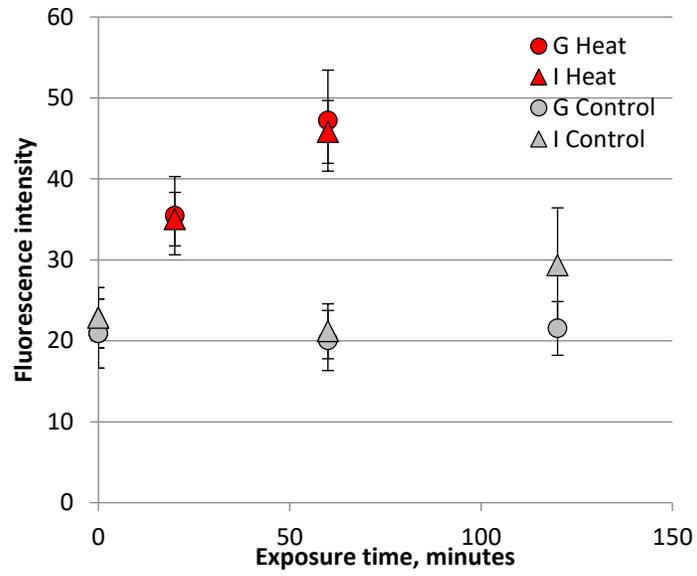


Figure 3. *Effects of heat on mitochondrial membrane potential.* Increase of tissue fluorescence (average of head, heart and antennae) after exposure to 37 °C-acclimated *Daphnia* from two clones, a heat-sensitive GB-EL75-69 “G” and heat-tolerant IL-MI-8 “I”.

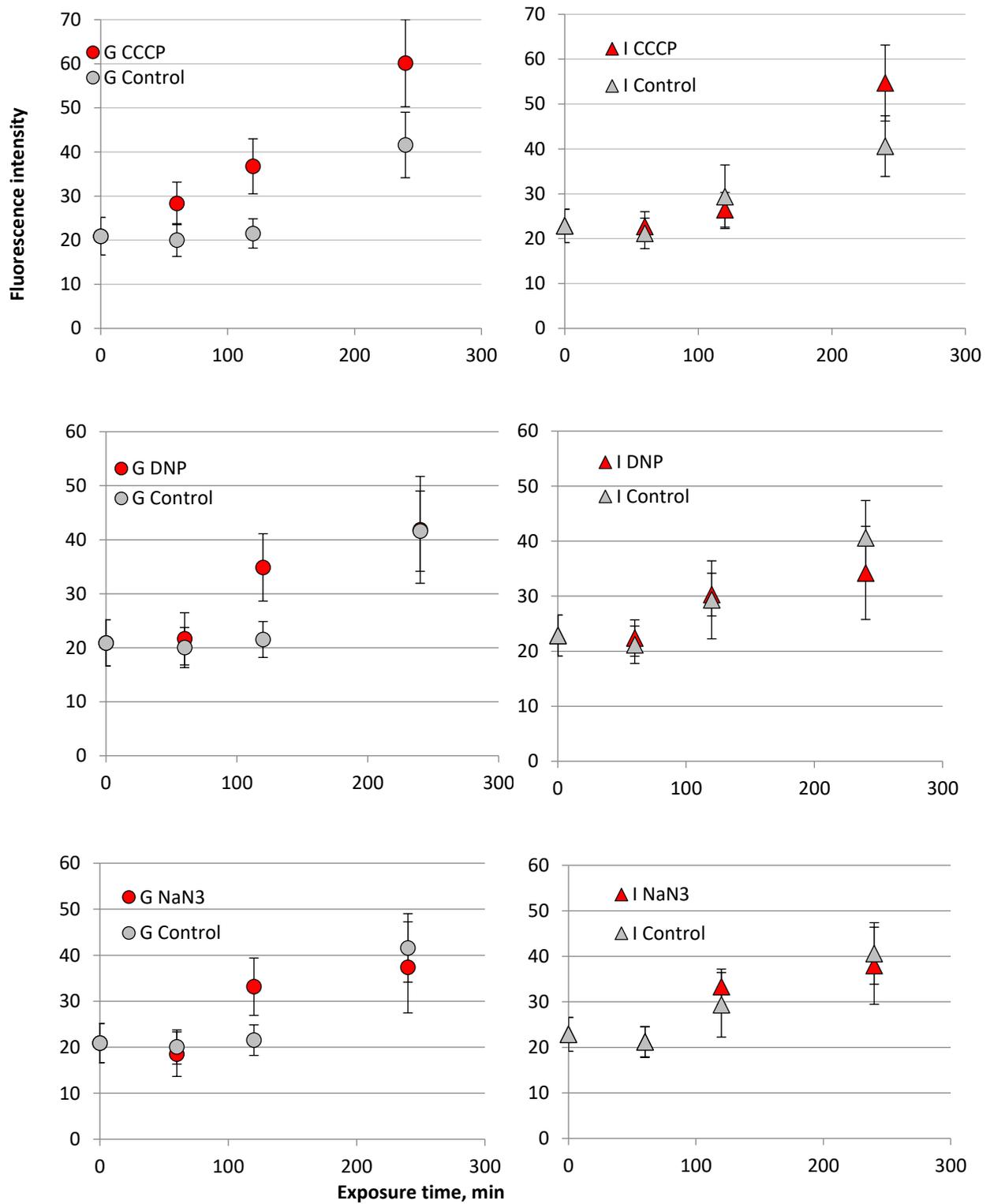


Figure 4. Mitotoxins Effects on the Mitochondrial Membrane Potential. The heat Sensitive (G, circles) and a heat tolerant (I, triangles) clones. The membrane damages were measured as the median fluorescent intensity. Mitotoxins: CCCP; DNP and NaN3.

Lactate Accumulation During Heat Exposure

As predicted, lactate accumulated in *Daphnia* tissues during 30 min exposure to lethal temperature 37°C (Fig. 5). However, this was only observed in 18 °C-acclimated *Daphnia*, but not in the 25 °C-acclimated ones. This possibly indicates a greater loss of aerobic respiration in the cold temperature acclimated *Daphnia* during high temperature exposure.

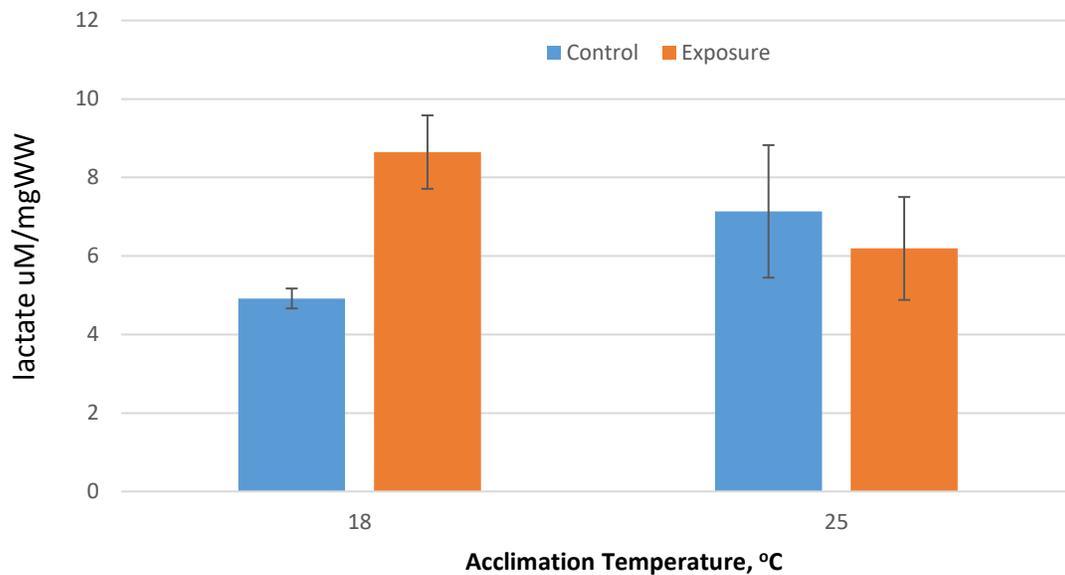


Figure 5. Lactate accumulation test in *Daphnia* acclimated to either 18 °C or 25 °C and exposed to the lethal (37 °C) temperature for 30 minutes. Blue bar represents the lactate concentration in unexposed control and orange bars represent the lactate concentration in heat exposed clones. Supplementary Table S3 for statistical results.

Temperature and Clonal Differences in CCCP Toxicity

The survival of juveniles from five different regions exposed to 1 μ M CCCP depends on the temperature at which the individuals and their mothers have been acclimated (Fig. 6). 15 °C-acclimated individuals showed no clonal differences in 24 h survival ($P>0.2$); 20°C-acclimated individuals showed a significant inter-clonal differences ($P<0.01$) which did not correlate with clones' temperature tolerance; and finally the 25°C-acclimated individuals showed highly significant differences among clones with the two most heat tolerant clones (IL, HU) having the lowest mortality and the two most heat-sensitive clones (FI, GB) showed the highest 24h mortality (Fig. 6). These differences are confirmed by the significant acclimation temperature X clone effect in both analyses: ANOVA with the 24h mortality as the response and proportional hazard model fitted to mortality schedule during the 2h exposure to the toxin (Table 3). Thus, a protonophore mitotoxin mortality mimics the detrimental effects of lethal temperate in acclimated genotypes.

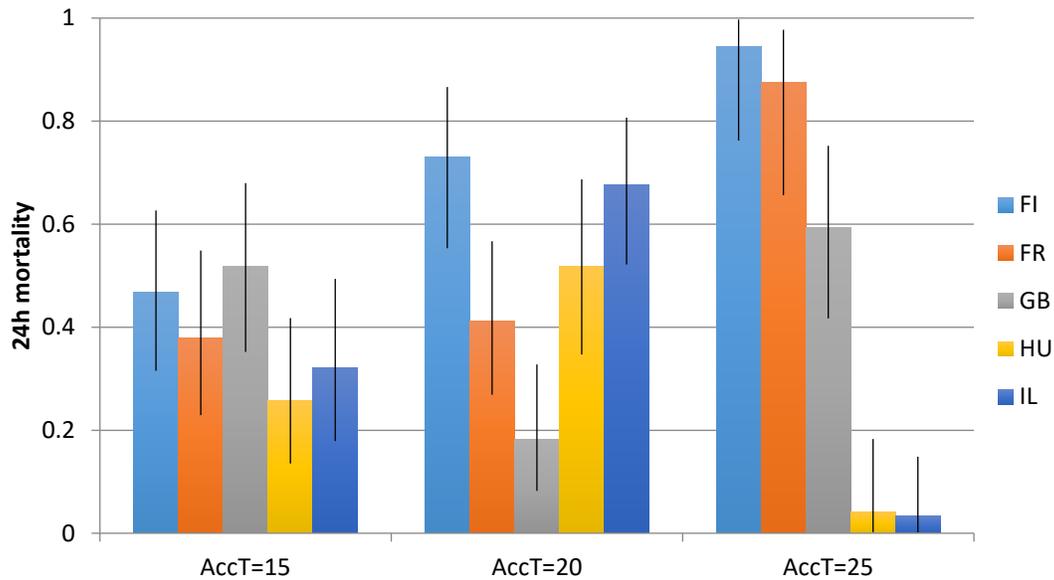
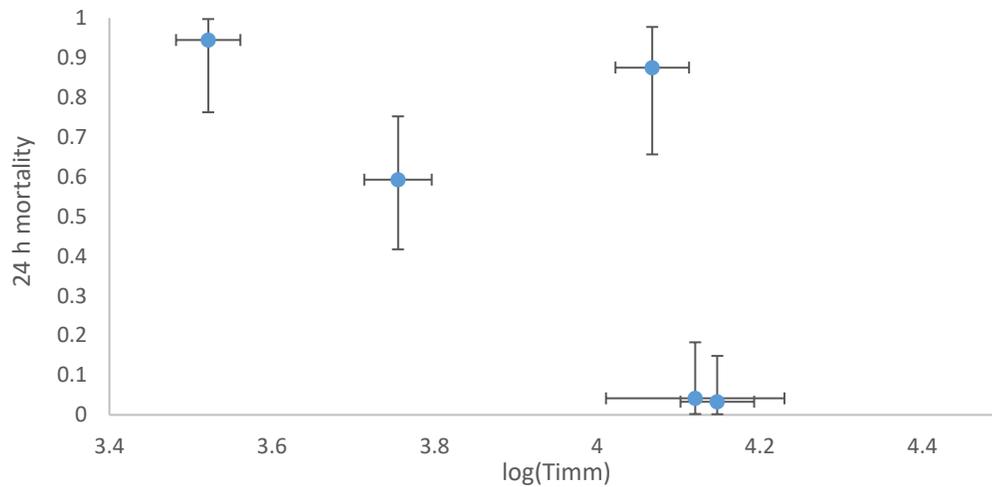
A**B**

Figure 6. Mitotoxins and acclimation effect on mortality. A: CCCP mortality test in temperature sensitive and temperature tolerant clones. The toxicology test in 5 clones (3 temperature sensitive and 2 temperature tolerant) with 3 acclimation temperatures (15 °C, 20 °C and 25 °C) for 24 hours period. **B:** Correlation of 24-h mortality with mean log-transformed time until immobilization (Timm) averaged across data from 5 different experiments in the same genotype acclimated to 25 °C (Coggins et al. 2017; Martin-Creuzburg et al. 2019).

Table 4: 3-way ANOVA of 24-h mortality between three acclimated temperatures and the effects of the CCCP and Effect Likelihood Ratio Tests.

3-way ANOVA of 24-h mortality with clones as a random factor nested within temperature tolerance levels (high vs. low)

Source	DF	MS	F Ratio	P
AccT	2	0.056	0.136	0.88
Ttolerance	1	0.541	3.069	0.18
Ttolerance*AccT	2	0.115	0.279	0.77
Clone[Ttolerance]	3	0.183	0.427	0.74
Clone*AccT[Ttolerance]	6	0.431	14.403	<.0001
Error	43	0.03		

Parametric Survival Fit Effect Likelihood Ratio Tests

Source	DF	L-R ChiSquare	P
AccT	2	6.273	0.0434
Clone	4	53.156	<.0001
AccT*Clone	8	95.651	<.0001

CHAPTER 4

DISCUSSION

We have reported several lines of evidence that the limits to lethal temperature tolerance are determined by the ability to retain mitochondrial function during exposure to heat. Firstly, tissue fluorescence in rhodamine 123-stained *Daphnia* increases during heat exposure, an indicative measurement of loss of mitochondrial membrane potential (Figs. 2, 3) and mito-toxins exposure mimics this effect (Fig. 4) in a heat-sensitive genotype. Secondly, exposure to mitotoxins with known protonophore mechanism of toxicity reduces heat tolerance in an acute temperature shift (Fig. 1). Thirdly, cold-acclimated *Daphnia* that is also temperature sensitive show accumulation of lactate in tissues during heat exposure. On the other hand, heat-tolerant warm-acclimated ones do not (Fig. 5). Finally, mortality due to exposure to the mitotoxin CCCP at the concentration close to LD50 at warm temperature correlated with temperature tolerance in *Daphnia* acclimated to the same temperature (Fig. 6).

Disruption of Proton Gradient on Heat Tolerance

The CCCP and DNP did have effect on the survival of the organism whereas NaN_3 did not (Fig. 1). Since, NaN_3 has no direct effect on damaging the proton gradient or on blocking the ATP production, the non-lethal doses of sodium azide perhaps have no effect on the heat tolerance. These indicate maintaining the proton gradient itself is the limiting factor of the temperature tolerance. Unlike the protonophore which particularly inhibits the ATP synthesis by disrupting the proton gradient, the sodium azide inhibits the function of complex IV or cytochrome c oxidase in ETC where oxygen consumption decreases by the inhibition of electron transfer through cytochrome c (Kake-Guena et al. 2017; Chen and Lesnefsky 2006). The

inhibition of the cytochrome oxidase increases the production of H₂O₂ or ROS and eventually reduce the mitochondrial membrane potential (Rahn et al. 1991). Moreover, it has stronger inhibitory function on ATPase stimulated by DNP which is not common characteristic of other uncouplers (Bogucka and Wojtczak 1966).

Even though we observed the effect CCCP and DNP on the heat tolerance but not in NaN₃ at the non-lethal doses, the dose effect relationship of these toxins on mortality is also not well known. If the relationship is more of a step function, then the non-lethal doses or doses below LC50 might not have effect perhaps it's because of the concentration of doses are way below to the threshold. The mitochondrial toxins at their different doses affect the survival differently. Regardless of no observed effects of sodium azide on survival in the present study, there is evidence that at lower concentration it stimulates the Mg²⁺ ATPase enzyme activity and at high concentration it inhibits the ATPase activity. On the other hand, at low concentration, it protects ATP-ADP exchange reaction against the effect of the DNP as well as of the reduced respiratory chain. But at high concentration, it inhibits the mitochondrial function by creating the proton leak (Bogucka and Wojtczak 1966).

Thermal Regimes on the Heat Tolerance

The cold acclimated heat-sensitive genotypes show significant differences in the damages of mitochondrial membrane potential exposed to the mitotoxins which is not the case for heat tolerance genotypes (Fig. 4). Moreover, anaerobic metabolism, a compensatory mechanism was significantly higher in cold acclimated genotypes than heat tolerant genotype (Fig. 5) due to the inability of cold-acclimated genotypes to sustain aerobic function at hypoxia caused by the lethal temperature. However, the damages to the mitochondrial membrane

potential by the exposure of lethal temperature are similar in both high temperatures acclimated and cold acclimated genotypes (Fig. 3), which is the indication of having other possible underlying mechanisms besides the mitochondrial membrane potential which are affected by the broader effects of high temperatures.

Observed Plasticity

Phenotypic plasticity is the changes in the developmental programs to adapt to the altered environment (Travis 1994). However, their heritability has been a controversial term in the evolutionary perspective. The genotypes phenotype varies which is also called the reaction norm such as morphology, physiology and life history traits as a function of the environmental variation (Woltereck 1909). The plasticity is adaptive when the population place themselves to a new optimum phenotype where that phenotypes increase the chance of fitness as well as the evolution of that traits in new environment. This type of plasticity is a likely product of selection in past to the environmental variation and has the advantageous of having broader tolerance and higher fitness across multiple different environments (Ghalambor et al. 2007). On the other hand, non-adaptive plasticity is a nondirectional selection of the reaction norm in terms of the environmental variation. In our study, the plasticity was observed due to the acclimation effects. The retaining total mitochondrial capacity (F_{max}) (Fig. 5) and lactate accumulation in 25°C (Fig. 5) acclimated control genotypes may be the indication of the adaptive phenotypic plasticity due to the enhanced ability to perform both aerobic respiration as well as anaerobic metabolism during metabolic demands.

Importance of Proton Leak on Mitochondrial Balance

The proton leak is crucial for maintaining homeostasis in the mitochondrial function. The mitochondrial uncoupling protein such as UCP1, UCP2, UCP3 etc. perform both as the heat

generator and synthesis of ATP. While dissipating the proton gradient from the mitochondrial matrix to the intermembrane space, UCP1 generates heat needed for the optimal physiological activities. Being the channel protein, UCP2 and UCP3 regulate the ATP synthesis (Nedergaard et al. 2005; Rousset et al. 2004) by working parallelly with the ATP synthase. The uncoupling proteins lessen the production of ROS by reducing the membrane potential (Brand 2000). The moderate proton leak by these uncouplers ensure the homeostasis as it produces heat instead of more ATP. Acclimation to moderate increase of temperature enhance the expression of uncoupling proteins in order to alleviate the oxidative stress (Mueller et al. 2014).

Observed VS Expected outcome

In this study some of the predictions have been met, supporting the hypothesis of the critical role of maintaining membrane phosphorylation as the requirement for heat tolerance. The lack of effect of the ETC disruptor sodium azide on heat tolerance indicates that the non-lethal doses don't have effects proton leak or ROS production even though its inhibitory function on the cytochrome c oxidase. This is consistent with the previous findings (Coggins et al. 2017) that supplementation or disruption of antioxidant pathways does not lead to immediate changes in acute heat tolerance in *Daphnia*.

However, there are several expectations that have not been demonstrated in our data. We see no difference between temperature tolerant and temperature sensitive genotypes in the increase of fluorescence due to exposure to lethal temperature (Fig. 3) but the differences were observed only in temperature sensitive genotypes during the exposure of three mitotoxins used in this study (Fig. 4). Similarly, we do not see a consistent difference between cold- and warm-acclimated *Daphnia* in mitochondrial membrane potential measured as the K_m parameter of the Rh-123 saturation curve fitted to Michaelis-Menten equation (Table 3). This indicates the

differences are possibly due to the presence of 1. Other underlying mechanisms beside of the mitochondrial function, 2. The genetic differences or modifications in the cellular contents and their membranes, 3. The difference in the plasticity and selection of ambient traits.

Since temperature has the broader effects on every cellular function, other cellular properties may also have role in limiting the thermotolerance in *Daphnia*. For example, nuclear origin heat shock proteins (HP) are involved in alleviating the oxidative damage either caused by UV or cold (Cao et al. 1999). So, we cannot conclude unequivocally on the mechanistic explanation of the effect of mitochondrial function on heat tolerance. However, we can hypothesize that the lack of expected higher membrane potential in warm-acclimated *Daphnia* than in cold-acclimated ones may be caused by a compensatory mechanism that reduces membrane potential under conditions of high rate of redox reactions leading to the increased potential, but low oxygen availability makes it impossible to sustain electron transfer to reduce oxygen. Although not reaching statistical significance, the observed nearly 2-fold difference in the K_m parameter in the heart muscle between the 18°C-acclimated and the 25°C-acclimated *Daphnia* (Table 3) is suggestive of possible reduction of mitochondrial potential at the higher temperature due to shortage of oxygen. This is consistent with the intermediate and unaffected by heat exposure level of anaerobic metabolism in the 25°C-acclimated *Daphnia*, measured by lactate concentration in tissues (Fig. 5). Such reduction of potential may be maladaptive and caused by proton leak through the membrane disruption (Sommer and Pörtner 2004) or may manifest an adaptive uncoupling (Bryant et al. 2018) phenomena caused by up-regulation of uncoupling proteins. Similar compensatory mechanism might also explain the lack of differences in membrane potential and response to mitotoxins among genotypes differing in temperature tolerance that we predicted but did not observe in this study.

Future Direction of this study

Since this study provides some insights of the role of mitochondrial function in setting up the thermotolerance in *Daphnia*, it also has the future direction of the more comprehensive exploration in understanding the mechanism. There are questions that couldn't be answered in this study such as, can we detect not only the membrane potential but also the degree of oxidative damage? Can we detect the differences in respiration rate and ATP production with association of membrane potential? Can we explain the broader effects of protonophores on thermotolerance but not of NaN_3 mechanistically? Does FCCP have the same effects of CCCP on thermotolerance since both are protonophores?

Though the measurement of mitochondrial membrane potential by using the rhodamine 123 probe gives proximate ideas of the integrity, the detection of oxidative damages of tissues are yet unknown with this probe. We did see the effects of lethal temperatures on the disruption of mitochondrial membrane potential but exposure to different sublethal temperature would be useful in more depth understanding of the threshold of broader effects of temperature. Measurement of the differences in ATP/ADP and NADH production would be other tool that would correlate with the membrane potential as well. In terms of toxin effects, CCCP showed pretty mitochondrial site-specific effect, but there are other well-known mito-specific protonophore such as FCCP would be effective in understanding the mechanistic effect. Moreover, the exploration of molecular mechanisms of thermotolerance that include proteomic, lipidomic as well as genetic studies should give us the knowledge of the complex mechanisms.

Concluding remarks

The anthropogenic climate change has become the major ecological and environmental force over the natural variation in temperature of the planet (Parmesan 2007; Corlett 2015) that causes the shifts the natural ecosystems (Hoffmann et al 2015, McGill et al 2015; Hoffmann and Sgro 2011). The severe and rapid changes in temperature alongside with the heatwaves are considered as the prime threat to the composition of organisms and their biodiversity (Moss 2012; Urban 2015; Corlett 2015). During the last 50 years, temperature in the earth atmosphere has increased significantly and it is predicted the global temperature in next hundred year would increase by up to 4.8 C (IPCC Climate Change 2013). The rising global temperatures directly or indirectly affect all the living organisms and our study particularly focuses the effects on ectotherms. If we can better know the mechanisms of their survival and tolerance to the changing environment or habitat that is resulted from the fluctuation in their usual habitable temperatures, we will better be able to manage the environment in a sustainable manner. Our conclusions about the interplay between maintaining membrane phosphorylation during exposure to heat and heat tolerance adds to the understanding of the mechanisms that are involved in adaptation and plasticity in living organisms those the aquatic environment as their habitat.

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APPENDIX

Supplementary Table S1. Results of one-way ANOVA and Tukey test for the differences in time until immobilization among *Daphnia* exposed to varying concentrations of mitotoxins (Fig. 1), including vehicle control.

ANOVA							Tukey's test	
							highest concentration vs. all others	highest concentration vs. vehicle
Toxin	Source	DF	Sum of Squares	Mean Square	F Ratio	P	P	
CCCP	Toxicant, μ M	3	25.12	8.37	21.56	<.0001	0.001	0.001
	Error	91	35.35	0.39				
DNP	Toxicant, μ M	3	4.65	1.55	2.93	0.0374	n.s.	0.05
	Error	96	50.80	0.53				
NaN3	Toxicant, μ M	3	1.38	0.46	1.04	0.38	n.s.	n.s.
	Error	93	41.37	0.45				

Supplementary Table S2. Results of one-tailed t-test for the differences between control and exposed *Daphnia* (60 min exposure to 37 C or 120 min exposure to sublethal concentrations of mitotoxins).

Clone	Treatment:	t-test	d.f.	P
	Heat, 60 min			
GB		3.999	20.47	0.0003
IL		4.325	42.17	<.0001
	1 μ M CCCP, 120 min			

GB		2.017	17.61	0.03
IL		0.461	15.76	0.67
	1 uM DNP, 120 min			
GB		2.171	20.25	0.02
IL		0.003	28	0.5
	5 uM NaN ₃ , 120 min			
GB		1.856	25	0.04
IL		0.324	28	0.37

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