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Escaping the Arrhenius Tyranny: Metabolic Compensation during exposure to high temperature in Daphnia

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Escaping the Arrhenius Tyranny: Metabolic Compensation During Exposure to High Temperature in *Daphnia*?

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by

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ABSTRACT

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Poikilothermic organisms experience trade-offs by differential physiological demands generated by temperature extremes. Many such organisms exhibit acclimatory effects, adjusting their metabolism and physiology to recently experienced temperatures. One such acclimatory effect is metabolic compensation, the deceleration of biological rates below Arrhenius expectations. *Daphnia magna* is eurythermal, and if acclimated to mildly stressful temperatures first, survives longer in lethal temperatures. This study examined the effect of ambient temperature (5°C-37°C) and acclimation history (lifetime at 10°C or 25°C) on the oxygen consumption rates of 8 genotypes of *Daphnia* with high or low acute temperature tolerance. There were decelerations of respiratory rates across a temperature gradient when acclimated to 25°C or following short 8-hour acclimation to measurement temperatures. *Daphnia* exposed to a near-lethal temperature (35°C) with a 24-hour recovery period at 25°C-acclimation temperature showed no difference in respiratory control compared to unexposed 25°C-acclimated Daphnia. Genotypes showed no difference in potential compensatory ability.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Poikilotherms and the Constraints of Temperature

Poikilotherms must adjust their metabolism and physiology to fluctuations in environmental temperatures in order to survive because all their biological functioning is a direct product of ambient temperature's influence on chemical reaction rates. Since biological enzyme rates are largely influenced by thermodynamic law, temperature is often a major constraint for the habitat range of a poikilotherm. Different poikilothermic organisms can exploit habitat temperatures ranging from below 0°C up to 50°C, yet the limit to the thermal range of tolerance for a given poikilothermic species is set by the amount of physiological plasticity. It is well established that some organisms are quite tolerant to the biological stress associated with temperature changes and their enzymatic Q10s are a direct function of their habitat temperature (Rao and Bullock 1954; Radmer and Kok 1978; Thornton and Lessen 2011; Arcus et al. 2016). However, there is considerable variation in biological rates with increases in temperature (Jacobs 1928; Huey and Kingsolver 2011). One type of plastic physiological response to elevated temperatures is reduced metabolic rates to compensate for high costs of maintaining energy homeostasis in the stressful temperature (Sokolova et al. 2012). Fish, like the shorthorn sculpin, *Myoxocephalus scorpious,* show considerable capacity to metabolically compensate to their thermal environment by fully restoring their resting metabolism and partial recovery of aerobic scope and feeding activity (Sandblom et al. 2014). However, specific mechanisms of such plastic responses are poorly understood. There is an increasing importance in understanding energy homeostasis as well as the physiological changes that occur within the maintainable levels of a stressor plasticity as the mechanisms that set the limit of stress tolerance (Sokolova et al. 2012).

Thermal Tolerance in *Daphnia*

Daphnia magna are excellent model organisms because of their cyclic parthenogenesis and diverse occupation of most of the northern hemisphere. Short generation times and robustness along a thermal gradient allow rapid acquisition of respiratory data across a broad temperature range with high resolution. Part of their robustness to temperature comes from their ability to acclimate to new ambient temperatures, even if they are mildly stressful (Paul et al. 2004). Furthermore, acclimation to higher, stressful temperatures increases their time of survival in lethal temperatures (Paul et al. 2004; Yampolsky et al. 2014; Geerts et al 2015). An addition benefit of the system is that specific genotype lineages can be obtained from specific locations of varying climates. Since genotypes can be isolated, and *Daphnia magna's* primary mode of reproduction is asexual, acclimation to higher temperatures can occur in the absence of selection. As such, *Daphnia* are ideal organisms to determine whether energy conservation (genotype alone) or compensation (some regulatory plasticity conserved across genotypes) is prevalent.

The causal mechanism for thermal acclimation in *Daphnia* is still not fully known, and intensive metabolic studies have not been coupled with the acclimation process. Prior work has investigated the role of antioxidants and membrane structure during acclimation (Coggins et al. 2017). While there are significant changes in both antioxidant capacity and membrane fluidity with higher temperatures, neither fully explains the mechanism for heat tolerance. Both of these findings do however corroborate with the gene regulation patterns found in heat acclimated *Daphnia pulex* and are useful to consider in the context of a metabolic study because any modification of the oxidative environment or membrane properties should have downstream effects on metabolic activity and functionality (Yampolsky 2014). For heat-acclimation in *Daphnia*, there are increases in antioxidant capacity and significant membrane polarity changes

with insignificant variation in lipid peroxidation (Coggins et al. 2017). This is likely due to less polyunsaturated fatty acid (PUFA) presence in higher temperatures and more antioxidant activity, both of which are beneficial to mitochondrial function because they are the respective main generators/targets and scavengers of ROS (Schonfeld et al. 2011). Excessive (2hr) exposure to a lethal temperature effectively sees antioxidant capacity reach its limit in *Daphnia*, so a shorter exposure without exceeding the antioxidant activity limit may ensure survival and at least partial recovery of metabolic function. While the relationship between lipid structure and respiration in *Daphnia* is untested at present, a diet supplemented with liposomes of increasing amounts of polyunsaturated fatty acids effectively eliminates the enhanced temperature tolerance effect achieved by heat acclimation (Martin-Creuzburg et al. unpublished data; Figure 5). High levels of unsaturation in mitochondrial membranes causes higher free radical damage and compromises membrane integrity leading to inefficient energy return and eventually the release of cell death signals (Pamplona et al. 2000;Al-Gubory 2012). In fact, bioaccumulation of UFAs in the mitochondrial membrane is associated with decreased longevity (Pamplona et al. 2000). Perhaps changes in regulation of respiration rate may be an important compensatory mechanism to manage the direct and indirect generation of ROS to combat compromised mitochondrial membranes, though this is difficult to disentangle from drops in respiration just due to the structural damage creating inefficiency and eventually an energy deficit. However, any adjustment to organismal respiration rates likely stems directly from changes at the mitochondrial level, and modulation of membranes can physically gate metabolic processes. Results from previous studies that the author was involved in focused on the roles of membrane structure and oxidative environment (Figures 5,6) and how they can be linked to respiratory activity are discussed later.

Part of understanding the physiological processes involved in energy homeostasis across temperatures requires the differentiation of constitutive defenses and inducible ones. Often, organisms have constitutive adaptations for a small range of thermal habitats that allow for enhanced metabolic efficiency in temperatures relevant to the organism. Antarctic fish (*Notothenia rossii*) have high ATPase activity in low temperatures compared to tropical fish (*Amphiprion sebea*) because they somehow reduce activation energy for ATPase compared to tropical counterparts (Johnston & Walesby 1977). A *Daphnia* population resurrected from ephippia initially laid 40 years prior which had lower thermal tolerance than the current population from the same lake, showing evidence for rapid evolution to temperature changes (Geerts et al. 2015). Conversely to local adaptation of constitutive physiological changes, some crustaceans acclimated to temperatures above or below their normal range and respectively cooled or heated to the opposite acclimation temperature show no irreversible changes to excitatory junctional potentials within the 10°C acclimation range (Stephens & Atwood 1982). *Drosophila* can sustain metabolic rates in cold environments comparable to the rates observed at room temperature, but selection for higher metabolic rates does not appear to occur in cold acclimated populations (Alton et al. 2016). Exact physiological mechanisms for thermal tolerance can be difficult to tease apart, but reversibility of reaction rates is vital to differentiating plastic acclamatory effects from local adaptation to a certain temperature even if the mechanism is initially poorly characterized.

Plastic Responses to Temperature

In cases when a poikilotherm can acclimate to temperature fluctuations higher than its norm it is often ambiguous whether the organism is simply maintaining temperature-dependent energy homeostasis (conservation) or adjusting some physiological parameter(s) to offset energy

demands (compensation) after the onset of respective threshold shifts in temperature (Sokolova et al. 2012). The former process suggests any differential ability to tolerate heat stress among individuals is due largely to differential quality in genotypes. More robust genotypes will experience a temperature in which their respiratory systems begin to physically fail and they eventually die, just like those maladapted to high temperature environments. The only difference being that adapted genotypes are able to sustain structural integrity and meet their environmentally-determined energetic demand longer and therefore survive longer in high temperatures. The latter process would suggest that thermal acclimation is not just a direct consequence of a genotype's biogeographic history (Chopelet et al 2008). Compensation implies that reaction rates will decrease not because of better constitutive protection, but because a shift in temperature signals for inducible elements to regulate energetic output and expenditure despite the rather ubiquitous influence temperature has on chemical reaction rates (Sandblom et al. 2014). If constitutive adaptations to maintain fully temperature-dependent metabolism is the predominant mechanism for thermal acclimation, biochemical reaction rates should match Arrhenius expectations with environmental temperature until they reach a state of respiratory failure. Conversely, in the case of compensation, changes in metabolic activity or physiological parameters will conform to Arrhenius predictions until a transitional temperature is reached that induces physiological changes to offset the costs of high energetic demand (Sokolova et al. 2012). Inevitably, when an organism reaches the lethal limits of its tolerance range (a second transitional temperature), conservation strategies will be prioritized simply because energetic demand becomes an impossible constraint.

Based on the Arrhenius equation, biological rates typically double with a 10-degree increase in temperature (Rao and Bullock 1954). The inverse relationship between enzymatic

rates often does not hold strictly true. Rates can often decelerate outside expectations with temperature increase causing “breaks” from linearity in traditional Arrhenius plots (Truhlar & Cohen 2001). While there are clear temperature ranges for organisms where Arrhenius expectations are meet, there is increasing evidence that these expectations do not hold true outside of those ranges (Kavanau 1950; Barnes et al. 1968; Leenson 1999). Thermal performance curves (TPCs) can be plotted which show performance of specific rates across a temperature gradient. The advantage of these plots is that they characteristically have absolute performance maxima that reveals the optimum temperature for the rate being studied, while the width of the curve reveals the thermal breadth of the rate (Schulte 2015). Since thermal performance curves do not “break” when biological rates decelerate possibly showing metabolic compensation), these plots are useful in showing trends of biological rates with increasing temperature that otherwise may be unnoticed in other plots.

If metabolic compensation is uncoupled from genotype quality as a plastic trait that has not been canalized, it is difficult to imagine both processes happening simultaneously because conservation maintains temperature dependent rates until they are unsustainable, while compensation still allows other processes to occur by differential regulation of metabolic activity (Guderley and St-Pierre 2002). If metabolic compensation is occurring, acclimation and subsequent measurement of metabolic activity across a thermal range will either shift the temperature of optimal performance or change the shape of the performance curve to reach that optimal temperature (Schulte et al. 2011). This not to say that locally adapted constitutive defenses and plastic mechanisms that are not under selective pressure are mutually exclusive. In fact, some form of damage control must be coupled with compensation in some organisms, as evidenced by the full recovery of only certain components of metabolism in heat-acclimated

Myoxocephalus scorpious (Sandblom et al. 2014). To reiterate, showing metabolic compensation in an organism will require not only testing for the effects of temperature on the metabolic rates of both locally adapted and temperature-acclimated organisms, but damage to metabolic systems at temperature extremes must also be assessed.

Differentiating Compensation and Damage

In regards to metabolic damage, it is important to consider the functional efficiency of mitochondria such as substrate conversion rates and electron transport chain (ETC) activity. Regulation of ETC activity is critical to longevity and stress response because at reproductive maturity it suppresses heat shock response accelerating senescence (Labbadia et al. 2017). Mild downregulation of ETC activity stops suppression of heat shock response and increases vitality and lifespan, with effects exceptionally notable after the onset of sexual maturity (Labbadia et al. 2017). Heat shock response is one of many cascading factors that likely aid in acclimation, so downregulation of antagonistic metabolic activity can be adaptive and regulated by means other than purely physical damage. Furthermore, ETC to cellular respiration ratio is an already established quantification for acclimation in *Daphnia* (Simcic & Brancelj 1997). Interestingly, short-term hypoxia can enhance survival time in lethal temperatures in *Daphnia* (Coggins et al. 2017; Figure 6), and hypoxia inducible factor directly interacts with heat shock factors (Baird et al. 2006). Given heat shock factor 1's prominent involvement in ETC activity (Labbadia et al. 2017), mitochondrial function and respiration again seem to be very relevant areas for investigation thermal tolerance. Coupling information about mitochondrial performance, like ATP and lactate generation, with organismal respiration at different temperatures will provide better resolution when distinguishing between respiratory failure and compensation because compensation should maximize respiratory efficiency and allow at least partial recovery to

metabolic function after short exposure to a lethal temperature while failure will see substantial and irreversible decreases in respiration and substrate conversion. Mechanistic insight can be gained about metabolic compensation by examining mitochondrial performance, and oxygen consumption by the whole organism should not only provide some evidence of compensation if it exists but also will allow inference about mitochondrial oxidative performance.

Studies of Metabolic Compensation

There is evidence of metabolic compensation in various poikilotherms, both terrestrial and aquatic in both low and high temperature extremes. Eastern newts, *Notophthalmus viridescens*, have been shown to adjust metabolic enzyme activity such as creatine kinase and citrate synthase to enhance locomotor performance in cold temperatures (Mineo & Schaeffer 2015). Marine gastropods from a subarctic region experience less metabolic rate depression in extremely cold temperatures than their temperate counterparts, but exhibit no ability to compensate when exposed to warmer temperatures (Sokolova & Pörtner 2003). An Antarctic amphipod, *Gondogeneia Antarctica*, with a low thermal tolerance range exhibits no changes in metabolism when exposed to combinations of salinity and temperatures from 0 to 2.5°C, but enters a conservation phase at 5°C (Gomes et al. 2013). Three species of invasive blue mussels exhibit generally significant compensation to higher temperatures by adjusting heart rate, with the degree of compensation matching their respective habitat ranges (Braby & Somero 2006). Potential compensation in the opposite direction has also been addressed as a prior study with *Daphnia magna* found that clones locally adapted to colder temperatures have higher respiratory rates at 15^{o}C than those adapted to warmer environments (Chopelet et al. 2008). Southern Catfish, *Silarus meridionalis*, have been shown to seasonally compensate by adjusting the oxidative capacity of tissue-specific mitochondria (Yan & Xie 2015). Higher oxidative capacity

occurred in colder months with a simultaneous reduction in their upper thermal tolerance limits, outlining a clear trade-off between efficiency of metabolic performance and reactive oxygen species (ROS) generation.

Of course, there are cases where there appears to be no compensation during acclimation. The long-jaw mudsucker, *Gillichthys mirabilis*, for example, can acclimate to different temperatures, but there has been no evidence of compensation in resting metabolic rates or heart rates (Jayasundara & Somero 2013)**.** The difficulty in definitively declaring there is no compensatory mechanism in play largely depends on the selection of physiological parameters chosen to investigate. In general, basal energy requirements and cardiorespiratory activity are much more flexible than the upper tolerance limits of stress in an organism (Sandblom et al. 2016)**.** Using organisms that can tolerate a wide range of a given stress are ideal for a compensatory study because there is more resolution in their transitional temperatures before a lethal range is encountered (Giomi and Portner 2013).

Metabolic Compensation in *Daphnia*?

Just as there is conflicting literature about metabolic compensation in other organisms, there is a paucity of evidence for compensation in cladocerans, despite *Daphnia's* remarkable and well-characterized ability to tolerate a wide range of temperatures (Paul et al., 2004)**.** The information that is present is contradictory. In fact, one of the few studies regarding changes in *Daphnia pulex's* metabolic capacity at different temperatures reported very low plasticity (Jose et al. 2009)**.** In contrast, a more recent look at the functional genomics of *Daphnia pulex* during thermal acclimation shows downregulation of metabolism, suggesting there may indeed be compensatory mechanisms in play (Yampolsky et al. 2014)**.** While specific mitochondrial functions were up-regulated by temperature, almost all gene expression involving DNA

replication and DNA repair activity decreased in heat-acclimated Daphnia. The highly differential gene activity in regulatory pathways during acclimation to high temperature was only observed in clones locally adapted to warmer environments, suggesting that differences in thermal tolerance may be at least in part to this differential capacity for such regulation. On the other hand, mitochondria-specific functions, including citric acid cycle, oxidative phosphorylation, and steroid biosynthesis, were up-regulated in both northern and southern clones, suggesting a universal regulation of mitochondria and membrane-specific activity. A similar study in zebrafish shows downregulation of all catabolic pathways during heat acclimation (Vergauwenet et al. 2010).

Predictions

If clones either locally adapted or acclimated to high temperatures exhibit exponential increases in respiratory rates before transitioning to a range of high temperatures in which respiration is independent of temperature (before reaching a critical, lethal temperature), then *Daphnia* potentially metabolically compensates to high temperature. Figures 1B, C show potential patterns in oxygen consumption that may be seen with acclimation, but any decrease in respiration with high temperature that isn't presumably metabolic depression could be a compensatory response. Acclimation may either additionally or alternatively shift the critical temperature, though this seems less likely (Sandblom 2016). Alternatively, without compensation, TPCs should be largely be temperature-dependent throughout with increases in respiration directly corresponding to increases in temperature until the critical temperature is exceeded (Figure 1A). In this case, any differential performance at high temperature should be explained by differences in acute thermal tolerance among clones.

A regression of locally adapted or acclimated to high temperature clones is expected to be nonlinear and independent of temperature (for at least a small range of temperatures) in acclimated clones. A curvilinear and temperature-independent response in the cold-acclimated *Daphnia* could be metabolic compensation to low temperature if higher metabolic activity occurs at low temperature because it is beneficial for performance in cold environments (Guderley and St-Pierre 2002). Potential patterns of metabolic compensation can be corroborated if respiration returns to the same levels as controls in 25˚C following a 24-hour recovery period after a 1-hour exposure to 37 ˚C. High amounts of irreparable respiratory damage after exposure to 37˚C will corroborate whether metabolic compensation is happening because it implies that high temperature acclimated *Daphnia* do not compensate but are instead able to partially reduce heat-induced damage for a short time.

This study aims to address the question whether metabolic compensation in *Daphnia magna* plays a role in heat tolerance. Specifically, we aimed to find out if oxygen consumption increases with increasing temperature in accordance to Arrhenius expectations regardless of *Daphnia* genotypes' local adaptations (e.g. origin from different climates) and of *Daphnia* temperature histories. We hypothesized that temperature-dependent profile of in oxygen consumption will be different in *Daphnia* acclimated, prior to the measurements, to different temperatures. Namely, if there are patterns indicative of metabolic compensation, we expect that high temperature (25˚C) acclimated *Daphnia* will show stronger metabolic compensation that low temperature (10˚C) acclimate ones. Finally, we aimed to find out whether the observed reduction of oxygen consumption at high temperatures can be reversed or is caused by irreversible damage.

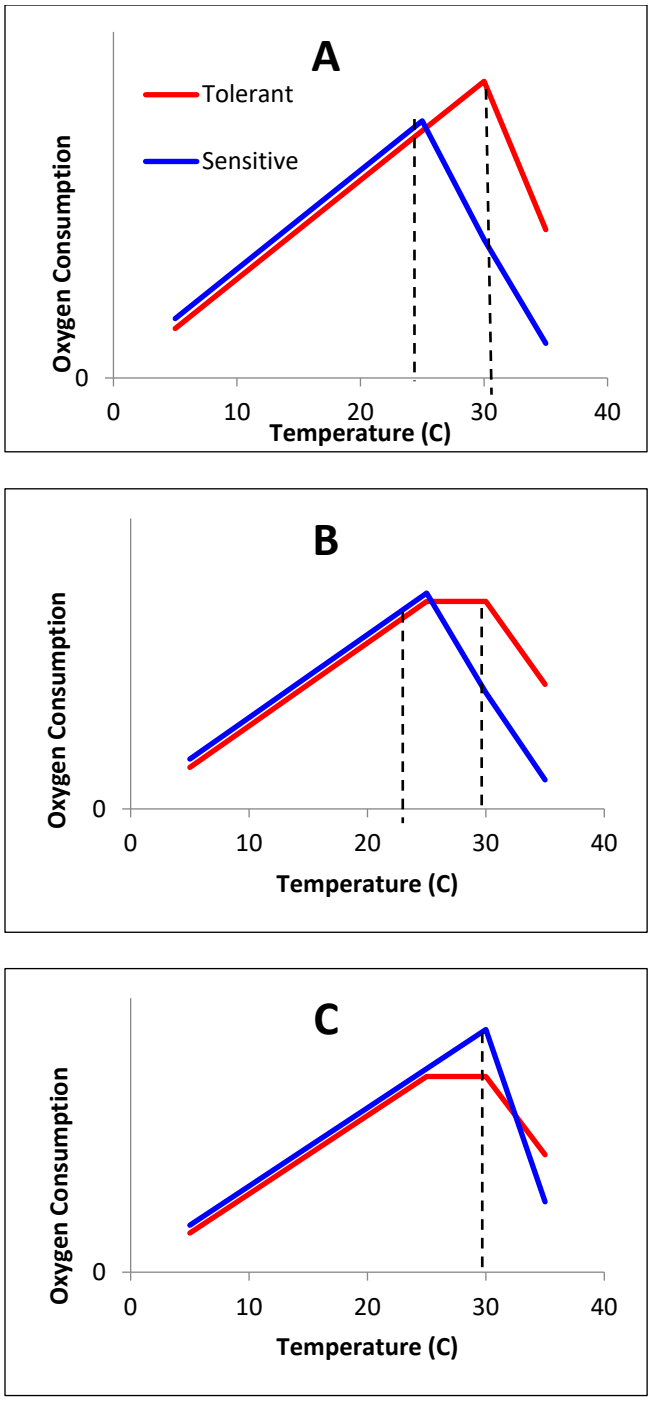

Figure 1. *Predictions for Respiratory Regulation in high and low heat-tolerant clones* (by adaption or acclimation). *a*. Respiratory failure across all clones with a single, irrecoverable transitional temperature in each type of individual; in heat-tolerant individuals this temperature is higher. *b*. Compensation in high tolerant clones in which reaction rates are sustained after the first transition temperature until reaching a lethal transition. Heat tolerant clones have a higher critical temperature. *c*. Compensation in high tolerant clones. Critical temperature is the same across clones.

CHAPTER 2

MATERIALS AND METHODS

Daphnia Cultures

Lineages of *Daphnia magna* from 6-8 different genotypes, 3-4 with high and 3-4 low acute temperature tolerance (Table 1), were maintained at 20°C with a 12:12 LD cycle in 100 mL flasks of COMBO medium (Kilham et al. 1998) at the density of 6-10 individuals per flask. All cultures were fed the green algae *Scenedesmus obliquus* supplemented with essential minerals and vitamins to a final concentration of 50,000 cells per individual per flask every two days. *Scenedesmus obliquus* offers complete nutritional supplementation for *Daphnia magna* as long as it is supplemented with B-vitamins (Mehdipour et al. 2011). The amount and type of food per individual was chosen to promote somatic growth without overinvestment into reproduction because the aim of the study was to only influence respiration rates by temperature in healthy, well-fed individuals rather than starved ones or clutches of eggs the mothers carry which undoubtedly have their own respiration rate. *Scenedesmus obliquus* was also chosen to avoid the addition of PUFAs which influence reproduction and thermal tolerance (Schlotz et al. 2012; Martin-Creuzburg et al. unpublished data). COMBO medium and flasks were replaced every 4 days to prevent waste buildup and to keep density constant.

Table 1. *Location and Thermal Tolerance of Daphnia Genotypes Used*. Genotype ID is presented along with habitat type, geographic coordinates, and temperature tolerance characterized by survival time after immediate exposure to a lethal temperature.

CloneID	Type of Habitat	Latitude	Longitude	Acute Temperature Tolerance
FI-FSP1-16-2	summer rock pool	60° 10.062"	25° 47.677"	Low
GB-EL75-69	year-round pond	51°30′26″	-0°7′39″	Low
IL-MI-8	Mediterranean pond	31° 42' 52.42"	35° 3' 3.38"	High
IR-GG1-7	lake	37° 54' 54.92"	46° 41' 58.29"	Low
DE-S3-3	pond	48° 48' 189.6"	9° 10'23.18"	High
FR-SA-1	Mediterranean pond	43° 27' 37.06"	4° 39' 09.83"	High
HU-HO-2	pond	46° 47' 50.03"	19° 08' 17"	Low
HU-K-6	lake	46° 47' 33.3"	19° 10' 53.84"	High

Acclimation

Adult *Daphnia* from the 20°C lines were transferred to either 10°C, 15°C, or 25°C (+/- 1°C) incubators and reared for 2 additional generations before any experimental work was conducted (Yampolsky et al. 2014). To obtain the 10°C- and 25°C-acclimated *Daphnia* of approximately the same age simultaneously for common garden experiments the 25°C acclimation lines were set up with a delay. Temperature ramping was achieved by keeping the *Daphnia* at a starting temperature for measurement (e.g., 5°) overnight prior to the measurements with a daily 5°C increase to the next measurement temperature.

Respirometry

Measurements of oxygen consumption, were conducted using two 4-channel Firesting O2 Fiber Optic Oxygen Meters (PyroScience, Aechen, Germany). Both sensors were placed inside an incubator set to assay temperatures ranging from 5°C to 37°C. For a reliable signal for each measurement, three *Daphnia* were placed in a specialized 4 mL vial (4ml Ox Vial, PyroScience) which constitutes an experimental replicate in all experiments to be described. Measurements were carried out in closed systems at 5 second intervals for a duration long enough to cover either 3 hours (5°C and 10°C for the shared history experiment) or 90 minutes (any other temperature during all other experiments) of usable data. This measurement range ensured at least a 0.5mg/L drop in oxygen concentration at each temperature (typically a 1-1.5 mg/L drop and never below 5mg/L at the end of the measurement). This allowed a sufficient amount of oxygen consumption for reliable detection without to inducing hypoxia. To ensure circulation across the measurement area of the vials, lids were modified with an additional airtight space for a magnetic stir rod bringing the total volume of the chambers to 5 mL. To prevent damage to the *Daphnia*, the open side of the stirring space was covered with a single layer, 59% open area nylon mesh that still promoted circulation. To avoid any potential temperature fluxes in the incubator and avoid increased risk of water contamination, all vials were submerged in a 4 L dry bath of Lab Armor beads (Lab Armor LLC, Cornelius, Oregon). An external temperature probe for each Firesting system was placed on either end of the bath to ensure homogenous temperature between channels. The bath was placed on top of a magnetic stir plate with separate stirring sections with the same stirring intensity for each vial.

COMBO medium was aerated for at least 8 hours in each assay temperature for use in experimental vials. The aerated medium was passed through a sterile 0.2 μm disk filter into each

chamber to minimize potential bacterial contamination in the experimental vials. No food was added to the chambers, and *Daphnia* were transferred to temporary flasks of same temperature as their acclimation or current temperature before transfer to experimental vials. One control vial containing only sterile COMBO medium was measured for the full duration of each assay temperature in each experiment. Vials were cleaned with 70% ethanol each day and calibrated by factory specifications at the start of every temperature change. To further reduce variation, only adult female *Daphnia* without eggs, or eggs in the first stage of development were used for measurements. To achieve this, replicate sets of *Daphnia* were staggered in experiments by egg development stage.

Shared History Experiments

Two experiments were conducted in which there was no difference in temperature history between *Daphnia* used in the experiments. *Daphnia* were first raised at 20˚C for multiple generations before adults were weighed (mg wet mass-blotted on filter paper to remove excess water) on an analytical balance and transferred to 5˚C for 2 days with subsequent measurement of oxygen consumption at 5˚C. After measurements were completed for an assay temperature (2 replicate sets of daphnia per clone, roughly 6-8 hours of measurement), *Daphnia* were transferred to new flasks and acclimated for 8 hours to a gradual 5˚C increase in temperature. During the acclimation period which was from birth to 1st clutch of eggs, *Daphnia* were fed on their normal 2-day schedule and were reweighed before each measurement period if there was mortality in a replicate. The 5˚C measurements continued sequentially to 30˚C, at which point many replicates had died. To avoid an imbalanced design in higher temperatures, the experiment ended after measurement at 30˚C. It should be noted that genotypes DE-S3-3 and FI-FSP1-16-2 were not used in this preliminary study because these cultures were not synchronized with the

others for the timing of this experiment. The goal of this experiment was to avoid allowing a lifetime acclimation effect and to simply get some idea of how respiration looks across most of *Daphnia magna's* thermal range. These experiments were conducted over a few days starting in relatively low temperatures, so any aging effect was minimal.

To further test shared acclimation history especially at the higher range of thermal tolerance, *Daphnia* were reared for 2 generations at 15˚C before oxygen consumption was measured in 5˚C increments from 15˚C to 30˚C. An additional measurement was taken at the absolute limit of thermal tolerance, 37˚C. The sequence of measurements and 8-hour acclimations followed the same methodology as the other shared history experiment, but 3 replicates were measured per clone because there were less assay temperatures and measurement times were all 90 minutes. Genotype FI-FSP1-16-2 was still not synchronized for experimental use at this time, so it was not included in this study. Again, the goal here was to avoid acclimation history differences and to further increase resolution on expected respiratory rates at each assay temperature. Measuring first at their acclimation temperature should also have prevented any potential temperature shock that could influence initial respiration rates.

Acclimation Temperature Experiment

Daphnia from 8 genotypes (Table 1) were acclimated to either 10°C or 25°C and remained in their acclimation temperature until use in a single oxygen consumption measurement (3 *Daphnia*/respiration vial) in each assay temperature in which there was no time allowed to acclimate to the new temperature. Following the single measurement, a Daphnia were weighed as a group and no longer used in the experiment. Assay temperatures consisted of 10°C, 15°C, 20°C, 25°C, 27.5°C, 30°C, 32.5°C, and 35°C, but these temperatures were not measured

sequentially. Alternating between high and low assay temperatures daily prevented an aging effect on assay temperature since the experiment required 16 days to complete. More resolution was sought between 25°C and 35°C because this was the flattest region in preliminary thermal performance curves generated from the shared history experiments. 3 experimental replicates (3 *Daphnia/*replicate) per clone per acclimation temperature per measurement temperature were targeted, and this was achieved apart from a few cases in which 2 replicates were used in some combinations where counts of *Daphnia* were low.

Recovery Experiment

Following the acclimation temperature experiment, 2 replicates from the 8 genotypes and from the same 25°C acclimated cohort were weighed then exposed to 35°C for 1 hour. 35°C is also within the temperature tolerance limit for all clones used, but a 1-hour exposure prevents high mortality in 25°C acclimated *Daphnia*. 10°C acclimated *Daphnia* were not used in this experiment because all 10°C replicates measured at 35°C during the acclimation temperature experiment were dead by the end of the measurement. After the 1-hour exposure, 1 replicate was placed back in 25°C for 24 hours while the other replicate was placed at 20°C. *Daphnia* were fed on their normal schedule and reweighed during the 24-hour recovery period. After 24 hours, both replicates were placed in 25°C and oxygen consumption was again measured for the normal duration. As a control for this experiment, *Daphnia* exposed to 35°C were compared to 4 replicates of 25°C acclimated *Daphnia* from the same cohort, but without exposure to the lethal temperature.

Regressions and Statistical Analysis

Using JMP statistical software (SAS Institute, Cary, North Carolina), dissolved oxygen content was plotted (mg/L) over time (seconds) for each replicate vial at the assay temperature in which it was measured. Once plots were finished, a default point was chosen to determine where to start using data for analysis. To avoid noise from equipment and to allow *Daphnia* adequate time to acclimate to vials, the first 960 seconds of each run were not included in analysis. The default cutoff of each run was 5,400 seconds. Each regression was manually inspected to ensure that data used in analysis was starting from the maximum point of oxygen content in the vial and that regressions were not influenced by nonlinear anomalies in the data. In cases where regressions were clearly not representative of oxygen consumption patterns, the start and end points for analysis of that replicate were manually adjusted. Manual adjustments were rare and did not influence the outcome of the following analyses. Slopes from each regression (mg/L/s) was normalized by wet weight (WW) of each replicate and then averaged for each assay temperature and treatment, a method used with other zooplankton (Gomez and Packard 2010). Final measurements were reported as μgO_2/min/mgWW and was used as a response variable in all subsequent analyses. All subsequent regression terms and model effects were tested against an α of 0.05.

Shared history experiments were analyzed with a three-way analysis of variance (ANOVA) using assay temperature, its square (to test for nonlinearity), and acute thermal tolerance (Table 1) of the clones. Relevant interactions between acute thermal tolerance and linear and nonlinear assay temperature effects were also included. A repeated measures analysis was not incorporated despite using the same replicates throughout the experiments because their placement in vials was randomized for every measurement. An alternative model in which clones

were a random nested effect in acute thermal tolerance revealed a clone by assay temperature interaction, but this effect was not significant after Bonferroni multiple testing correction and was ultimately not included. To further emphasize the lack of respiratory differences among locally adapted clones in the same acclimation temperature, the 5°C and 15°C data were combined with experiment as a random blocking effect.

For the differential acclimation temperature experiment, each acclimation temperature was analyzed as a mixed effects model with a 2^{nd} degree polynomial regression. Assay temperature and its square were fixed effects, while clones were considered random.

For the recovery experiment, a two-way ANOVA was performed. Treatment was a fixed effect and included 35°C, recovery at 20°C or 25°C (R20 and R25), and unexposed controls from 25°C (C25). Clones grouped by acute temperature tolerance were again included as a fixed effect and an interaction between treatment and acute temperature tolerance is included in the model.

CHAPTER 3

RESULTS

Shared History Experiments

Daphnia reared at 20°C then acclimated to 5°C for a short 2-day span before temperature ramping show significant differences in oxygen consumption both between assay temperatures ($p = 2.43E-07$, Table 2A), and response to those temperatures was significantly nonlinear ($p = 5.73E-03$, Table 2A). With clones classified by acute temperature tolerance as a fixed effect, neither the individual effect nor its interactions suggesting either a local adaptation by assay temperature effect or differential nonlinearity by local adaptation effect were significant in this experiment (Table 2A). As mentioned in methods, an alternative approach considered clones as a random effect nested within acute thermal tolerance classification, and for this experiment there was a clone by temperature interaction, but it is not significant after Bonferroni adjustment for multiple testing.

Again, for *Daphnia* fully acclimated to 15°C before temperature ramping from 15°C to 37°C, there was significant effect of assay temperature and nonlinearity ($p = 3.05E-09$, $p = 1.24E-06$, Table 2B). There was also no significant effect of acute thermal tolerance or its interactions in this experiment. The alternative nested approach showed no significant clonal effects.

Table 2. *Shared History ANOVA Models*. T denotes assay temperature, T^2 is nonlinear response to assay temperature, Ttolerance_acute is characterized acute temperature tolerance (high or low). Bold F and p values indicate significance at an $\alpha = 0.05$ confidence interval. Random Variation associated with clones is not different from zero.*a.* Individual F-test for the experiment starting at 5˚C. *b.* Individual F-test for the experiment with 15˚C-acclimation. *c.* Both shared history experiment data combined with experiment as a random block (and insignificant) effect.

	A 5˚C to 30˚C Assay Temperature Ramping Model F test			**B** 15˚C to 37˚C Assay Temperature Ramping Model F test			**C** Combined Temperature 5˚C to 37˚C Assay Temperature Ramping Model F test		
Source	MS	F Ratio	p	MS	F Ratio	p	MS	F Ratio	p
T	0.00168	**32.6128**	**2.4E-07**	0.0013	**48.5747**	**3E-09**	0.00242	**54.81**	**1.30E-11**
T^2	0.00042	**8.1184**	**0.0057**	0.0008	**29.1733**	**1.2E-06**	0.00072	**16.35**	**8.79E-05**
Ttolerance_acute	7E-05	1.364	0.25	1E-05	0.3859	0.54	0.00002	0.43	0.51
Ttolerance_acute *T	8.6E-08	0.0017	0.97	1E-05	0.362	0.55	5.47E-08	0.0012	0.97
Ttolerance_acute *T^2	6.1E-05	1.1803	0.28	1E-07	0.005	0.94	1.32E-06	0.03	0.863
Experiment (Block effect)							6.43E-06	0.1459	0.70
Error	5.1E-05			3E-05			0.005952		

Since measurements between the two experiments were close, and the same effects were significant in both (Table 2), data from the two experiments were combined. This manipulation did not change the outcome of the experiment as assay temperature and nonlinearity were both significant effects (Figure 2, Table 2). The random block effect representing the experiments was not significantly different (Table 2).

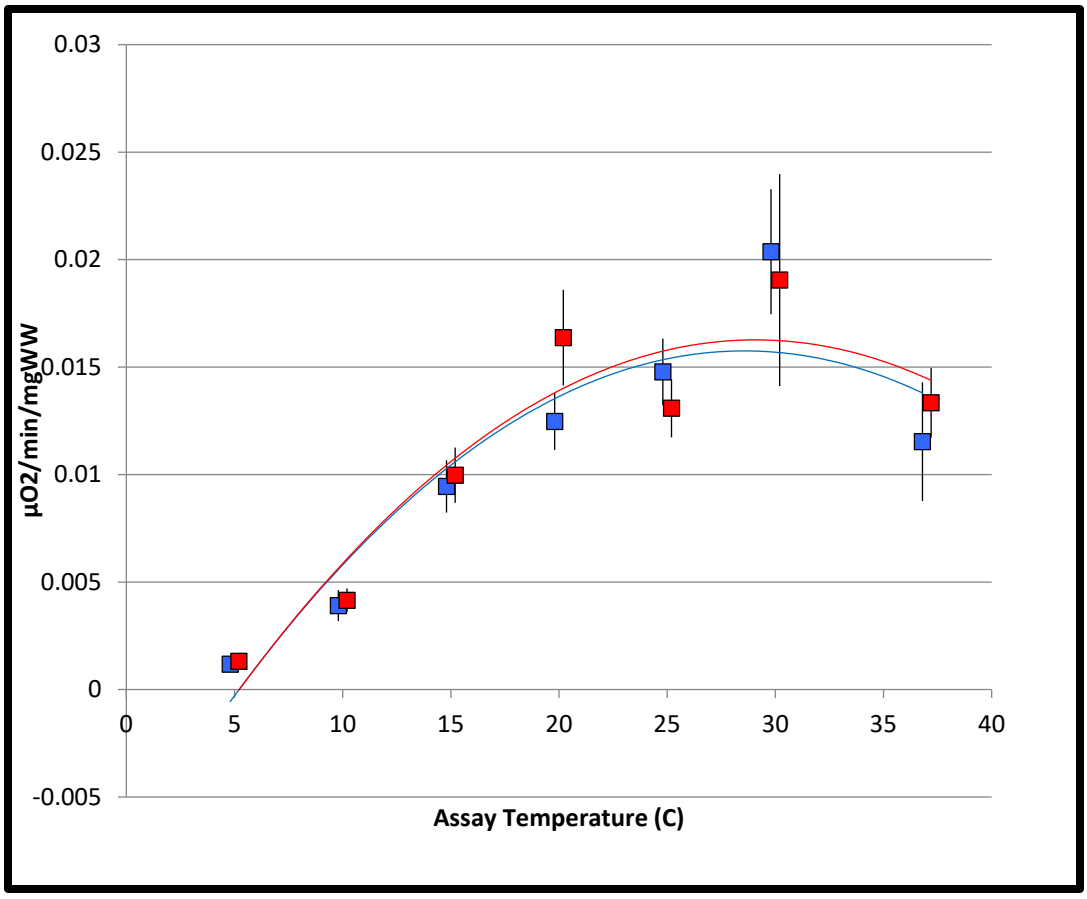

Figure 2. *Oxygen consumption during a gradual temperature ramp-up with 8-hr acclimation to assay temperatures*. Blue squares represent mean oxygen consumption (µO2/min/mgWW) at each assay temperature for clones characterized with low acute thermal tolerance. Red squares represent mean oxygen consumption in clones with high acute tolerance. The respectively colored trendlines represent the nonlinear, 2nd degree polynomial relationship of oxygen consumption and assay temperature for each set of clones. Vertical bars represent standard error of the means.

Acclimation Temperature Experiment

Unsurprisingly, the oxygen consumption of *Daphnia* acclimated for 2 generations to either 10°C or 25°C and subsequently plunged into a measurement temperature between 10°C and 35°C was explained by the measurement temperature in a regression analysis, regardless of

acclimation temperature (Table 3). While the linear term fully describes the relationship between temperature and respiration in 10°C-acclimated *Daphnia,* a nonzero, nonlinear parameter is significant in describing the same relationship for 25°C-acclimated *Daphnia* (p = 4.00E-04, Table 3, Figure 3). Clone type was included as a random effect in the model for either acclimation temperature but had no significant effect on the regressions. All 10°C-acclimated clones were killed during or dead shortly after exposure to 35°C. To be conservative, weights were measured immediately after the respiration measurement only in *Daphnia* still moving or dead, but not already decaying/bloating.

Table 3. *Fixed Effects for Polynomial Regressions at 2 Acclimation Temperatures.* AssayT is the fixed linear parameter for assay temperature, while assayT*assayT represents a nonlinear parameter. Bolded F and p terms indicate significance at an $\alpha = 0.05$ confidence interval. Random Variation associated with clones is not different from zero.

	10˚C Acclimation			**25˚C Acclimation**		
Source	Parameters	F Ratio	p	Parameters	F Ratio	p
assayT	1	**54.9913**	**1.00E-04**	1	**42.0613**	**1.00E-04**
assayT*assayT	1	0.2076	6.49E-01	1	**12.8884**	**4.00E-04**

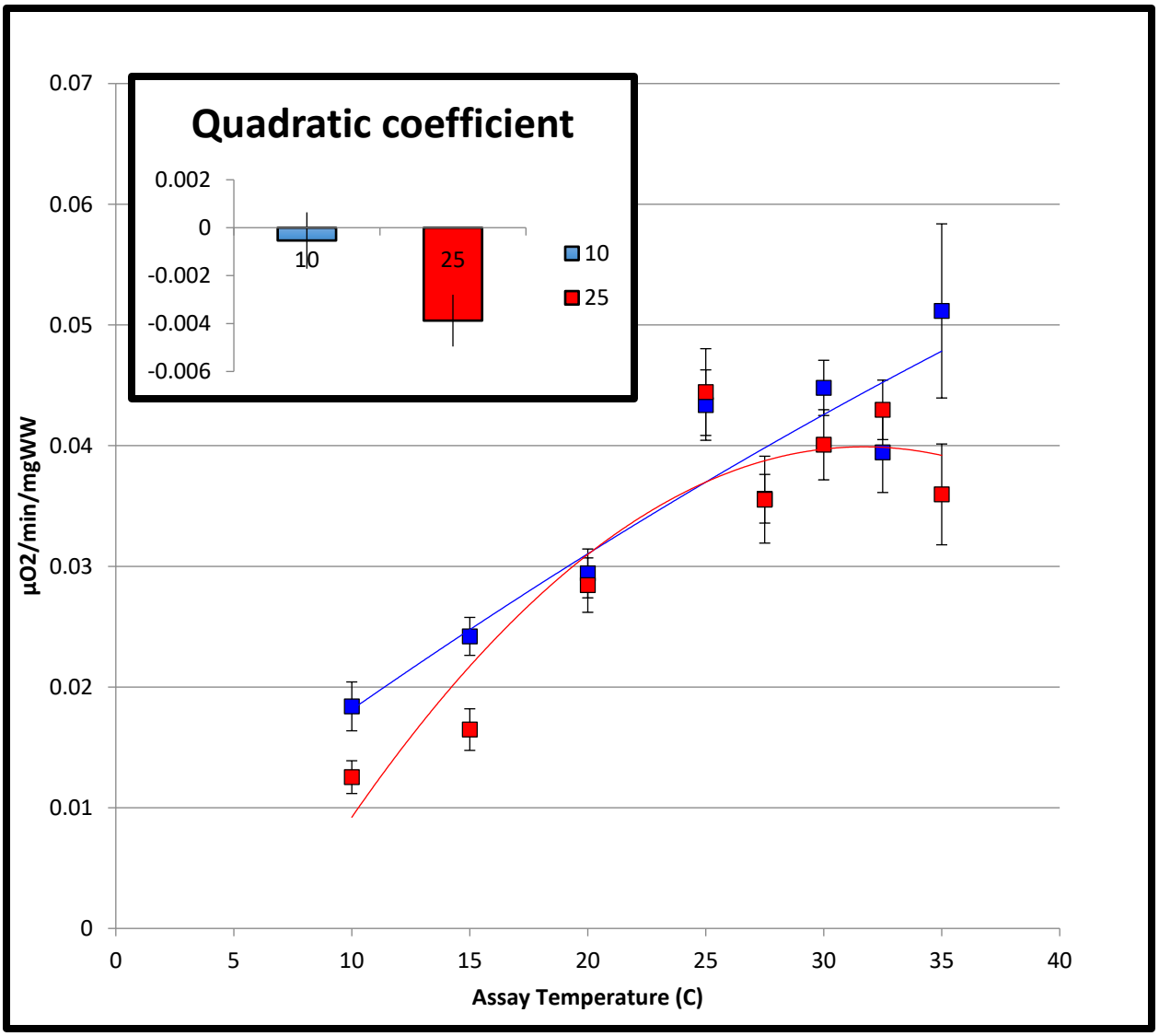

Figure 3. *Oxygen consumption during acute exposure to assay temperatures by acclimation temperature.* Blue squares represent mean oxygen consumption (µO2/min/mgWW) at each assay temperature for clones acclimated to 10°C. Red squares represent mean oxygen consumption in clones with high acute tolerance. The respectively colored trendlines represent the near-linear relationship between temperature and respiration for 10°C acclimation while the 2nd degree polynomial line represents the nonlinear relationship temperature and respiration for 25°C acclimation. The insert shows that the estimated quadratic coefficient is no different from 0 for 10°C acclimation but is nonzero for 25°C acclimation. Vertical bars represent standard error of the means.

Recovery Experiment

Recovery conditions (treatment) were a significant effect on oxygen consumption ($p = 1.30E-03$, Table 4) while, again, there was no effect of clones grouped by acute temperature tolerance. *Daphnia* reared at 25°C and exposed to 35°C for 1 hour exhibited lower respiration at 35°C than compared to unexposed Daphnia from 25°C or those allowed to recover for 24 hours at 25°C ($p = 6.00E-04$, Figure 4). Those allowed 24-hour recovery at 20°C exhibit an intermediate respiratory rate while, importantly, there was no difference between those allowed to recover at 25°C and those raised at 25°C without lethal temperature exposure (Figure 4).

Table 4. *Two-way ANOVA Model for Recovery of Respiratory Rates After Exposure to 35°C.* Treatment represents recovery conditions for 25°C-acclimated Daphnia following exposure to 35°C or normal conditions without exposure to 35°C. Ttolerance_acute is characterized acute temperature tolerance (high or low). Bold F and p values indicate significance at an $\alpha = 0.05$ confidence interval. Random Variation associated with clones is not different from zero.

Source	SS	F Ratio	p
Treatment	0.000161	6.4992	**1.30E-03**
Ttolerance_acute	1.24E-06	0.1495	0.70
Treatment*Ttolerance_acute	4.07E-06	0.1638	0.92
Error	0.000298		

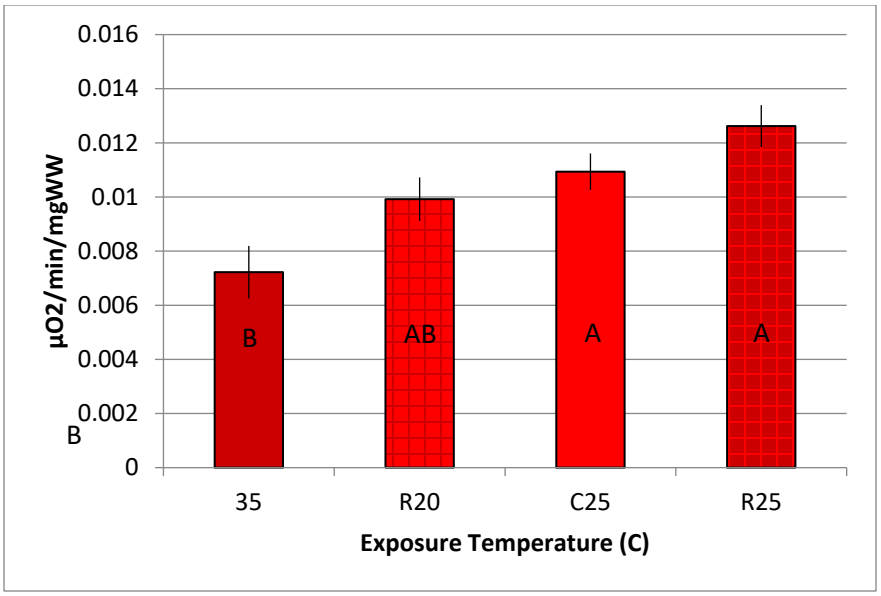

Figure 4. *Full oxygen consumption rate recovery following exposure to 35°C.* From left to right the columns represent oxygen consumption of 25°C -acclimated *Daphnia* at 35°C, 25°C following recovery from 35°C at 20°C, no exposure, or recovery at 25°C. Vertical bars represent standard error. Letters over the columns represent significant differences (at α=0.05) among treatment effects based on Tukey-Kramer HSD.

CHAPTER 4

DISCUSSION

Potential Evidence of Metabolic Compensation

In all but lifetime low temperature acclimation (10°C), *Daphnia* exhibit thermal performance curves indicative of metabolic compensation (Figures 2 and 3). As predicted for metabolic compensation, individuals acclimated to high vs. low temperature show different shape of and metabolic rate relationship with current assay temperatures across the thermal range. We see no evidence for different critical temperatures between 10°C- vs. 25°C-acclimated *Daphnia* (Figure 1 A, B, C). We do, however, observe a stronger curvilinearity of the reaction norm in the 25°C-acclimated individuals (Table 3, Figure 3), indicating a sustained metabolic rate over the 25-30°C subcritical range, over which the 10°C-acclimated *Daphnia* continue to increase their respiration rate with temperature. While the difference in curvilinearity between acclimation temperatures is not fully conclusive evidence for compensation, the high mortality of 10°C-acclimated *Daphnia* at 35°C and the high mortality in the 5°C shared history experiment at even lower temperatures suggests a benefit of 25°C-acclimation.

More characterization of routine metabolic rates (RMR) across temperatures in *Daphnia* will be needed to determine the exact aerobic scope of the organism (Schulte 2015). RMR alongside maximum metabolic rate data can be used to determine not only aerobic scope but whether maximum rates are oxygen-limited at higher temperatures and whether resting metabolic rates and maximal rates are under the same constraints (Schulte 2015). While it's likely that physiological processes of arthropods are more oxygen-limited at lethal temperatures (Giomi and Portner 2013; Verberk et al. 2016) even with the minor drop of oxygen saturation

experienced at 35°C, which could account for a drop in respiration at 35°C, 10°C-acclimated *Daphnia* had some of the highest respiratory rates in the experiment and those rates increase linearly with temperature (Figure 3). *Daphnia* raised at intermediate temperatures (15°C and 20°C) also show the same nonlinear response to gradual increasing temperature (Table 2, Figure 2). Given this result, timing of acclimation likely only influences the breadth of aerobic scope rather than shifting it toward a certain temperature range. Interestingly, respiration rates were around twice as high at all comparable measurement temperatures in the acclimation temperature than in the shared history experiments. Both shared history experiments were indeed separate experiments, yet both have respiratory rates in the same range. Perhaps acclimation to 10°C pushes for higher metabolic rates overall, while acclimation to 25°C primes metabolic rates to quickly adjust to changes in temperature.

Surprisingly, given the linear nature of their respiratory response, 10°C-acclimated *Daphnia* do not appear to compensate in the same way but in the opposite direction to 25°C-acclimated *Daphnia* (Figure 3). There is the possibility that if measurement range extended to lower than 10°C and that more resolution was available in the colder temperature range, a nonlinear response to cold temperature may have been found. However, measurement times for adequate resolution would require too much time within the range of temperatures tested already as the *Daphnia* would have died from aging before every temperature could be tested. Metabolic cold adaptation predicts higher respiration in organisms from cold climates and this is often the case (White et al. 2012). In fact, 10°C-acclimated *Daphnia* appear to have higher oxygen consumption rates in temperatures below 20°C than their high temperature-acclimated counterparts (Figure 3). Perhaps gene regulation differences in clones locally adapted to cold environments account for this difference (Yampolsky 2014). However, acclimation was a

stronger effect because there were no significant effects of clones in the model, regardless of habitat, suggesting that adjustment of respiratory rates to temperature is not experiencing selective pressure.

No Evidence for Genetic Variation or Local Adaptation

All experiments in this study show a curvilinear response to increasing temperature depending on acclimation history. This response is indicative, but not unequivocal proof of metabolic compensation. The study also finds that, at least for metabolic compensation of respiratory rates, there is no effect of genotype grouped by acute temperature tolerance (Table 2). Metabolic compensation of respiratory rates appears to be plastic, i.e., not canalized and not under the influence of selection. This is surprising, given the continuous focus on temperature adaptation in freshwater poikilotherms. There is of course strong evidence for adaptation to thermal environments. (Johnston & Walesby 1977; White et al. 2012; Geerts et al. 2015). As already noted, locally adapted genotypes of *Daphnia* have differing acute thermal tolerance and highly differential gene expression during exposure to stressful temperatures (Yampolsky 2014), yet, metabolic compensation effect appears to be constrained. One may hypothesize that there are multiple pathways involved in response to thermal stress and some pathways for at least some organisms will not be found by testing for selection because they are not adaptive as there is nothing for selection to act on yet.

Perhaps studies that see a lack of adaptation to thermal environment are either byproducts of much larger system-wide physiological shifts which are adaptive, or perhaps the trait of interest is integral but not under selection, as is evidenced by this study. One study suggests that some bivalves do not exhibit metabolic compensation because the benefits simply do not outweigh the costs of thermal compensation of biological rates (Lurman et al. 2014).

Given the findings presented here, however, evidence needs to be produced that there is indeed a fitness cost associated with metabolic compensation and that there is something for selection to act upon, which is sometimes the case (Williams et al. 2016).

Understanding how overall metabolic systems react to temperature changes is likely necessary to understand the scope of metabolic compensation (Ruoff et al. 2007). Understanding the system can help link adaptive responses to fully plastic ones. In fact, selection for a seemingly metabolically costly trait, like antibiotic resistance, can mobilize and restructure entire metabolic networks in unexpected ways (Handel et al. 2013). There is also evidence that metabolic rate robustness to fluctuation is important for adaptive circadian systems (Johnson & Egli 2014). Since the potential metabolic compensation reported here appears to be genetically constrained, it is possible that the mechanism relies on highly conserved, important pathways that are essentially fixed.

Alternatively, the patterns of plasticity reported here are not beneficial at all and a really only signs of physiological dysfunction with increasing temperature. This alternative would easily explain the lack of an effect by local adaptation because respiratory dysfunction would simply not be adaptive. More work is needed to determine whether the response to ambient temperature seen here is truly metabolic compensation, so alternative plastic pathways that could additionally explain the lack of genetic variation in oxygen consumption with increasing temperature are discussed later.

Mitochondria and Membrane Regulation

Mechanistically, metabolic compensation in *Daphnia* makes sense when coupled with information and evidence of membrane restructuring with temperature changes. Membrane

restructuring and fluidity adjustment occurs during temperature acclimation in *Daphnia*, and a diet high in PUFAs reduces their thermal tolerance (Coggins et al. 2017; Martin-Creuzburg et al. unpublished data). While no explicit respiratory damage is shown here (Figure 4), and lipid peroxidation is not predictive of thermal tolerance in *Daphnia,* lipids are major signalers involved in heat stress management and heat shock response (Balogh et al. 2013; Torok et al. 2013). Modification of membrane lipids is likely a major induction pathway for generalized temperature responses, and membrane lipids significantly influence transient receptor potential channels (Balogh et al. 2013; Torok et al. 2013). Similar modulation probably occurs across the mitochondrial membrane, directly influencing energetic output, but not by means of explicit mitochondrial damage. Mitochondrial proton leaks which alter membrane potential and energetic output can in fact be adjusted by temperature response (Guderley & St-Pierre 2002).

A seminal study by Labbadia et al. shows that the repression of heat shock response is a programmed event in aging that can be prevented by mild mitochondrial perturbation which drastically improves acute heat and stress response even in old age (2017). Furthermore, mitochondrial membrane function has a direct relationship with HSF-1, so perhaps thermal plasticity arises from induction of HSF-1 and downstream perturbance of mitochondrial potential such as deliberate proton leaks to uncouple oxidative phosphorylation in stressful temperatures. Better understanding of this pathway will likely lead to a better understanding of plastic metabolic compensation to temperature.

Alternative Plastic Responses to Temperature

While all experiments conducted in this study show a reduction in respiration at the onset of high temperatures without a loss of respiratory control, the mechanisms at play are poorly understood. Metabolic compensation suggests that a reduction in oxygen consumption with

increasing temperatures will be caused by beneficial physiological changes in the organism. However, as suggestive for compensation as these data are, this study was not a mechanistic investigation, so compensation is not yet a forgone conclusion. Below two alternative, but not mutually exclusive, mechanisms are discussed that may generate the same effect on oxygen consumption with increasing temperature in a passively plastic and not necessarily beneficial manner.

Changes in membrane structure and fluidity happen in concurrence with changing temperature, and these changes also differ by acclimation temperature (Coggins et al. 2017; Martin-Creuzburg et al. unpublished data). Furthermore, oversaturation of PUFAs in acclimated *Daphnia* reduces survival in lethal temperatures. Time to immobilization (T_{imm}), or the time in which *Daphnia* can no longer swim, decreases with a diet supplemented with high levels of the essential fatty acid eicosapentaenoic acid (Figure 5; Martin-Creuzburg et al. unpublished data). Perhaps any metabolic regulation *Daphnia* is capable of is explained by the membrane pacemaker theory of metabolism (Hulbert & Else 2005) in which membrane potential and subsequent metabolic rates are directly dictated by membrane structure, which also changes with temperature. Lethal temperature thresholds may coincide with irreparably damaged membranes.

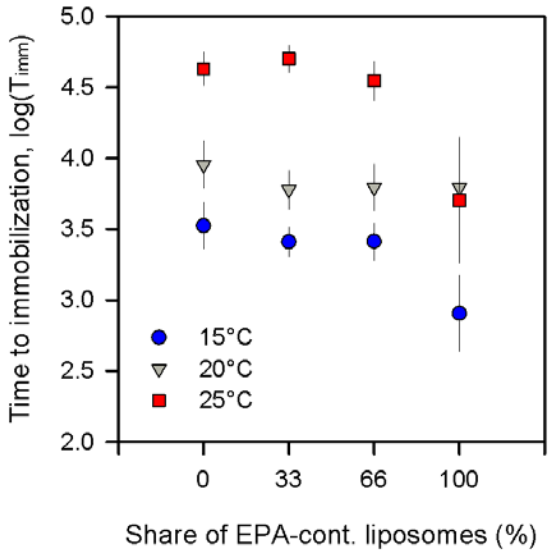

Figure 5. *Effects of acclimation temperature and dietary EPA supply on acute high temperature tolerance.* The x-axis is the dietary ratio of EPA-containing liposomes. Temperature tolerance is represented as time to immobilization at 37°C (log-transformed Timm). Vertical bars represent standard error.

Hypoxia, like membrane fluidity and structure, is innately tied to the aquatic thermal environment because dissolved oxygen content in water is dependent on temperature. Hypoxic responses often include downregulation of cellular respiration to meet energy demands (Michiels 2004). Hypoxia inducible factor (HIF) levels increase in the absence of its antagonist which requires molecular oxygen to activate, so perhaps mitochondrial activity is sensitive to even small changes in HIF, in which case increasing temperature could result in downregulation of metabolism. It is possible that even the relatively minor drop in dissolved oxygen saturation at 37°C is enough to limit full aerobic scope of an organism as predicted by the oxygen and capacity limitation of thermal tolerance (Verbec et al. 2016). However, short term exposure (90 minutes) to hypoxia (>50% DO) increases T_{imm} only in 28°C acclimated *Daphnia* (Figure 6;

Coggins et al. 2017), so perhaps HIF-induced cascades and potential subsequent metabolic regulation are more prominent in heat-acclimated *Daphnia*.

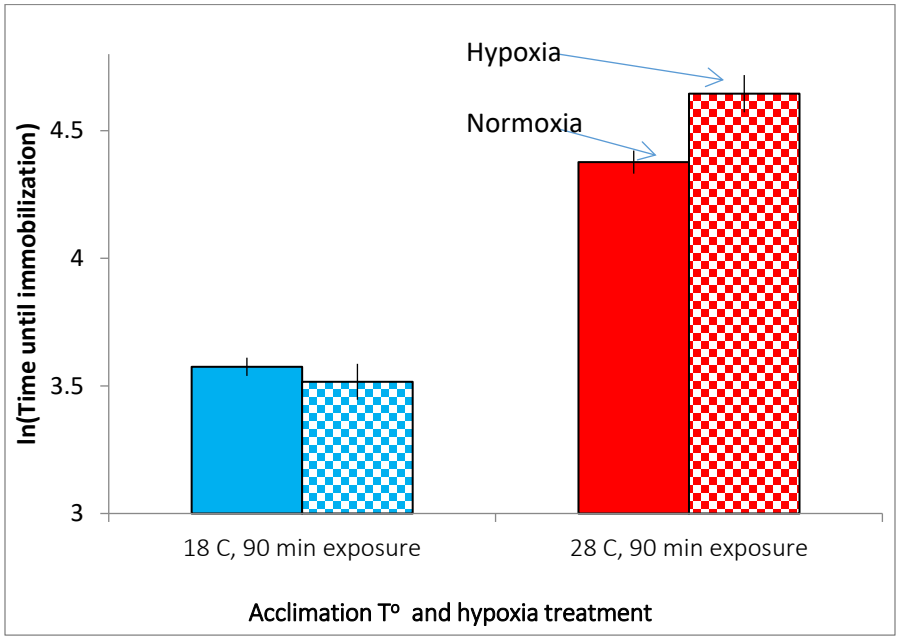

Figure 6. *Effects of acclimation temperature and exposure to hypoxia on time to immobilization at 37°C.* Acclimation temperatures are 18°C (blue) and 28°C (red). Exposure to hypoxia (<50%DO) occurred for 90 minutes in the checked blue and red bars. Time to immobilization was natural log-transformed. Vertical bars represent standard error.

Membrane fluidity mechanics and sensitive responses to oxygen content in habitats could potentially generate the same respiratory responses evident for metabolic compensation, but a key distinction is determining how much respiratory damage is occurring with exposure to high temperatures. If membrane fluidity regulation is the sole mechanic of respiratory response to high temperature, there will be a point in which membranes simply irreparably melt. Likewise, hypoxic responses can only be sustained for so long before irreparable oxidative stress is incurred, perhaps by the onset and buildup of anaerobic products.

Suggested Future Work

An immediate follow-up to this study should simply investigate at respiration in samples throughout the process of acclimation. To show more evidence of metabolic compensation in the case of high temperature acclimation, *Daphnia* acclimated over their lifetime to 20°C then placed in higher temperature (25°C-30°C) should initially have higher respiratory rates that will decelerate with acclimation to the new temperature. Cold acclimation (5C-10C) should initially show lower respiratory rates that increase with acclimation.

The recovery experiment should be repeated but with a focus on ATP and lactate production during and after exposure. This will give more insight into whether respiratory compensation is occurring by uncoupling activity in mitochondria. A potential way to investigate the relationship between degrees of lipid unsaturation, mitochondrial function, and metabolic compensation would be to again link a respiratory rate recovery experiment to mitochondrial output, but with the additional treatment of a diet high in PUFAs. If the addition of PUFAs prevents recovery, more evidence exists regarding the importance of membrane regulation and its role in both thermal tolerance and modulation of mitochondrial function. Testing the effect of direct perturbance of mitochondrial membrane potential on temperature tolerance will also be useful. Mild mitochondrial toxins may yet be another useful treatment in a future respiratory rate recovery experiment. Manipulation of HSF pathways directly by RNAi or indirectly by induction of other pathways (like hypoxic response) will be useful to determine if metabolic compensation occurs within conserved pathways.

Finally, characterization of aerobic scope is promising find further evidence for metabolic compensation. Different physiological traits may have different thermal optima and critical temperatures (Clarke et al. 2013;Jayasudara and Somero 2013). Characterizing thermal

performance of antennae beats and heart rates in addition to respiration can help further show potential compensatory responses. *Daphnia* are known to exhibit temporary antennae paralysis to urethane, so basal respiratory rates in the absence of locomotion can also be investigated. Characterization of lactate production in different assay temperatures after different acclimation regiments or as a recovery experiment will be useful because *Daphnia* show increased lactate production just before reaching their critical temperature (Verberk et al. 2016).

Conclusion

This study demonstrates that metabolic compensation of respiratory rates at high temperature potentially occurs in *Daphnia* acclimated to high, but not low temperature. Reduction of metabolic rate at the critical (35C) temperature is recoverable, possibly indicating that it represents adaptive plasticity and not higher thermal damage. Despite sampling from geographically distinct habitats and including genotypes known to be different in their temperature tolerance, the observed metabolic compensation seems to be genetically constrained, indicating that it may play a role in plastic response, but not in local adaptation to high temperature. Alternately, this study outlined how acclimation can shape the onset of physiological dysfunction in *Daphnia* as temperatures increase. More work will be needed to further characterize this potentially acclimation-dependent compensatory response to high temperatures, but a foundation for such work has been established here.

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