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Genetic Variation in Long-Term and Short-Term Physiological Changes in Daphnia magna During Acclimation to High Temperature

bret l. coggins
East Tennessee State University

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By

Bret L. Coggins

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Bret L. Coggins	Date
Dr. Lev Yampolsky, Thesis Mentor	Date
Dr. Thomas Laughlin, Reader	Date
Dr. Aruna Kila	Date
Dr. Aleksey Vasiliev, Reader	Date

ABSTRACT

The aquatic zooplankton crustacean *Daphnia magna* must be able to tolerate thermal stress in order to survive their native shallow ponds that are susceptible to drastic seasonal and diurnal temperature fluctuations as well as to globally increasing temperatures. Survival in such variable environments requires plastic responses that must include fundamental aspects of *Daphnia* biochemistry and physiology. Adaptive response to selection favoring such plastic phenotypes requires the presence of genetic variation for plastic response in natural populations. Adverse effects of elevated temperature on aquatic organisms are diverse and so are their plastic responses; among the most severe challenges aquatic organisms face when exposed to heat is the elevated oxidative stress. In this work we focused on short-term and long-term responses of *Daphnia* to temperature changes that increase its resistance to oxidative stress.

Daphnia acclimated to stressful but non-lethal temperature (28ºC) show longer survive during exposure to a lethal temperature (37ºC) than those acclimated to the optimal temperature (18ºC). Short-term reciprocal switches between 18ºC and 28ºC result in intermediate temperature tolerance. These changes are accompanied by mirroring changes in total antioxidant capacity indicating the increased antioxidant capacity as a possible causative mechanism for heat tolerance gained from acclimation.

The analysis of 6 geographically distinct genotypes representing a range of temperature tolerance levels shows a genetic difference in response to short-term and long-term acclimation as well as in the effect of antioxidant capacity on temperature tolerance. These results indicate a significant degree of local adaptation in heat and oxidative stress defenses in *Daphnia* and provide a better understanding of adaptive responses of this zooplankton crustacean to rising temperatures.

INTRODUCTION

An important component for an organism's survival in a dynamic environment is an equally dynamic method of response. A particularly important constraint for all ecological systems is temperature, which can effectively dictate the ranges of various populations of organisms. This abiotic factor is especially a concern if global temperature trends continue to increase. Under the selective pressure of high temperature, which in aquatic environments also entails less oxygen saturation, organisms must exhibit some immediate plastic or long-term evolutionary response to avoid extinction. Plastic and evolutionary responses aren't necessarily mutually exclusive, however, because some predisposed genotypes might simply withstand temperature more than others independent of plastic response, if it indeed occurs.

Fundamentally, high temperature can be damaging due to increased production and particle movement of reactive oxygen species (ROS) which can pass the capacity of an organism's antioxidant systems leading to oxidative stress, an imbalance between increased metabolic demand versus lower oxygen availability, the liquefying of cellular membranes, or denaturation of proteins. Likely, all these issues are present in tandem, but organisms that can overcome some of these factors will be more tolerant of higher temperatures, if such factors are responsible for the ill effects of high temperature. For example, if an organism responds to ROS by increasing the production of antioxidants, it should tolerate higher temperatures (Becker et al. 2011).

Another common way to quantify oxidative stress is to examine lipid peroxidation (LPO) specifically because free radicals often attack polyunsaturated fatty acids which signals a cascade of cellular response, but is also cytotoxic (Ayala et al. 2014). This is supportive of the hypothesis that LPO is explicitly a measure of damage, so as stress caused by LPO increases, organismal adaptation decreases. In fact, there are highly specific HPLC assays to check for LPO as a form of damage (Oexle et al. 2016). There are alternative views of the roles LPO, however. Another common idea is that LPO are homeostatically maintained in the sense that stress and adaptation are always relatively equal. LPO levels and antioxidant activity appear to be in a consistent positive feedback mechanism throughout the normal aging process (Rodriguez-Martinez & Ruiz-Torres, 1992). Another alternative is that LPO actually benefits the organism in the sense that as stress increases, adaptability also increases. Specifically, the formation of intermediates for lipid peroxidation such as Malondialdehyde, 4-hydroxyalkenals, and other reactive electrophile species, are important for healthy cell functioning (Farmer & Mueller, 2013; Cohen et al. 2013). LPO may also be important for ion homeostasis during neuronal degeneration (Mattson, 1998). Despite being a common quantification of oxidative stress, lipid peroxidation is variable dependent upon the type of stimulus and isn't the only form of oxidative stress (Barata et al. 2005). While either

antioxidant capacity or lipid peroxidation can be used to study oxidative stress, using both in tandem can provide a clearer picture. For example, it is necessary to consider both these aspects when studying aging (Ortiz-Rodriguez & Wiegand, 2010). Specifically, when regarding the different roles LPO appear to have in stress response, having other means to measure damage may be quite necessary to fully understand the temperature tolerance mechanism. Despite the wealth of biochemically-focused studies crediting the signaling and maintenance potential of LPO, most ecological studies place negative emphasis on LPO. This appears to be an unfortunate discrepancy between specializations approaching the same components from unnecessarily dissociated backgrounds.

The zooplankton *Daphnia* is an interesting model system for thermal tolerance because of its physiological plasticity to changing temperatures. Given that the organisms have short generation times and exhibit cyclic parthenogenesis, sample sizes are large and variability is more controllable. The genome of *Daphnia magna* and *Daphnia pulex* has been sequenced, providing yet another layer of control (Colbourne et al. 2011). Some species are known to regulate temperature behaviorally as a response to levels of haemoglobin (Wiggins & Frappell, 2002). Haemoglobin itself is an antioxidant mechanism that can extend the range of many invertebrates (Weber & Vinogradov, 2001). In addition to molecular antioxidant measures, *Daphnia* ventilate themselves through constant appendage movement, which is variable with immediate oxygen conditions (Pirow & Buchen, 2003).

Most importantly, *Daphnia* are able to acclimate over time to higher temperatures, which increases their survival time to a lethal temperature. This acclimation effect involves of haemoglobin expression, but other antioxidant mechanisms likely play a role too (Williams et al. 2011). A common hypothesis for this phenomenon is based on oxygen limitation on a thermal gradient (Paul et al. 2004). Basing acclimation on oxygen limitations in regards to temperature poses some interesting dynamics such as reactions to ROS and viewing hypoxia as the limiting factor rather than temperature. In fact, some haemoglobin is induced by hypoxia, a low environmental concentration of oxygen, which is subsequently tied to temperature increase (Zeis et al. 2013). Of course, hypoxia induces other forms of antioxidant enzyme response like superoxide dismutase(SOD), catalase(CAT), and Glutathione-S-transferase(GST) (Trubenbach et al. 2012). Glutathione activity is especially useful as an assay of organismal oxidative stress. (Doyotte et al. 1997).

The phenotypic plasticity for temperature, or oxygen content, displayed by *Daphnia* can manifest in many interesting ways. One such way that the phenotype is flexible is, intuitively, its regulation of antioxidant molecules, like haemoglobin (Yampolsky et al. 2014). This response, again, is not limited to one particular type of antioxidant system because enzymes like glutathione and catalase are regulated too (Becker et al. 2011). Phenotypic changes in response to temperature are not limited to just molecular

response, however, as *Daphnia* size decreases and development speeds up in response to high temperature (Chopelet et al. 2008). Ultimately, a major constraint on organismal development in high temperatures is oxidative stress. The trade-off to survive this constraint is variable phenotypic response (Metcalfe & Alonso-Alvarez, 2010).

Daphnia magna are particularly suited for thermal tolerance studies because they inhabit small, shallow ponds across an expansive continental range that are susceptible to rapid and severe changes in temperature. Geographically distinct clones have different capacities for stress, depending on the environment they inhabit, but most have a capacity to acclimate. In fact, *Daphnia's* plastic response to temperatures that consistently rise each year may be causing rapid evolution for higher temperature tolerance (Geerts et al. 2015). A competition study with thermal stress as a variable indicates that population dynamics are an influential mechanism for the continued rapid evolution of thermal tolerance (Doorslaer et al. 2009). Essentially, plastic response can enhance genetic variability and aid in adaptation, if it is aligned with the direction of selection and compounded within succeeding examples of plasticity (Lind et al. 2015). This interaction has been further emphasized by the comparison of modern temperature tolerance data to data from 1973 (Henning-Lucass et al. 2016).

In short, organisms can exhibit flexible phenotypes in response to abiotic factors in their immediate environment, such as temperature. For temperature specifically, an organism's spectrum of response may include behavioral regulation, membrane bilayer restructuring, and antioxidant mechanisms, among other reactions. Antioxidant capacity is of particular interest due to the nature of ROS in higher temperatures. Given the high interest in antioxidant capacity, it is easy to find protocols for assaying antioxidants for experiments. Glutathione is even more specifically appealing as a point of focus because it is critical to many oxidative pathways. Its activity, along with that of other antioxidants, can be further induced by hypoxia, which is easy to simulate in a laboratory setting. Further, glutathione can be specifically inhibited, adding a separate layer of experimental control. *Daphnia magna* are exceptional models for plasticity simply by exhibiting plastic response to abiotic factors coupled with their ease of maintenance and controlled clonal replication. *Daphnia* are made even more interesting as models because different clones are geographically distinct and face different temperature ranges which leads to different innate capacities to respond to thermal stress. Essentially, they are manageable for physiological studies, but also viable for considering how evolutionary mechanisms interplay with an organism's proximal interactions with its environment.

One way other studies have not yet approached identifying the mechanism for the phenotypic plasticity of *Daphnia* to thermal stress is by comparing what kinds of physiological changes occur within a short-term acclimation (4 days: a typical molt cycle) versus a long-term acclimation to high temperature

for 2 generations. The benefit innate to this approach is that it provides some insight into how quickly *Daphnia* can actually acclimate, and any correlated physiological changes that happen with long-term acclimation only may be more easily distinguished as potential causative responses for acclimation. Specifically, we have studied how lipid peroxidation as well as total antioxidant capacity changes between 6 different geographically distinct clones of *Daphnia magna* as previously used by Yampolsky et al. (Table 1; 2014) each raised at either 18ºC and 28ºC with or without a short-term switch to the opposite temperature for a 4-day period. We hypothesize that possible causative mechanisms are those that change along the same patterns as both long-term acclimation and short-term. If lipid peroxidation is causative for tolerance, it will mirror long term and short-term changes during acclimation. The same can be said about total oxidation. Given the focus on oxidative stress, hypoxia is included as a separate experimental factor to induce antioxidant response. Specifically, hypoxia is known to up-regulate glutathione synthesis in *Daphnia* (Becker et al., 2011). Additionally, individual levels of glutathione were more directly manipulated by increasing GH or inhibiting its synthesis with buthionine sulphoximine (BSO). If antioxidant capacity is critical to organismal success in higher temperatures, then increasing glutathione levels should increase temperature tolerance, while inhibiting glutathione should eliminate or reduce any tolerance. If this hypothesis is correct, we expect to see differential response to glutathione manipulations similar to the effect of short-term exposure to hypoxia.

We are particularly interested in the analysis of genotype by environment interactions of the 6 clones because there is little data on genetic variation in the physiological responses to environmental changes affecting temperature tolerance. In particular, we will test the hypothesis that the observed clone-specific effects on total oxidation capacity and temperature tolerance are related to clone's differential ability to synthesize or utilize glutathione.

METHODS

Daphnia magna

Six different clones of *Daphnia magna* were used throughout the study. These clones were chosen to represent a wide geographic and thermal tolerance range (Yampolsky et al. 2014). Their locations of origin are listed in Table 1. The *Daphnia* were kept in 200 mL flasks filled with artificial pond water COMBO medium (Kilham et al. 1998) and fed using a *Scenedesmous* algal culture with added vitamins and micronutrients to an algal-cell density of 100,000. The *Daphnia* cultures were fed every other day and the medium was changed every 4 days.

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

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

Acclimation

Individuals from each of the 6 *Daphnia magna* clones were separated into two different temperature treatment groups, unacclimated and acclimated, and placed into corresponding incubators. The unacclimated *Daphnia* were kept at 18ºC, while the acclimated *Daphnia* were kept at 28ºC. *Daphnia* were kept and maintained in these conditions for 2 generations, in COMBO water medium with a consistent light-dark cycle. *Daphnia* were kept in 200 mL plastic tapered flasks with no more than 5 adults of the same clone type per flask. Juveniles were kept at a density of 10 individuals per flask. Individual flasks were fed and maintained as described above.

Thermal Tolerance

After the 2 generation or 4-day acclimation period, individuals from each clone in the temperature treatments were place in separate 50 mL vials, containing COMBO water at their acclimation temperature. For each sample, an unexposed individual from the same group was put aside as a control for further assays. The vial's IDs were recorded specifically on paper, but were directly labeled 1-54 on the vials. The original IDs were then covered to avoid any bias. Additionally, vial placement in the chamber for a 37^{O}C water bath were randomized to avoid any bias for potentially hotter regions in the bath. The vials were almost completely submerged into the water bath. Once the *Daphnia* were in the water bath, there is a ramp-up period to 37^{O}C that takes roughly 10 minutes, at which point the temperature is kept stable. They were continuously monitored from the time the targeted temperature was reached until they became immobilized and that time was recorded (T_{imm}). Immobilization was considered to be when the *Daphnia* were unable to lift themselves. Once they were immobilized and had spent a minimum of 60 minutes in the bath, they were removed and their body size and clutch size were measured and they were checked for a heartbeat. The samples are then saved for downstream assays.

Switches (Short Term Acclimation)

To emulate a short term acclimation, individuals from both temperature groups were either upshifted or downshifted to the opposite group for a period of 4 days, which is around a typical molt cycle. Individuals were switched into corresponding temperatures by simply keeping them in their native flasks and temperatures and reciprocally placing them in the opposite incubators, allowing the temperature to increase or decrease over a period rather than risking shock from immediate submergence into the new temperature. Otherwise, the switched individuals received the same treatment as all other individuals in assays.

Hypoxia

A hypoxic environment was simulated by continuously boiling combo water, until it was heavily depleted of oxygen, then carefully poured into 50 mL flasks, while still hot. Vials were filled to the brim and holes were drilled into the caps and fashioned with a 1000-µL pipet tip. The pipet tip is necessary to break the surface tension of the medium to prevent any oxygen-rich bubble formation. The pipet tip is filled, then a small microcentrifuge tube is fashion to seal the tip. Oxygen content was checked in an openly exposed beaker after a period of cooling because the O_2 probe in use could not fit inside the flasks and the temperature after boiling is much higher than the range of the probe itself. However, even after open exposure and cooling, average readings were around 5 mg, which is considered hypoxic. These vials are airtight and prepared a day in advance to allow sufficient cooling. Individuals are added to the vials by quickly removing the tip and placing an individual inside before replacing the tip and any displaced medium. Hypoxic vials were only used directly before the tolerance assay, and were either given no acclimation period or a short 90-minute period. After, individuals were exposed to the same water bath as described above. To avoid running bias, hypoxic vials were always run with normal ones in the assay.

Lipid Peroxidation and Total Oxidation

The Image-iT® kit was used to measure the lipid peroxidation in the *Daphnia*, using BODIPY 581/591 as the fluorescent reporter (Pap et al. 1999; Beretta et al. 2006). Samples were prepared as either unexposed controls or those that underwent the tolerance assay in some form. Samples were placed in reinforced Eppendorf® tubes filled with 100 µL of deionized water and small, uniform scoop of 0.15 mm cubic zirconium beads roughly 1/3 the biomass of a single adult *Daphnia*. The samples were then homogenized using a bead beater. Homogenized samples were placed in a black 96-well plate for use in a fluorescent plate reader. The oxidized lipids fluoresced at 528 nm and reduced at 590 nm. The plate is first measured without the sensor to get a calibration. Once the sensor is added, the plate was incubated for 30 minutes and measured as a ratio of oxidized-to-reduced sensor (R30m) and ran again, then incubated again for 24 hours (R24h) for total sample oxidation due to exposure to air. All incubations are kept in the dark to avoid degradation of the sensor. The measurement after the first 30 minutes measures the total amount of lipids in the sample and after 24 hours, total oxidation is reflected. This method is not as precise as a newly described HPLC method in a Oexle et al. study, but allows us to consider other quantifications of oxidation not possible with the specificity of HPLC (2016).

Glutathione and BSO

In order to quantify the role of glutathione pathway in temperature tolerance and antioxidant properties in Daphnia we attempted to manipulate the tissue concentration of glutathione by either maintaining Daphnia in the medium with glutathione or by blocking glutathione biosynthesis pathway by means of buthionine sulfoximine (BSO; Anderson 1998). We expect these manipulations to affect total oxidation, but not lipid peroxidation because glutathione pathway is more likely to protect water-soluble molecule than lipophilic substances specifically peroxidized by lipid peroxides. Glutathione (GH) supplementation experiment was conducting in Daphnia acclimated to 18ºC (in order to detect predicted increase in temperature tolerance; BSO inhibition experiments were conducted in 28ºC-accllimated Daphnia (in order to detect the predicted decrease in temperature tolerance). For the GH supplementation experiments either juveniles 3-4 days of age or adults 7-10 days of age were placed in 200 mL flasks containing COMBO medium with either 0.1 mM glutathione, or 0.05 mM or 0 (control) of glutathione. Ten juveniles or 5 adults per flask were used in this experiment. After 4 days of this exposure temperature tolerance and Image-iT® oxidation were measured as described above. For BSO inhibition juvenile Daphnia (<2 days of age) were placed in 30 mL glass vials (10 individuals in each) containing 1, 0.5, 0.2, 0.1 or 0 (control) mM BSO. Flasks were checked daily for mortality. At day 5 of the experiment the individuals (now pre-adults) were harvested and their temperature tolerance and Image-iT® oxidation was measured. Similarly, adult Daphnia 7-10 days of age were placed into 50 ml flasks (3 individuals in each) with either 0.5 mM, 0.1 mM or 0 BSO added and maintained in these conditions for 4 days prior to temperature tolerance and oxidation measurements. A survival curve was constructed to show the effect of BSO additions to samples (fig 1). Juvenile individuals were included in these experiments in order to detect possible effects of GH and BSO accumulation in tissues during somatic growth.

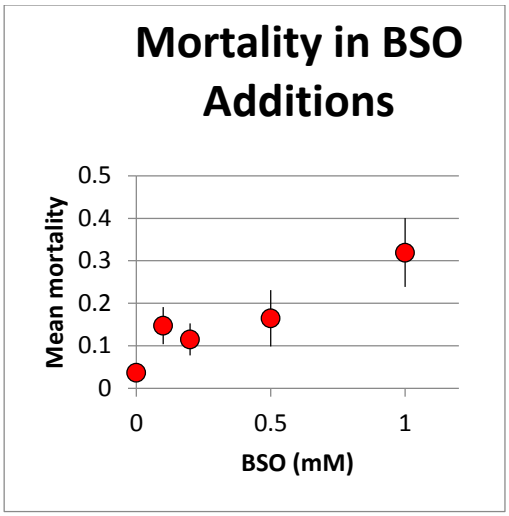

Figure 1. Increased mortality in higher concentrations of BSO.

Statistical Analysis

Experiments were analyzed as individual samples as a general linear model on JMP® software. The models were created using the traditional method of analysis. Clone type as well as dates of the experiments were treated as random effects and all interactions of clones with categorical effects were treated as random effects. Each source of variance was tested as an ANOVA with 95% confidence. Any probability for the F ratio less than 0.05 was considered significant.

RESULTS

To start, all tables following table 2 use the abbreviated headings listed in that table. Additionally, for the sake of space, source of variance is simply reduced to "source". To avoid any potential confusion, lipid peroxidation is an equal term to R30m, and total oxidation is equivalent to R24h. These terms are simply abbreviated forms of the assay time to determine either value. Total oxidation should not, however, be confused as an equivalent term to total antioxidant capacity because it is actually the inverse of total oxidation. Total oxidation is a raw value of the assay that was not converted for the purpose of reporting the results, but will be discussed later in an expanded capacity in terms of antioxidant defense.

Acclimation Temperature

As expected from the premise of this study, acclimation temperature was a very significant term for temperature tolerance at 37ºC (Table 2A, fig 2A). T_{imm} was also influenced by clone by acclimation temperature interaction. As notable on Table 2A, dates of tolerance tests were considered as a block effect for generalized T_{imm} data to account for any incongruities with the water bath, which was indeed significantly different. In contrast to the proposal that oxidation is an important component to an organism's thermal tolerance, acclimation temperature had no bearing on lipid peroxidation or total oxidation (Table 2B, 2C). However, the clone by acclimation temperature interaction becomes a significant difference for total oxidation (Table 2C, P=0.0067). Figure 2B makes this effect more visible as different clones have different ranges of total oxidation based on acclimation temperature.

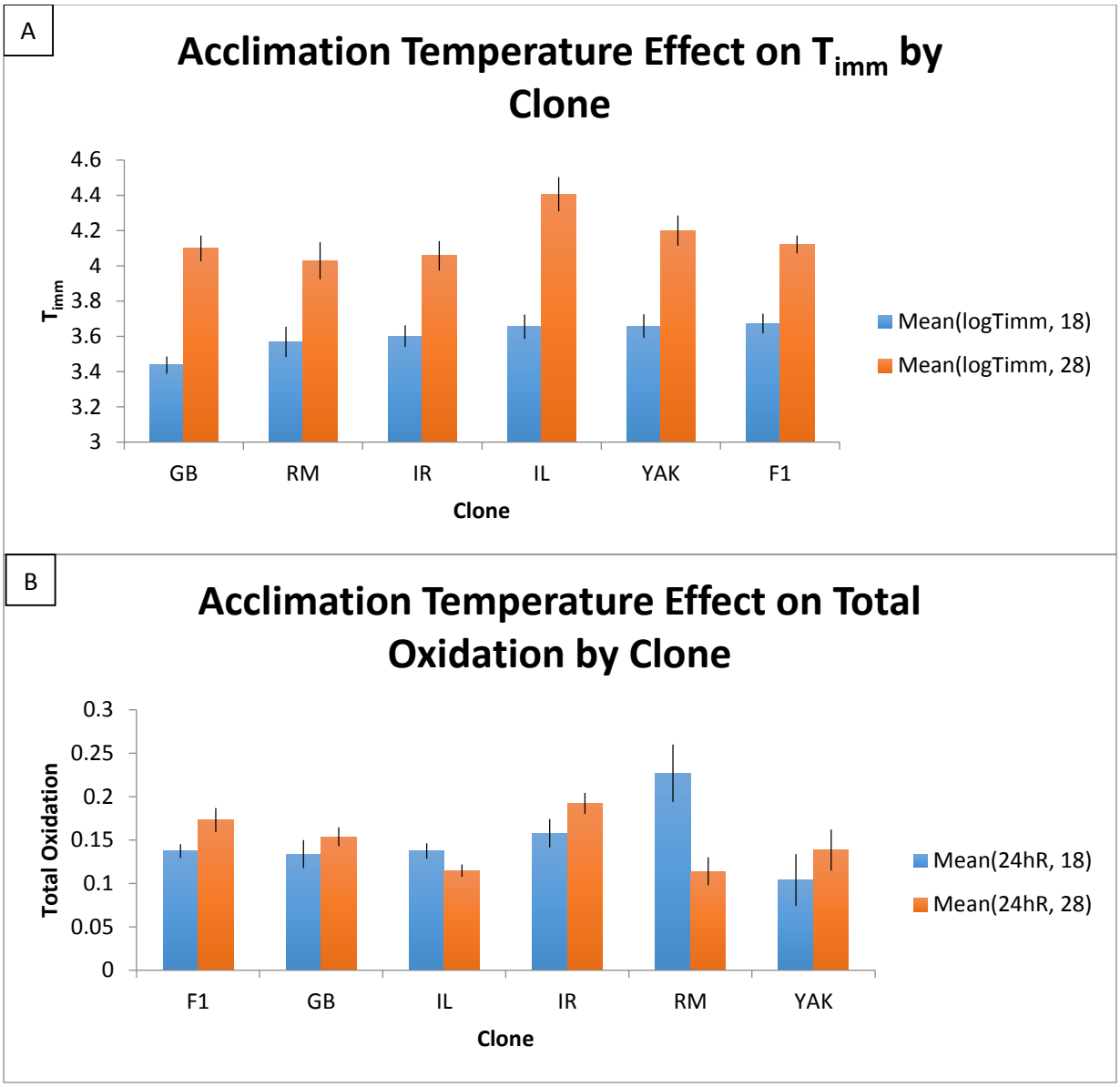

Figure 2. effect of acclimation temperature and clones on T_{imm} (A) and on total oxidation (B).

Table 2. Difference in acclimation temperature and clone interaction for time until immobilization (A), no effect of acclimation temperature for Lipid peroxidation (B), and effect of different clonal response to acclimation temperature on total oxidation (C).

A — T_{imm}

Source of variance	Mean Square (ms)	Degrees of freedom (df)	F ratio (F)	Probability (P)
AccT	21.860	1	134.44	**<.0001**
Clones	0.4400	5	2.34	0.19
AccT*clones	0.1900	5	2.43	**0.04**
Date(block)	1.5500	9	20.16	**<.0001**
Error	0.0771	264		

B — **LPO**

	No Exposure				**1h Exposure to 37ºC**			
Source	MS	df	F	P	MS	df	F	P
AccT	0.0003	1	0.10	0.76	0.0019	1	3.03	0.12
Clones	0.0010	5	0.36	0.86	0.0018	5	3.17	0.12
AccT*clones	0.0028	5	1.66	0.26	0.0006	5	0.46	0.80
Error	0.0017	7			0.0012	29		

C — **Total Oxidation**

	No Exposure				**1h Exposure to 37ºC**			
Source	MS	df	F	P	MS	df	F	P
AccT	0.0013	1	0.38	0.56	0.00003	1	0.01	0.94
Clones	0.0014	5	0.41	0.82	0.00308	5	0.67	0.67
AccT*clones	0.0034	5	1.13	0.43	0.00461	5	4.03	**0.0067**
Error	0.0030	7			0.00114	29		

Switches

After establishing a premise with long-term acclimation, focus shifted to a comparison of time allowed for acclimation. To reiterate, long-term acclimation is acclimation at 18ºC or 28ºC for multiple generations, whereas short-term acclimation is a 4-day switch to the opposite acclimation temperature. When examining the effects of these 4-day switches on temperature tolerance, either LPO or total oxidation was considered as a co-variable to either acclimation temperature (Table 3). With LPO as a co-variable to acclimation temperature, no term gives any significant difference to thermal tolerance. However, if total oxidation is considered instead, then switching, oxidation for 24 hours, and all possible 2-way and 3-way interactions, including those with clones, become significant terms for 28ºC-acclimated individuals only (Table 3). Conversely, no effect was found in the 18ºC-acclimated samples, or from clones alone at 28ºC. Figure 3 shows a notable increase in total oxidation after samples were moved from 28ºC to 18ºC for 4 days.

Table 3. The effect of 4-day switches on temperature tolerance in either 18- or 28ºC-acclimated *Daphnia* with either Lipid peroxidation (R30m) or total oxidation (R24h) as covariables.

		18ºC				**28ºC**			
	Source	MS	Df	F	P	MS	df	F	P
R24h	switch	0.0525	1	0.69	0.42	0.4062	1	10.91	**0.0193**
	clone	0.1159	4	1.59	0.33	0.1122	4	1.67	0.32
	24hR	0.0447	1	0.59	0.46	0.2463	1	24.51	**0.0011**
	switch*clone	0.0725	4	0.96	0.47	0.0672	4	6.68	**0.0115**
	switch*24hR	0.0451	1	0.59	0.46	0.3482	1	34.66	**0.0004**
	clone*24hR	0.03	4	0.39	0.81	0.1068	4	10.63	**0.0027**
	switch*clone*24hR	0.0342	4	0.45	0.77	0.0628	4	6.25	**0.0139**
	Error	0.0758	10			0.01	8		
R30m	switch	0.0283	1	0.44	0.52	0.0118	1	0.64	0.45
	30minR	0.0145	1	0.19	0.67	0.0133	1	0.74	0.41
	switch*30minR	0.0036	1	0.05	0.83	0.006	1	0.33	0.58
	clone*30minR	0.0405	4	0.54	0.71	0.0185	4	1.03	0.45
	switch*clone*30minR	0.0166	4	0.22	0.92	0.0627	4	3.49	0.06
	clone	0.1411	4	2.68	0.18	0.0282	4	0.55	0.71
	switch*clone	0.0528	4	0.69	0.61	0.0511	4	2.85	0.1
	error	0.0755	10			0.0179	8		

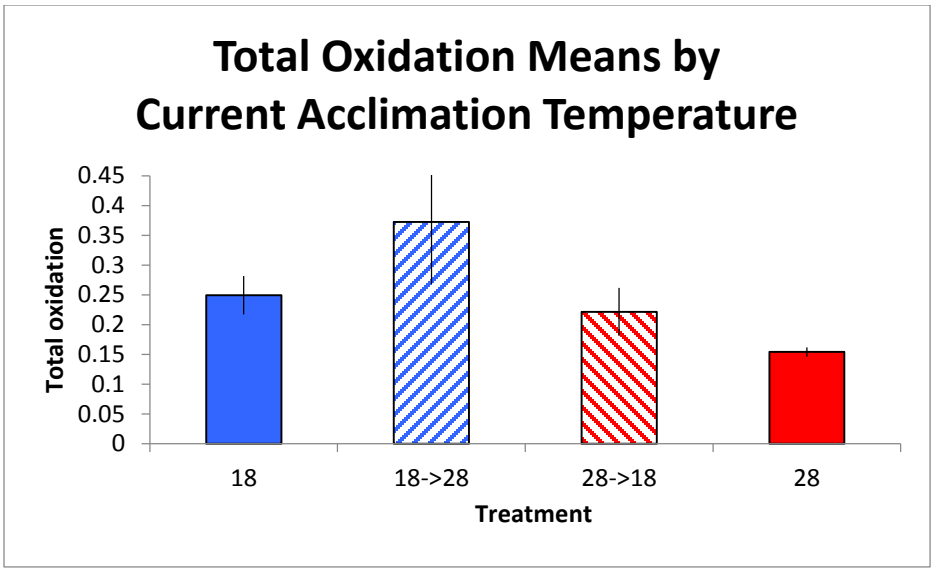

Figure 3. Total oxidation in short-term and long-term acclimation treatments.

Comparing switch data in regards to time until immobilization shows a trend of tolerance to high temperature being lost within 4-days of being switched to 18ºC, but not fully gained by switching to 28ºC within the same amount of time (fig 4A). After performing a regression analysis of the short-term and long-term acclimations by T_{imm} and total oxidation, there is a suggestive heterogeneity of slopes between consistently acclimated individuals at 28ºC and those that were switched down to 18ºC (fig 4B), while no such trend exists for individuals that started in 18ºC (fig 4C). However, the correlation wasn't quite significant (P=0.09), so a larger sample size would be necessary to confirm this relationship at 28ºC.

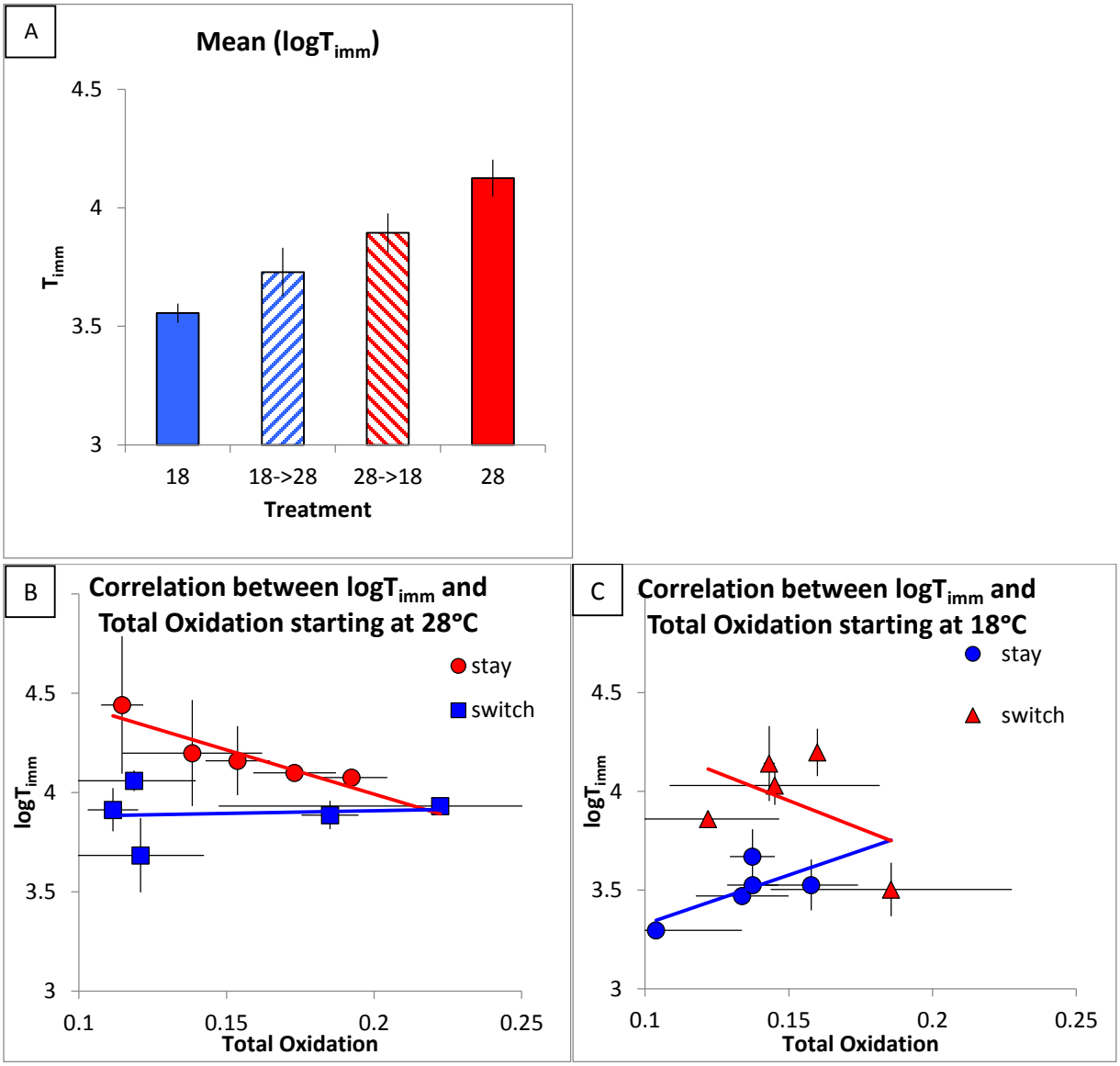

Figure 4. Effect of long-term and short-term acclimations on temperature tolerance (A), heterogeneity of slopes in individuals whose acclimation started at 28ºC fit to T_{imm} and total oxidation (B), but a less clear effect in 18ºC (C).

Hypoxia

Hypoxia trials included exposure to hypoxic conditions for 90 minutes prior to exposure to 37ºC and created a significant difference in survival for those acclimated at 28ºC (Table 4). While clones were a significant term for temperature tolerance, there does not appear to be any difference in their response to hypoxia (Table 4).

Table 4. Effect of exposure to hypoxia for 90 minutes on temperature tolerance.

	18ºC				**28ºC**			
Source	MS	df	F	P	MS	df	F	P
Hypoxia	0.0057	1	0.03	0.86	0.7575	1	26.42	**0.0014**
Clone	0.1088	5	0.63	0.69	0.3147	5	12.68	**0.0072**
Hypoxia*Clone	0.1723	5	1.59	0.19	0.0248	5	0.09	0.99
error	0.1079	36			0.2679	35		

GH and BSO

Following the indirect manipulation of antioxidants, we directly enhanced or inhibited GH concentrations in *Daphnia* adults and juveniles. As with hypoxia, clones were a significant source of difference for T_{imm} in adults and juveniles (Table 5). There was no effect of clone and GH/BSO exposure in any case nor was there any difference among clones' on T_{imm} after exposure to BSO (though this could be due to higher mortality; fig 1). Exposure to any amount of GH or BSO had no effect on T_{imm} (Table 5, fig 5). There is a consistent effect of BSO additions having much higher T_{imm}, but this is an artefact of separate experiments in which GH-exposed *Daphnia* were kept at 18ºC (in the hopes of enhancing temperature tolerance without acclimation) and BSO-exposed *Daphnia* were kept at 28ºC (to lower tolerance despite prior acclimation).

Table 5. Effect of added glutathione or buthionine sulfoximine on time until immobilization in *Daphnia* adults (A) and juveniles (B).

A					
Adults	**GH**				
	Source	MS	df	F	P
	GHmM	0.0117	2	0.30	0.7455
	clone	0.1303	5	3.38	**0.0441**
	GHmM*clone	0.0383	10	0.82	0.6104
	error	0.0466	30		
	BSO				
	Source	MS	df	F	P
	BSO>0	3E-07	1	0	0.9989
	clone	0.2462	5	1.58	0.3146
	BSO>0*clone	0.1561	5	1.29	0.2896
	error	0.1212	38		

B					
Juveniles	**GH**				
	Source	MS	df	F	P
	GHmM	0.0433	2	1.4269	0.2839
	clone	0.2471	5	8.1408	**0.0026**
	GHmM*clone	0.0304	10	0.9435	0.4986
	error	0.0321	82		
	BSO				
	Source	MS	df	F	P
	BSO>0	0.0481	1	2.2731	0.1826
	clone	0.0905	5	4.3239	0.067
	BSO>0*clone	0.0209	5	0.8737	0.5071
	error	5.5058	0		

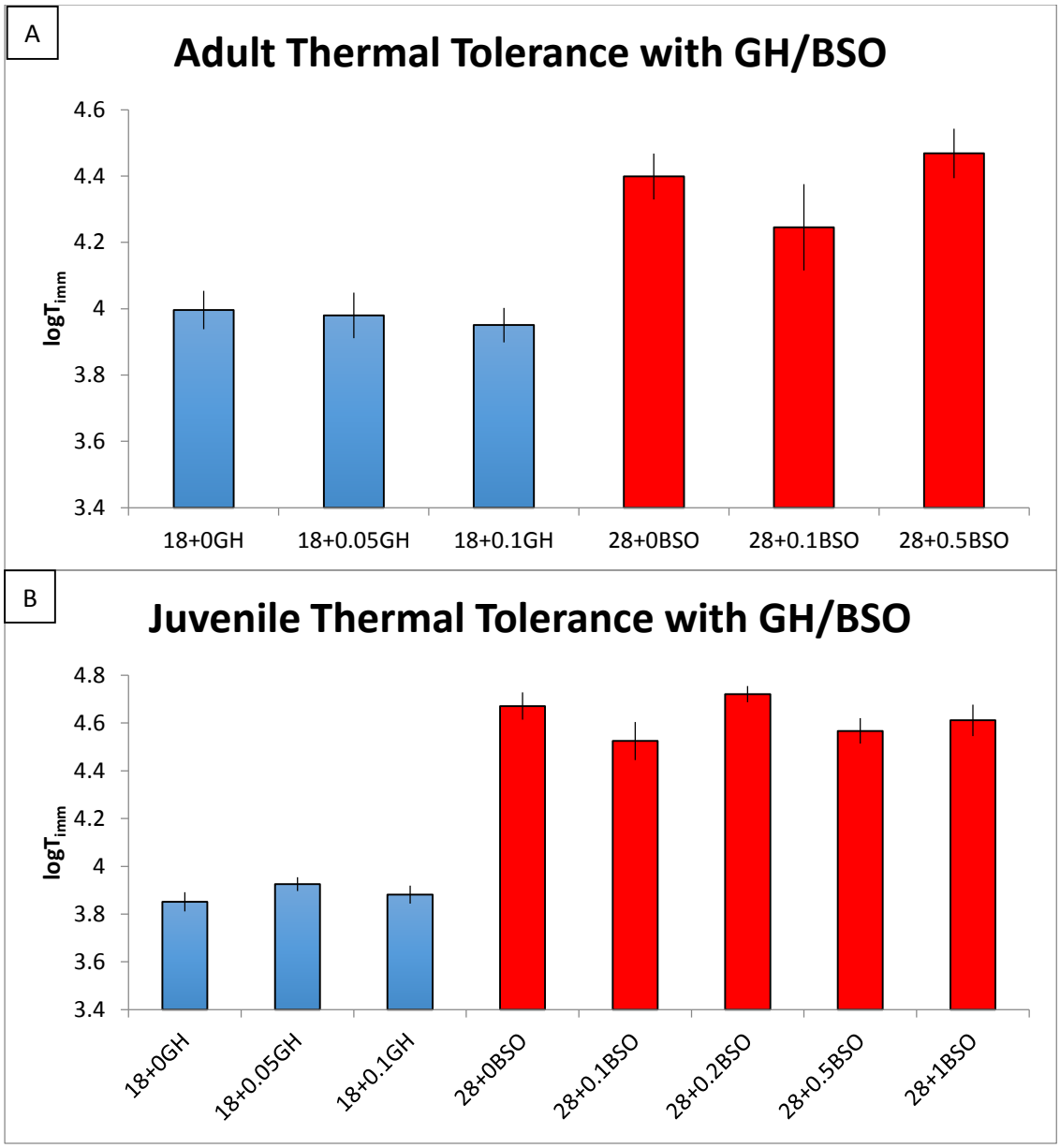

Figure 5. No effect of glutathione or buthionine sulfoximine exposure on temperature tolerance in *Daphnia* adults (A) and juveniles (B).

Table 6. Effects of glutathione concentration on lipid peroxidation and total oxidation in *Daphnia* adults (A) and juveniles (B).

A									
	R30m								
		No exposure				exposure to 37°C for 1h			
	Source	MS	df	F	P	MS	df	F	P
Adults	GHmM	0.0020	2	0.87	0.45	0.0001	2	0.15	0.86
	clone	0.0015	5	0.66	0.66	0.0013	5	2.73	0.07
	GHmM*clone	0.0023	10	1.60	0.15	0.0004	10	0.36	0.96
	error	0.0014	30			0.0012	30		
	R24h								
		No exposure				exposure to 37°C for 1h			
	GHmM	0.0243	2	5.90	**0.0198**	0.0111	2	3.80	0.06
	clone	0.0041	5	1.00	0.46	0.0042	5	1.43	0.29
	GHmM*clone	0.0041	10	1.05	0.43	0.0029	10	0.77	0.65
	error	0.0039	30			0.0037	30		

B									
	R30m								
		No exposure				exposure to 37ºC for 1h			
	Source	MS	df	F	P	MS	df	F	P
Juveniles	GHmM	0.0072	2	4.24	**0.0461**	0.0010	2	0.56	0.59
	clone	0.0032	5	1.90	0.18	0.0065	5	3.73	**0.0361**
	GHmM*clone	0.0017	10	1.09	0.40	0.0017	10	1.01	0.45
	error	0.0016	35			0.0017	36		
	R24h								
		No exposure				exposure to 37°C for 1h			
	GHmM	0.0480	2	13.86	**0.0013**	0.0254	2	8.06	**0.0079**
	clone	0.0045	5	1.29	0.34	0.0062	5	1.97	0.17
	GHmM*clone	0.0035	10	0.76	0.67	0.0031	10	0.75	0.68
	error	0.0046	35			0.0042	36		

Given glutathione's role as an antioxidant, the effects of GH addition were tested in regard to LPO and total oxidation. For adult *Daphnia*, GH exposure only caused differences for total oxidation in samples that were not exposed to 37ºC (Table 6A). Adults with no exposure to 37ºC had lower total oxidation if GH was present, though the amounts used did not matter (fig 6B). Juveniles responded differently, however, as individuals not exposed to 37ºC were different not only for total oxidation, but also for LPO (Table 6B, fig 6C, 6D). Figure 6C shows a difference in LPO in the juveniles not exposed to 37ºC if the GH concentration was 0.1 mM, while figure 6D shows that total oxidation, regardless of exposure, decreases with the chosen additions of GH. Clones were another significant term for juvenile LPO, at least for those exposed to 37ºC (Table 6B).

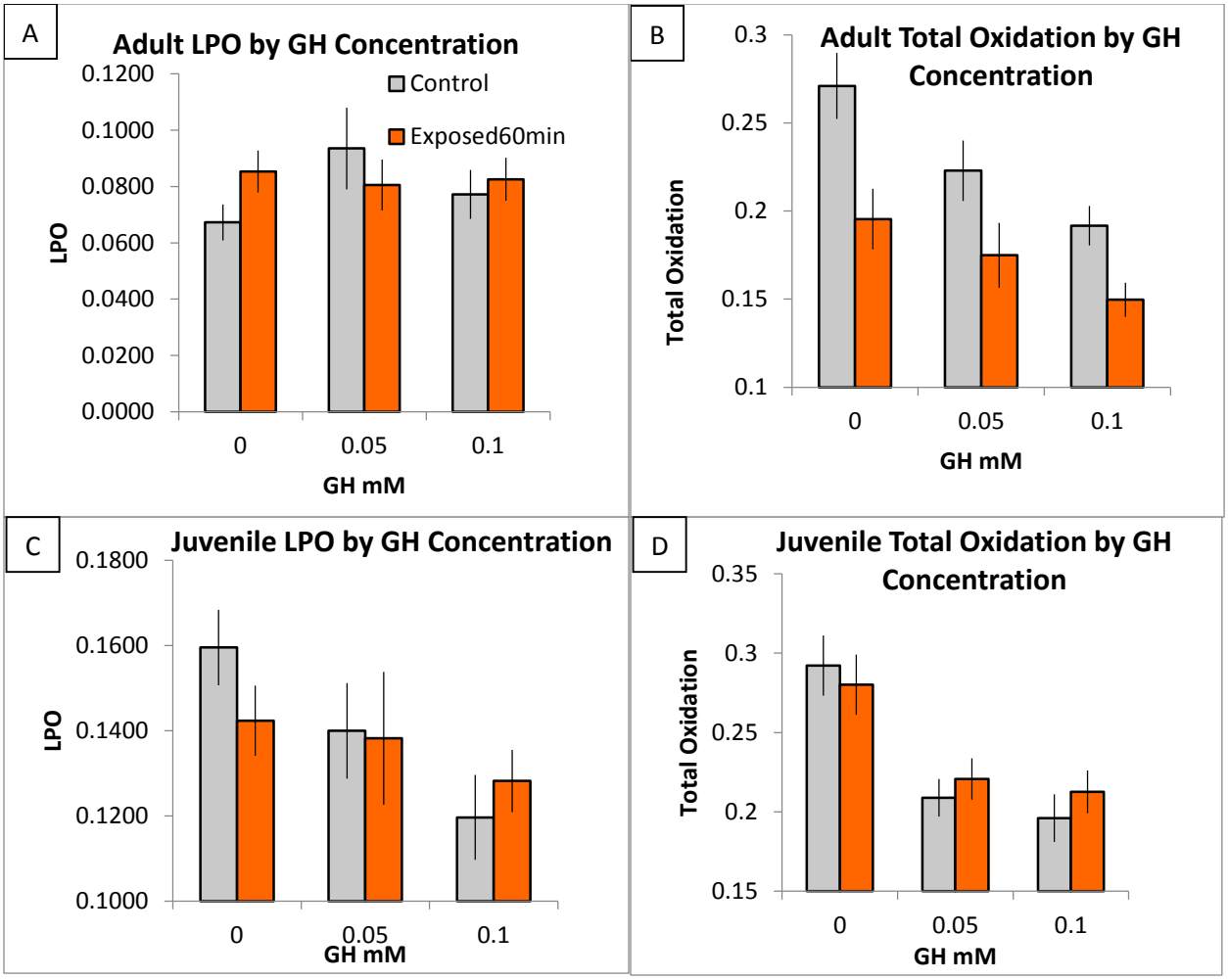

Figure 6. Effects of Glutathione concentration on Lipid peroxidation and total oxidation in *Daphnia* adults (A,B) and juveniles (C,D) with exposure to 37ºC for 60 minutes or no exposure.

Table 7. Effect of buthionine sulfoximine addition to total oxidation in adult *Daphnia* after 120 minutes of exposure to 37ºC.

Adult Total Oxidation with Exposure to 37ºC for 120 minutes				
Source	MS	df	F	P
BSO>0	0.02208	1	37.98	**0.0003**
clone	0.00896	5	18.81	**0.0029**
BSO>0*clone	0.00048	5	0.075	0.996
Error	0.00636	38		

When analyzing the effect of BSO additions on adults and juveniles, the only test with significant effects was for total oxidation in adults exposed to 37ºC for 120 minutes (Table 7, Fig. 7B), although the same trend is suggested also for the unexposed adults (Fig. 7B). Specifically, there was a significant difference among clones and a significant increase in total oxidation in Daphnia exposed to BSO (Table 7), but no clone X BSO interaction. No such effect was observed in juveniles, possibly because of mortality that occurred at high BSO concentrations (Fig.1; see Discussion).

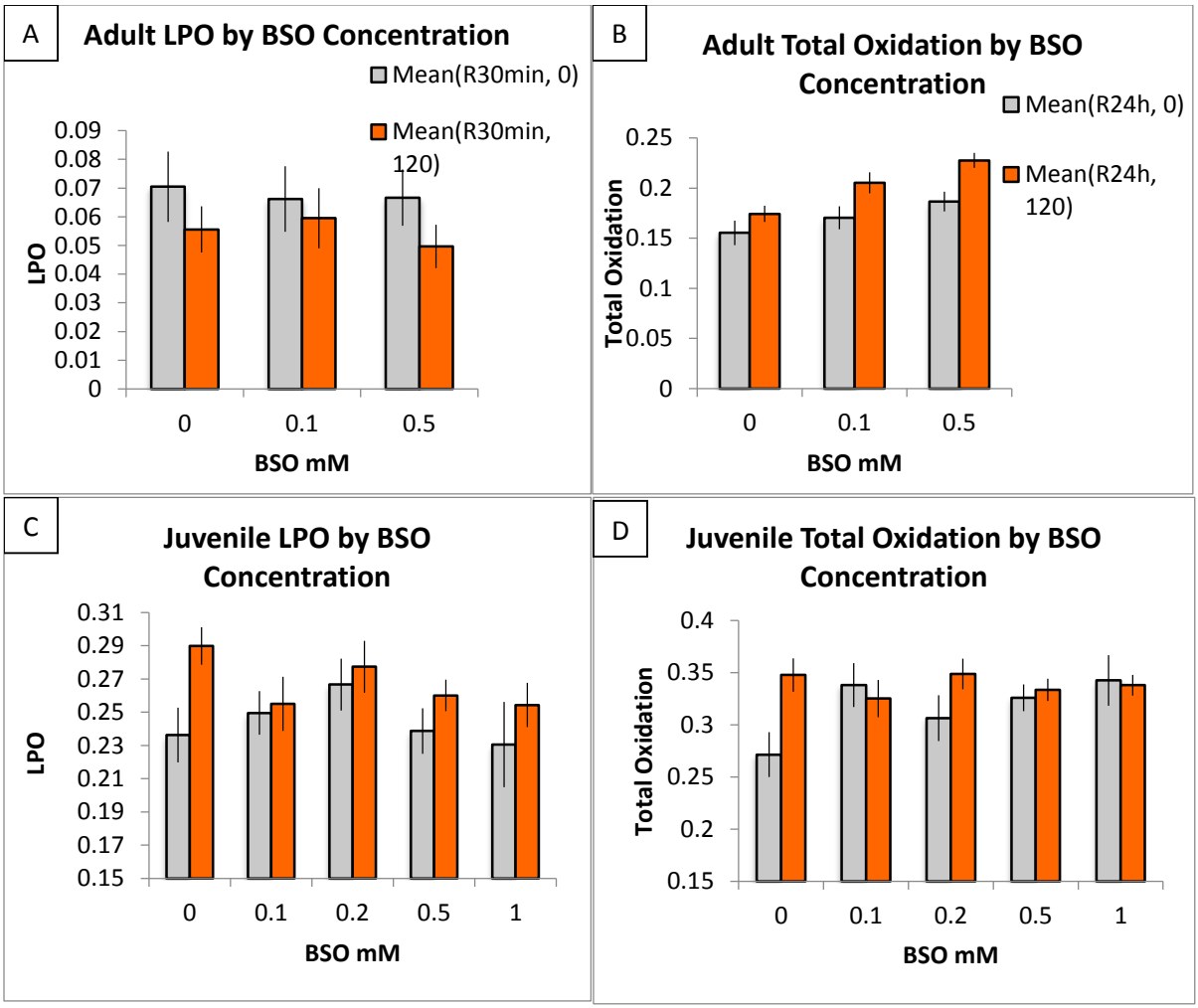

Figure 7. Effects of buthionine sulfoximine concentration, up to 1 mM, on lipid peroxidation and total oxidation in *Daphnia* adults (A,B) and juveniles (C,D) with exposure to 37ºC for 120 minutes or no exposure.

DISCUSSION

We successfully provide more evidence that *Daphnia* acclimated to higher temperature have a higher tolerance to lethal temperature based on a significantly increased T_{imm} in 37ºC (Table 2A, fig 2A). Based on the same data, there is also indication that clone interactions with acclimation temperature are a significant effect for temperature tolerance and total oxidation. IL clones gain more of advantage from prior acclimation than other clones, at least in terms of T_{imm} (fig 2A). This advantage is not clearly linked to antioxidant capacity, at least for this clone, because their total oxidation levels do not change very much with acclimation. RM clones, however, responded to acclimation temperature with a much higher antioxidant response (fig 2B).

Assuming that physiological changes that reflect long-term acclimation and short-term acclimation in *Daphnia* are causative mechanisms for heat tolerance, antioxidant capacity appears to be a significant factor. Based on the temperature switch experiment, T_{imm} was highest in long-term acclimated individuals at 28ºC, lowest at 18ºC, and intermediate with switches (fig 4A). In terms of total oxidation, those from 28ºC had the highest antioxidant capacity (fig 3), but following a 4-day switch, over half their capacity had been lost. Interestingly, the same cannot be said about switches form 18ºC, in which there was no significant change in antioxidant capacity. Essentially, acclimated individuals lose their antioxidant capacity within 4 days in a milder temperature, while unacclimated individuals do not gain an increased capacity in the same amount of time. Our analysis also suggests that clones interact differently to not just the acclimation, but to the temperature switch as well.

The lack of significant change in LPO among most treatments and experiments practiced here seems to support that either LPO is under constant equilibrium with antioxidant activity because it remains relatively unchanged while total oxidation does not. The alternative, and not mutually exclusive hypothesis that LPO is beneficial on some levels, such as in signaling and the cascading roles of its intermediates generated by ROS interaction, cannot be supported or negated to any degree within the scope of this study (Farmer & Mueller, 2013; Cohen et al. 2013). However, it is important to note that this lack of difference among treatment groups makes LPO a poor measure of oxidative stress, especially considering the significant responses generated concerning total oxidation levels.

The six clones used in this study have been chosen from a set of clones studied by Yampolsky et al. to represent the opposite ends of the range of temperature tolerance (2014). Not surprisingly when analyzed at each acclimation temperature separately (Fig. 2) they show a significantly different T_{imm} (ANOVA $F=3.28$; $P<0.008$ for 18ºC and $F=4.89$; $P<0.0005$ for 28ºC; see also clone effects in Tables 4, 5 and 7). However, when analyzed at two temperatures together and tested against the interaction term,

clonal effect is not significant (Table 2A). This indicates that clones respond differently to the acclimation to high temperature (clones by temperature interaction term in Table 2A: $P<0.05$). Furthermore, there is a significant clones by temperature interaction in the effect on total oxidation capacity (Table 2C), indicating that different clones have different ability to increase synthesis of antioxidants in response to exposure to elevated temperature. When 28ºC-acclimated *Daphnia* were switched short-term to 18ºC, their temperature tolerance dropped (Fig. 3A; Table 3), but it dropped differently in different clones (Table 3, $P<0.0115$). The importance of total oxidation status on temperature tolerance is further corroborated by the fact that there also was a significant clone by total oxidation interaction in the effect on temperature tolerance in the short-term switch experiment (Table 3, $P<0.003$). Thus, we demonstrate that the correlation between acclimation temperature and oxidative status on the one hand and temperature tolerance on the other hand is genotype-specific.

As predicted, we observed increased temperature-tolerance in 28ºC-acclimated *Daphnia* after 90 minutes of exposure to hypoxia (Table 4). There was no indication of any differences among clones in the strength of this effect (Table4: interaction temp $P>0.9$). Therefore, the observed clone-specific responses to long-term and short-term changes in temperature are not related to glutathione metabolism. Though, it should be considered that 90 minutes may not have been an adequate amount of time to substantially increase the total amount of glutathione, at least not by the time the tolerance assay was conducted because we showed that antioxidant capacities, or at least their effects, don't accumulate highly over short term periods.

This is also observed in the experiments in which we attempted to manipulate total antioxidant capacity by either supplying *Daphnia* with glutathione or blocking its synthesis by glutathione synthase inhibitor, BSO. As expected, we see reduced total oxidation in both juvenile and adult *Daphnia* exposed to GH addition (Table 6, fig 5). However, such exposure did not result in any change in temperature tolerance (Table 5, fig 4), indicating that if antioxidant capacity is indeed critical for temperature tolerance, it is not based specifically on glutathione. There were also no clones by glutathione interactions detected in any of these experiments, again, indicating that the observed clone-specific responses are not linked to glutathione metabolism. If hypoxia does indeed stimulate GH metabolism but still increases heat tolerance, hypoxia either stimulates other mechanisms alongside GH synthesis, or GH has an effect on tolerance that was not adequately recognized by our statistical analysis.

While specific antioxidants causative for heat tolerance were not identified in this study, we have shown that antioxidant response occurs along the same timeframe that long-term acclimation to higher temperature, implying that oxidative defenses are necessary for heat tolerance. Clones also appear to have differential capacities to respond to the abiotic influence of temperature. The differential effects between

clones found here include those to long-term (table 2A, fig 2A) and short-term (table 3) acclimation temperatures, as well as total oxidation (table 3, fig 2B). Given that plastic response in this case varies by genotype, more careful consideration of genetics and biogeography should be considered in physiological studies of environmental response, while specific plastic physiological mechanisms should not be immediately lumped together as a singular component in evolutionary analysis.

To conclude, the plastic phenotypic response of *Daphnia magna* is necessary to ensure their continued occupation of temporary ponds which are susceptible to daily and seasonal temperature fluctuations as well as ever-increasing global climates. These pressures effectively select for strong plastic response over long exposures to high temperatures (Geerts et al. 2015; Henning-Lucass et al. 2016). A large component of thermal plastic response is defense against ROS, in which Daphnia respond to acclimation to a stressful temperature by upregulating their total antioxidant capacity rather than maintaining LPO exclusively. We consider antioxidant capacity to be more indicative of thermal tolerance because it must be built up over a long-term exposure to a stressful temperature. Additionally, given the potential metabolic costs of high antioxidant levels, this capacity appears to be readily lost within a short period if the organism is removed from the stressful environment. The important role of antioxidants is also emphasized by the increased survival in lethal temperatures after exposure to hypoxia. This study fails to adequately define the specific activity of GH as an antioxidant during acclimation, so at this point, no specific antioxidants' roles have been identified for thermal tolerance. Our analysis of 6 geographically distinct genotypes shows a genetic difference in response to short-term and long-term acclimation as well as in the effect of antioxidant capacity on temperature tolerance. These results indicate a significant degree of local adaptation in heat and oxidative stress defenses in Daphnia and provide a better understanding of adaptive responses of this zooplankton crustacean to rising temperatures.

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