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The Role of Copper in the Oxidative Stress Response of Chlamydomonas reinhardtii to Heat Shock

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Abstract

In response to environmental stress cells produce reactive oxygen species (ROS), which can cause molecular damage in the form of DNA breaks and modification to lipids and proteins. On the other hand, ROS are important in cell signaling to induce differential gene expression, metabolic changes and thus, adaptation to different environmental conditions. The hypothesis of this project is that the ROS response and its modulation by transition metals are a common denominator of different environmental challenges, including radiation and microgravity during spaceflight, directly applicable to the unicellular green alga *Chlamydomonas reinhardtii* grown in spaceflight experiments. The goal of this thesis was to characterize the response of *C. reinhardtii* to stress by examining biochemical and gene expression changes using heat shock. The heat shocked (42 °C) *C. reinhardtii* exhibited an increase in ROS in both inside the cell and in the supernatant, paralleled by a selective regulation of ion transporters, specific for copper. These results provide new insights about the cellular response to stress and its possible mediation during long-term environmental pressure and provide a basis for understanding the evolution of multicellularity.

Table of Contents

List of Figures

List of Tables

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Abbreviations

ATP	Adenosine triphosphate
BIOS1	Biosphere 1
BIOS 2	Biosphere 2
BIOS 3	Biosphere 3
CELSS	Closed Ecological Life Support System
COPT1	Copper Transporter 1
C. Reinhardtii	*Chlamydomonas reinhardtii*
CRR1	Copper Responsive Regulator 1
CTR1	Copper Transport Regulator 1
CTR2	Copper Transport Regulator 2
CTR3	Copper Transport Regulator 3
Cu	Copper
CuREs	Copper Response Element
DCF	2', 7' Dichlorodihydrofluorescein
DCFDA	2', 7' –dichloroflurescin diacetate
DCFH	2', 7' Dichlorodihydrofluorescin
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ESA	European Space Agency
HSF1	Heat Shock Factor 1
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
OD	Optical Density

NADPH	Nicotinamide adenine dinucleotide phosphate
NANOSIMS	Nanoscale Secondary Ion Mass Spectrometry
PAM	Pulse-Amplitude-Modulated
qPCR	Quantitative Polymerase Chain Reaction
RCK1	Receptor of Activated Protein Kinase C
ROS	Reactive Oxygen Species
RT	Reverse Transcription
SBP	Squamosa Binding Protein
SOD	Superoxide Dismutase
STS	Space Transportation System
TAP	Tris Acetate Phosphate
TRIS	Tris(hydroxymethyl)aminomethane
V. carteri	*Volvox carteri*
VMP	Volvox Matrix Metalloproteases
WST	4-[3-(4-Idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
Zn	Zinc

Introduction

Life on Earth started in a reducing environment but the onset of oxygenic photosynthesis during the Great Oxygenation Event about 2.3 billion years ago changed the atmospheric composition.[1] The introduction and accumulation of oxygen in the environment was paralleled by a change in the bioavailability of transition metals as oxidized copper became soluble while oxidized iron became insoluble.[2]

Reactive oxygen species (ROS) are produced when molecular oxygen is reduced, often by reaction with transition metals to form superoxide O_2^{-}, hydrogen peroxide H_2O_2 and hydroxyl radical $HO^{\cdot}$, and these cause molecular damage in the form of DNA breaks, lipid peroxidation and protein carbonylation.[3] However, ROS are also produced during normal metabolism and are involved in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors and gene expression.[3] Both copper and iron are essential micronutrients, often involved in oxygen chemistry but with the potential to create ROS.[4] Therefore, it is critical for cells to control these metals to avoid cellular damage.

Unpublished data from work done in Dr. Oana Marcu's lab through the SETI Institute at NASA Ames Research Center suggest that under heat shock as a form of oxidative stress there was a change in elemental transport, specifically copper, in the multicellular alga *Volvox carteri*..This species evolved from the unicellular alga *Chlamydomonas reinhardtii,* through a series of colonial algae that are aggregates of one cell type, to functional and morphological differentiation of two cell types, somatic and reproductive.[5] Copper transport is differentially regulated in the somatic vs. reproductive cells of *V. carteri* in response to environmentally-induced heat stress, which raises the

question of whether copper metabolism is implicated in cell differentiation and the evolution of multicellularity.

Algal Uses

The single-celled photosynthetic organism *C. reinhardtii* is a green alga of particular interest for studying oxidative stress, and can be used as a model for both space biology and astrobiology research.[6] For astronauts to survive in outer space, closed life support systems are necessary to manage waste such as carbon dioxide, feces and urine and convert these bi-products into usable oxygen, nutrients and water mimicking Earth's environment.[7] On the International Space Station, there is an elaborate Environmental Control and Life Support System dedicated to creating a working habitat for the astronauts in space.[8] This current system, however, requires the continuous launch of fresh air, water and other equipment. Not only is this expensive, but on future deep space missions it is not possible to bring all of the water and air required based on volume and weight restrictions. Thus, regenerative life support hardware is necessary.

One possibility for regenerative life support is the use of algal bioreactors. Beginning in 1961, several different bioregenerative test beds have been investigated.[9] Biosphere (BIOS) 1-2 in the Arizona desert contained compartments allowing for gas exchange between CO_2-and-O_2 producing organisms, while BIOS 3 in Krasnoyarsk, Russia also included biological water purification and food production.[9] In 1991, for two years, a crew of eight was able to survive in BIOS 2 as the first long duration habitation by humans in a closed environmental system.[10] From BIOS 2, it became apparent that there were serious challenges in utilizing resources and creating limited weight and volume life support systems from small algae tanks.[10]

Chlamydomonas has been previously used for spaceflight research in Spacelab D1 as part of the Space Transportation System (STS)-61-A mission (1985), ALGAE experiments on the Russian Bion 10 satellite (1992), and the Russian and European Space Agency (ESA) Foton 10, 11 and 12 satellites (1994-1997).[11-12] Results from these experiments found in ESA Erasmus Experiment Archive suggest that the spaceflight environment affected the growth, cell division, circadian rhythm and phototactic behavior of *Chlamydomonas*.[13] Because algae are photosynthetic organisms, understanding their response to the environmental stressors experienced in space allows for manipulation of these systems to reach optimal oxygen production and ultimately help astronauts survive in space under strict resource weight restrictions.

Currently *Chlamydomonas* is also being studied for applications of terrestrial benefits, such as biomass production, and alternate fuels and energies. One such use is for the production of oil since algae have the ability to produce and store lipids that are very similar to vegetable oils.[14] While oil production is limited by the capacity of various strains, we found that environmental stress induced by the presence of bacteria can increase lipid production in our algal cultures. These results became the preliminary data in a currently funded DOE grant between NASA Ames Research Center and Lawrence Livermore National Lab beginning this past February, 2015, entitled "A Systems Biology Approach to Microbial Symbioses: How Algal-Bacterial Interactions Control Resource Allocation in Biofuel-Producing Communities." Other biotechnological applications for *Chlamydomonas* include the production of hydrogen as a clean energy source since the alga can make hydrogen under certain conditions, such as a lack of sulfur in the growth media.[15]

C. reinhardtii and the evolution of multicellularity

The genome of *C. reinhardtii* was sequenced and the information is available through the Phytozome database produced by the Joint Genome Institute and the University of California Regents.[16] While there are few differences in gene numbers between *Chlamydomonas* and *Volvox,* it has been shown that genes regulated by the environment in *Chlamydomonas* (e.g. *regA* gene is turned on during dark and sulphur depletion) can be coopted for functional differentiation in *Volvox* (gene *regA* is only expressed in somatic, but not in reproductive cells).[17] Thus, gene expression induced by environmental stress in the unicellular *Chlamydomonas* becomes fixed in evolution in the multicellular *Volvox*.[17] This mechanism is modulated by cellular redox balance particularly in photosynthetic organisms since the flux of electrons through the electron transport system has to be balanced with the rate of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH).[18]

Response of *C. reinhardtii* to oxidative stress

There is a wide array of data characterizing the response of *Chlamydomonas reinhardtii* to different environmental conditions such as heat and cold stress, oxidation from singlet oxygen and nutrient limitation.[19,20,21] One result of aging and stress is the accumulation of an autophagy-related ATG8 protein in *C. reinhardtii*.[20] Under stressful conditions or development, autophagy, a catabolic membrane-trafficking process, occurs where cells recycle proteins and organelles in the cytosol using the ATG autophagy related proteins.[20] Additionally, a new proteomic approach that involved full ^{15}N metabolic labeling and mass spectrometry shotgun proteomics with the ^{15}N standard over all time points was applied to the heat stress time course of *C. reinhardtii*.[19] A large

number (3433) of *C. reinhardtii* proteins were identified and 1116 of these were found in at least three out of the five time points of the heat shock course.[19]

The experimental time points for this thesis project were chosen to match up with those from Mühlhaus[19] (control 25 °C, heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180') allowing for direct comparison. This thesis work is original, since no previous studies have specifically focused on copper related genes or proteins in response to heat stress in *C. reinhardtii*. *C. reinhardtii* is also the first, unicellular member of the Volvocine lineage, followed by colonial species and the multicellular relative *Volvox carteri*, making this family of algae a good model for understanding the evolution of multicellularity in response to environmental stressors.[22]

Results from my previous two summers (2013 and 2014) in the Marcu lab at NASA Ames Research Center showed that the production of ROS contributes to the heat stress response in *C. reinhardtii*. Unexpectedly, there was a ten-fold increase in the levels of ROS in the supernatant compared to the levels of ROS inside the cell, suggesting that this increase came from ROS produced in the cell wall or extracellular matrix (ECM) of *C. reinhardtii*. Such a finding is interesting because the expansion of the ECM is considered as one of the 12 physiological steps in the evolution of multicellularity . A genome comparison between *Volvox* and *Chlamydomonas* found very few differences between the two genomes.[5] One difference, however, was seen in the size of the *Volvox* matrix metalloproteases (VMP) protein family: 6 genes in *Chlamydomonas* vs. 48 genes in *Volvox*.[5] Thus, since VMP proteins bind copper and other metals in the extracellular matrix, we hypothesize that they may play a role in the evolution of multicellularity

through the sequestrations and availability of metals to regulate redox reactions within the cell.

Copper in *C. reinhardtii*

Previously, as a member of the Marcu lab, I found that in response to oxidative stress in the form of heat shock, *C. reinhardtii* displayed a selective regulation of copper transporter genes, and for this senior thesis completed a statistically significant number of replicates. This copper regulation paralleled an increase in ROS both inside the cells and in the supernatant.

Copper is a single-electron donor/acceptor and has the ability to displace iron as it is near the top of the Irving-Williams series which organizes reactivities of metals.[23] Copper is an essential micronutrient for most organisms, including *C. reinhardtii* because it acts as a catalyst of redox reactions as well as reactions involving molecular oxygen (such as reactive oxygen species).[24] As a result, too much copper is toxic, so copper transport is tightly regulated and highly selective.[25] Metallochaperones can bind copper and transport it to cellular compartments where it binds antioxidant enzymes such as superoxide dismutase, responsible for the breakdown of harmful reactive oxygen species.[23]

The model for understanding nutritional copper homeostasis focuses on copper response regulator 1 (CRR1), a transcription factor that binds to the GTAC motif of copper response elements (CuREs).[26] CRR1 has a Squamosa Binding Protein (SBP) to recognize the GTAC motif, and is normally a zinc (Zn)-dependent DNA-binding domain, but can also contain copper.[26] It is possible that copper might impact how well CRR1 binds DNA for transcriptional activation, either directly with copper ions or as an

interaction of CRR1 with another binding protein.[4, 27] *In vitro* experiments support a direct interaction of copper ions with CRR1 as well as the possibility of additional protein involvement. Although Cu(II) may play a role, reduced Cu(I) is believed to be the predominant intracellular form of copper due to high concentration of cellular reductants. Additionally, many other eukaryotic copper binding regulators bind Cu(I).[26]

Copper Transport Regulator (CTR) genes are the targets of CRR1-dependent signaling.[25] CTRs, specific for Cu(I) but not Cu(II) are the most prominent and only route for copper assimilation. The CTR family in *Chlamydomonas* includes Copper Transport Regulator 1 (CTR1), Copper Transport Regulator 2 (CTR2), Copper Transport Regulator 3 (CTR3) and Copper Transporter 1 (COPT1). CTR1 and CTR2 are located in the plasma membrane and are high affinity copper transporters (Figure 1). Based on the localization, pattern of expression, and magnitude of regulation in copper deficient *C. reinhardtii* CTR1 and CTR2 might be responsible for inducible copper uptake activity.[25] The diagram below (Figure 1) depicts possible steps of copper regulation in *C. reinhardtii* and signaling from the extracellular matrix to changes in gene expression in the nucleus.

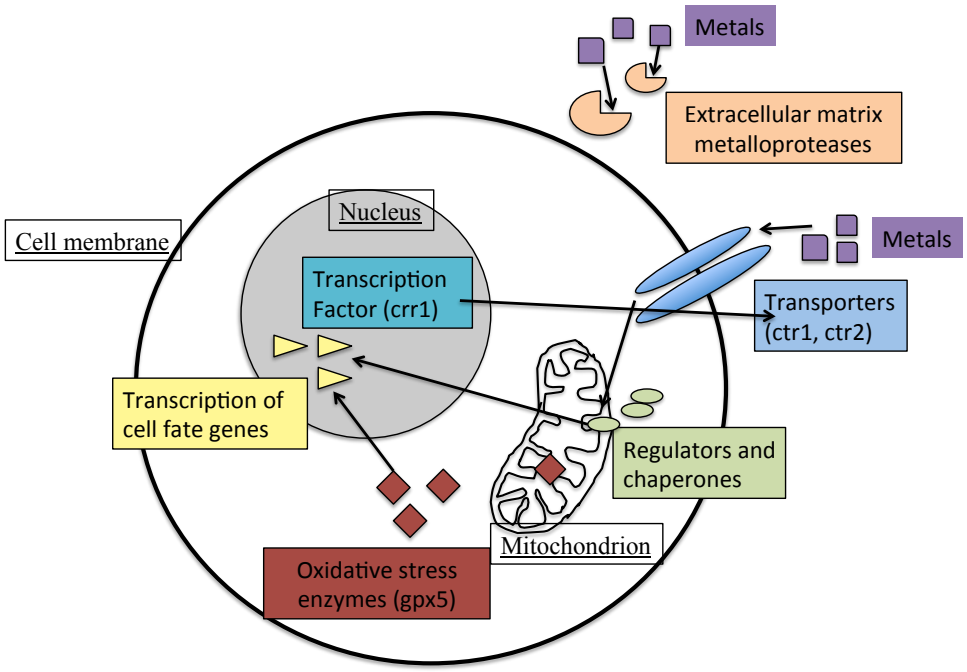

Figure 1. Redox-Metal Response Network. *The products of the genes involved in oxidative response are in distinct subcellular compartments. Each class could contribute to an interactive signaling cascade from the outside extracellular matrix, through the membrane, into the cytoplasm and mitochondrion and into the nucleus of the cell (arrows). Metalloproteases are in the extracellular matrix, transporters are in the cell membrane, regulators and chaperones are present in the cytoplasm and in mitochondrial intermembrane space while oxidative enzymes have cytoplasmic and mitochondrial activity. Figure created by Oana Marcu and Michelle Brann.*

Research Goals and Hypotheses

The initial goal for this project over the past two summers at NASA Ames Research Center was to characterize the response of *C. reinhardtii* to stress by examining metabolic, biochemical and gene expression changes using heat shock to monitor adaptive responses. The two driving hypotheses of the project were:

1. Reactive Oxygen Species are involved in the response to heat stress
2. Transition metals are involved in the modulation of the ROS response

The ROS levels were monitored by assays for the presence of intracellular and extracellular ROS. In order to assess the ROS response in relation to the physiology of the alga, metabolic changes were detected by Pulse Amplitude-Modulated (PAM) fluorometry to measure the efficiency of photosystem II. In general chlorophyll fluorescence indicates photosynthetic energy conversion in higher plants, algae and bacteria.[28] The design of PAM fluorometers allows for highly sensitive and specific measurements of chlorophyll fluorescence yield while being non-invasive. PAM fluorometers cause fluorescence excitation through a series of pulses of light.[29] Photosynthesis is measured after a saturating light pulse is sent out to shortly suppress the photochemical yield to zero, creating a maximum fluorescence yield.[29] The role of ROS in downstream intracellular signaling was assessed by gene expression, as determined by quantitative PCR (qPCR) of a cDNA template that was reverse transcribed (RT) from RNA extracted from *C. reinhardtii* under heat-shock experimental conditions.

The heat shocked (42 °C) *C. reinhardtii* shows a decrease in the efficiency of photosystem II, measured by pulse amplitude modulated fluorometry (PAM). This is paralleled by the selective regulation of ion transporters, specifically copper, and an increase in ROS in cells and supernatant. The working hypothesis is that the majority of the ROS detected in the supernatant is not originally excreted from the cell, but rather was produced in the outer cell wall or extracellular matrix of the algae and diffuses into the supernatant (Figure 2). Thus, the levels of extracellular ROS could be mediated by copper binding in the ECM. This hypothesis is supported by analogy with the

extracellular polymeric substance (EPS) of cyanobacteria which adsorbs metals, including copper. This mechanism allows cyanobacteria to remove metals from the surrounding environment by association to the cell surface.[30] In *Chlamydomonas,* the copper-modulated ROS in the extracellular matrix may be a first line of defense against environmental heat stress.

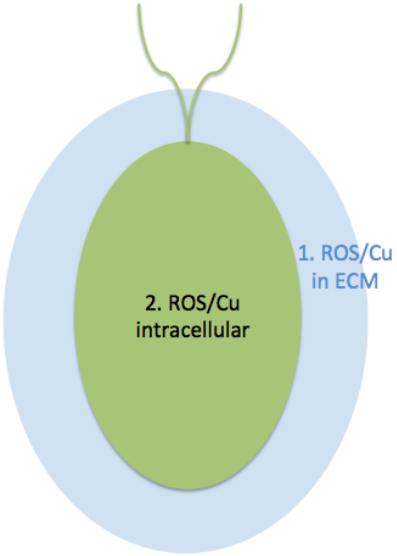

Figure 2. Two possible ROS responses to heat stress. *1. The immediate response to heat stress recruits copper and produces high, potentially toxic, levels of ROS in the ECM outside the cell. 2. In response to heat stress, intracellular ROS which are normally kept low by activity of scavenging enzymes, act as signaling molecules.* *Figure by Oana Marcu.*

The additional goal for this senior thesis was to understand the implications of copper transport in response to stress at the gene expression level as well as the enzymatic level. Copper is a metal cofactor of superoxide dismutase (SOD), an enzyme responsible for the breakdown of ROS. If the immediate response to stress in *C. reinhardtii* recruits copper regulation to mediate toxic levels of ROS in the ECM, the second line of response to heat stress allows ROS to act as signaling molecules for a

specific response at gene expression level (Figure 2). Such results provide new insights about the cellular response to stress and its possible mediation during long-term environmental burden (including spaceflight), and provide a basis for a greater understanding of the evolution of multicellularity in the Volvocine lineage.

Materials and Methods

Buffers

Tris Acetate Phosphate (TAP) Media is adapted from Gorman, D.S., and R.P. Levine (1965)[31]. Each 1L contains 2.42 g Tris(hydroxymethyl)aminomethane (TRIS), 25 mL of TAP Salts (15 g NH_4Cl, 4.0 g $MgSO_4 \cdot 7H_2O$, 2.0 g $CaCl_2 \cdot 2H20$, water up to 1L), 0.375 mL of Phosphate Solution (28.8 g K_2HPO4 14.4 g KH_2PO4 water to 100 mL) 1 mL of Hunter's trace elements, 1 mL glacial acetic acid and water up to 1 L. The medium was titrated to pH 7.0 with HCl. Hunter's Trace Elements were purchased commercially and contain zinc, copper and iron. The Cell Lysis Buffer contained 10mM TRIS pH 8, 150 mM NaCl, 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100.

Culture Growth

Chlamydomonas reinhardtii were grown in an incubator at 25ºC on a 16/8-hour light/dark cycle in TAP media containing Hunter's Trace Elements. The culture was transferred into fresh media every 3 or 4 days when the cultures were in stationary phase (Optical Density $(OD)_{750} > 0.8$) or when more culture was needed for additional experiments (experimental cultures used in the growth phase, $OD_{750} < 0.7$)

Gene Expression from RNA Extraction and RT-qPCR

Twenty-five mL of *C. reinhardtii* culture (OD_{750} .268 with 7,800,000 cells/mL and total of 195,000,000 cells) was collected for RNA isolation . After dark-adapting for ten minutes, a twenty-five mL culture was exposed to heat in a 42 °C water bath every 30 minutes for 2 hours. At the end of two hours, all cultures (control 25 °C, heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180') were flash frozen for RNA extraction with the Molecular Bio PowerPlant RNA Isolation kit with DNase. Once

extracted, gene expression was tested with Qiagen Quantitect SYBR Green RT-PCR kit using 100 ng of RNA for each 10µL reaction and the Illumina qPCR machine.

ROS Biochemical Assay

One-half mL of *C. reinhardtii* culture (with 9,180,000 cells/mL) was incubated with 1 mM 2',7' –dichlorofluorescein diacetate (DCFDA) to determine the levels of ROS In the presence of ROS, the non-fluorescent 2', 7' Dichlorodihydrofluorescin (DCFH) becomes oxidized to highly fluorescent 2', 7' Dichlorodihydrofluorescein (DCF) [32]. After dark adapting for ten minutes, four 0.5 mL cultures (for media and culture both with 0.01 mM neocuproine in methanol and methanol control) were exposed to a 42 °C water bath in the dark every hour for three hours, including a 30 min time point. At the end of three hours, all cultures (control 25 °C 0, heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180' for both *C. reinhardtii* culture with 0.01 mM neocuproine in methanol and methanol control were collected by centrifugation for 2 minutes at 10,000 x g. One hundred µL of supernatant from the cultures and 100µL of the media were distributed in triplicate in the well plate. For the culture, the remaining supernatant was removed and resuspended in 200 µL 2X cell lysis buffer and 200 µL media (TAP) or 400 µL 1X cell lysis buffer. One hundred µL of the cell pellet in triplicate was added to the cell plate along with a blank containing 50 µL cell lysis buffer and 50 µL media before recording fluorescence (480 nm-excitation and 530 nm-emission). The cell pellet was normalized to the blank and the supernatant to the media to time 0 of the heat shock to calculate fold change.

PAM Fluorometry

A white light XY diving PAM (Waltz) was used to provide saturated pulses (intensity 8, gain 1) every hour to 300 µL of *C. reinhardtii* culture (9,180,000 cells/mL) at either 25 °C or 42 °C that had been dark adapted for 10 minutes. The *C. reinhardtii* contained 0.001, 0.01, 0.1 or 0 mM neocuproine dissolved in methanol in triplicate. The photosynthetic efficiency, YII in the PAM software, was plotted against time to determine the kinetics of *C. reinhardtii* in response to heat shock.

SOD Assay

Cell BioLabs Oxiselect SOD Assay

One-half mL of *C. reinhardtii* culture (with 9,180,000 cells/mL) was used for the SOD assay. After dark adapting for 10 minutes, four 0.5 mL cultures (for media and culture both with 0.01 mM neocuproine in methanol and methanol control) were exposed to a 42 °C water bath in the dark every hour for three hours including a 30 min time point. At the end of three hours, all cultures (control 25 °C 0, heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180' for both *C. reinhardtii* culture with neocuproine and without) were collected by centrifugation for 2 minutes at 10,000 x g. The supernatant was discarded and the cells were lysed with 125 µL of 2X cell lysis buffer and 125 µL water. Seventy-eight µL of the cell lysate was combined with 2 µL Chromagen and 10 µL 10X SOD Assay Buffer from OxiSelect Superoxide Dismutase Activity Assay (Cell BioLabs). The wells were mixed, incubated at 37 °C and the absorbance recorded every 5 minutes for 1 hour at 490 nm. The values were normalized to the blank, time zero of incubation for the assay and time zero of the heat shock to generate fold change.

Dojindo SOD Assay Kit - WST

The *C. reinhardtii* SOD activity was determining using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazoil]-1,3 benzene disulfonate (WST-1), a water soluble tetrazolium sodium salt, to detect superoxide radicals generated by the enzyme xanthine oxidase over a range of SOD concentrations. One unit of SOD activity was defined as the amount of enzyme inhibiting 50% of WST-1 photoreduction.

Three different *C. reinhardtii* concentrations were tested, at starting OD_{750} of 0.67, 0.14 and 0.079, equivalent to 3.67 $x10^6$, 7.34 $x10^5$ and 2.74 $x10^5$ cells per well. After dark adapting the *C. reinhardtii* culture for 10 minutes, the cells were collected by centrifugation for 2 minutes at 10,000 x g. The supernatant was discarded and the *C. reinhardtii* cells were lysed with 50 µL of 1X cell lysis buffer.

Twenty µL of the cell lysate in duplicate was combined with 200 µL WST Working Solution and 20 µL of xanthine oxidase for samples. For sample blanks, 20 µL of *C. reinhardtii* was combined 200 µL WST and 20 µL of dilution buffer instead of xanthine oxidase. Additionally, for the coloring without inhibitor blank, xanthine oxidase was tested without sample and for the reagent blank no sample or enzyme was used. All plate wells were mixed, incubated at 37 °C and the absorbance at 450 nm was recorded every 1 minute for 20 min. SOD activity, or inhibition rate was calculated using the equation [(S1-S3)-(SS-S2)]/(S1-S3) x 100 where S1 was the coloring without inhibitor blank, S3 was the reagent blank SS was the *C. reinhardtii* sample with xanthine oxidase and S2 was the *C. reinhardtii* sample without xanthine oxidase. All values were taken as the linear slope over the 20 minute incubation period in the plate reader.

Data Analysis and Normalization

For all ROS Assays, the fold change for the cell pellet was calculated by dividing each heat shock time point by the control time zero. For the supernatant at all time points, the TAP media with either with methanol or neocuproine at the corresponding time point was subtracted. When measuring ROS without neocuproine, the fold change was calculated by dividing each time point (heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180') by control 25 °C, the culture that experienced no heat shock. When using the neocuproine chelator, the fold change for each time point (control 25 °C 0', heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180') between the culture with the neocuproine and that with just the methanol control was calculated before normalizing to control 25 °C 0', the culture without any heat shock.

All pairs of means were compared using a student t-Test in excel. $P < 0.05$ was considered statistically significant.

Results

In order to determine the implications of copper transport in response to oxidative stress at the gene expression level as well as the enzymatic level, the levels of ROS and gene expression of specific copper transporter genes were measured during a heat shock (42 °C) time course. These ROS values were compared to those detected from *C. reinhardtii* culture in the presence of 0.01 mM neocuproine, a copper chelator, using the same heat shock time course. Values were normalized against the corresponding room temperature (25 °C) and solvent (methanol) controls for each experiment.

ROS is the mechanism of response to heat stress in *C. reinhardtii*

The mechanism of response to heat stress in *C. reinhardtii* was hypothesized to be the modulation of ROS levels. To detect changes in ROS, *C. reinhardtii* cells were incubated with DCF-DA which in the presence of ROS becomes oxidized to fluorescent DCF, measurable at wavelength 495 nm. Over the course of heat shock (42 °C) there was an increase in the ROS levels in the supernatant, detectable as early as 30 minutes (*Figure 3*, p=0.02). All supernatant values were normalized to the baseline ROS values from TAP media containing DCF-DA at each time point. The cell pellet values were normalized to a blank containing cell growth medium and cell lysis buffer. In the cell pellet, the fluorescence values increased by 180 minutes of heat shock but this difference was not statistically significant. (*Figure 3*, p=0.06).

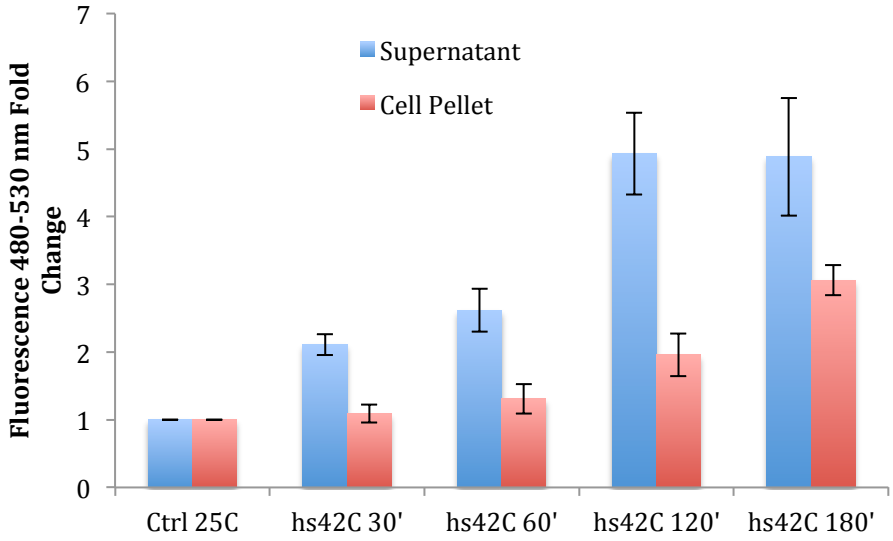

***Figure 3. ROS levels increase in response to heat stress in* C. reinhardtii.** *The level of Reactive Oxygen Species (ROS) increases in* C. reinhardtii *supernatant and cell pellet when exposed to heat (42 °C). 1 mM DCFDA was added to C. reinhardtii culture. In the presence of ROS, DCF-DA becomes oxidized to fluorescent DCF, detected at a wavelength from 480nm (excitation)-530 nm (emission). The fluorescence measurements for the supernatant were normalized to those from the media and the levels of the control at 25 °C. n=4 independent experiments. Error bars indicate standard error.*

	Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**
Date	6/26/14	7/9/14	7/14/14	7/21/14
Average Fold Change of Supernatant to Cell Pellet	10.217	13.651	15.189	11.498

Table 1. Raw fluorescence data demonstrates at least a 10 fold increase in the relative fluorescence of the supernatant compared to the cell pellet. *At each time point (control 25 °C 0', heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180'), the ratio of supernatant to cell pellet was calculated from the raw fluorescence values and averaged for each independent experiment (n=4).*

These results uncover an important role for ROS during heat stress, both extracellular as well as inside cells. From the raw fluorescence data, there was at least a

ten fold increase in the relative fluorescence of the supernatant compared to that of the cell pellet at each time point and for every independent experiment (*Table 1*). There was a large, ten-fold, difference between the raw fluorescence values of the supernatant compared to the cell pellet and less variation in cell pellet fluorescence compared to that in the supernatant. From this, a working hypothesis is that the majority of the ROS detected does not come from inside the cell to be excreted, but rather is produced in the outer cell wall or extracellular matrix of the algae and diffuses into the supernatant (Figure 2).

Gene Expression of *C. reinhardtii* under heat shock (42 °C)

To understand the difference in ROS levels between the cell pellet and supernatant and to fully characterize the response of *C. reinhardtii* to heat shock (42 °C), gene expression was monitored by RNA extraction and RT-qPCR. RNA was extracted using the Power Plant RNA isolation kit with DNase from Molecular Bio and tested with 10 µL reactions of Qiagen Quantitect SYBR Green RT-PCR kit on the Illumina ecocycler machine. The genes of interest included *crr1*, *ctr1* and *ctr2*. Heat Shock Factor 1 (*hsf1*) was used as a positive control. All expression levels were normalized to Receptor of Activated Protein Kinase C (*rck1*), the housekeeping gene, as a control for each heat shock time point (control 25 °C 0', heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180').

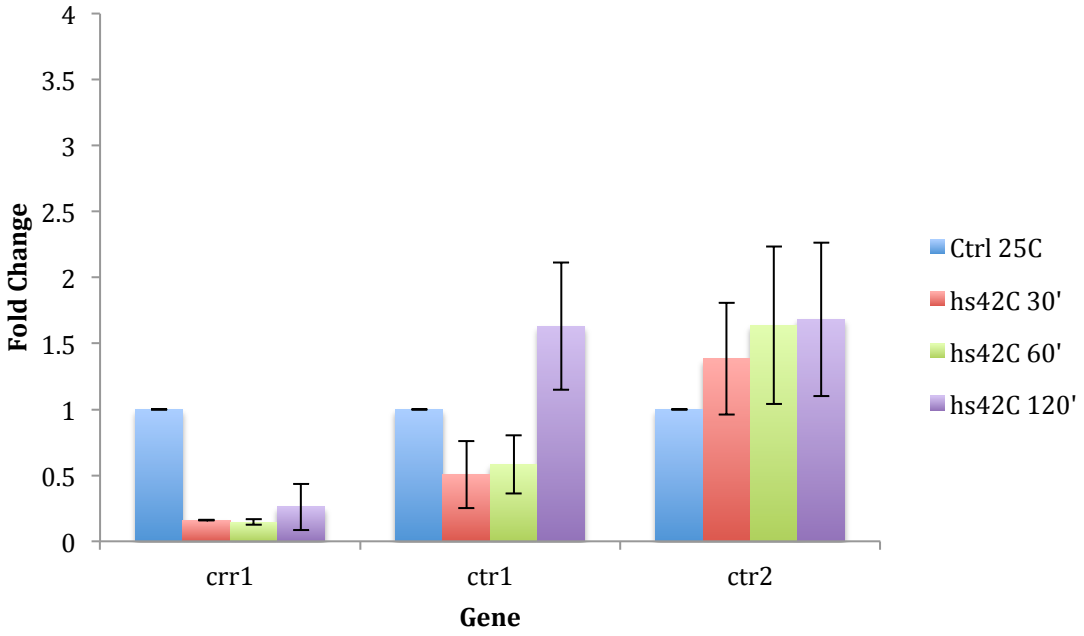

Figure 4. ***Gene expression of*** **C. reinhardtii** ***under heat shock (42 °C) conditions demonstrates selective regulation of crr1 and ctr1.*** *Crr1 and ctr1, but not ctr2 were down-regulated during exposure to heat shock, possibly to block copper from entering the cell. n=3-5 independent experiments.*

Over the course of the heat shock, there was a difference in expression of copper related genes, specifically *crr1*, *ctr1*, *ctr2* which are involved in activation and thus transport of copper in and out of the cell (Figure 4). The 180-minute time point was eliminated since RNA degradation, seen in the lower concentration of extracted RNA, caused large variability and little reproducibility in expression levels. Although not shown in Figure 4, *hsf1* was a positive control since 30 minutes of heat shock at 42 °C produced a 3 fold increase in expression levels compared to culture that experienced no heat shock (25 °C). Thus, it could be ensured that the expression levels seen in Figure 4 occurred when *C. reinhardtii* culture was under heat shock as a form of oxidative stress.

For *crr1*, a decrease in expression compared to no heat shock (25 °C) was detectable as early as 30 min (p=0.008). This decrease was paralleled by a decrease in *ctr1* but not in *ctr2* indicating that there was selective regulation between *ctr1* and *ctr2* during heat shock in *C. reinhardtii*. Since copper can bind with oxygen radicals inside the cell to form ROS and CTR1 transports copper into the cell, it is possible that the down regulation of *ctr1* was a feedback mechanism to block copper from entering the cell. Another explanation is that copper inside the cells acts as a co-factor for ROS scavenging enzymes such as SOD. With the down-regulation of *ctr1* and the possible block in copper import, SOD could lose activity thus causing an increase in ROS levels. The SOD activity inside the *C. reinhardtii* cells was tested (*vide infra*), but the results were inconclusive due to the sensitivity range of the assay. Changes in *ctr2* expression were not significant between the heat-shocked culture (at any time point) to that of the control (25 °C). Overall, these results suggest a selective role for *ctr1* in response to heat stress, and also open up question of whether *ctr2* is also regulated by *crr1* but under different environmental conditions.

The role of copper during heat stress

To uncover the role of copper in *C. reinhardtii* response to oxidative stress, ROS levels were tested in the presence of neocuproine, a Cu(I) chelator dissolved in 100% methanol. Copper (Cu) exists in two forms, Cu(I) and Cu (II) but reduced Cu(I) is the predominant intracellular form. The optimal 0.01 mM neocuproine concentration was determined from PAM Fluorometry titration experiments (Figure 5). PAM Fluorometry measures the efficiency of photosystems II and thus the photosynthetic physiology of *C. reinhardtii.* 0.01 mM neocuporine was found to be the concentration at which *C.*

reinhardtii responded to the lack of Cu(I), but would not die from toxicity (Figure 5). Although 0.001 mM neocuproine gave a similar effect in response to heat shock (42 °C), 0.01 mM was chosen since there was less variation across the different plate wells

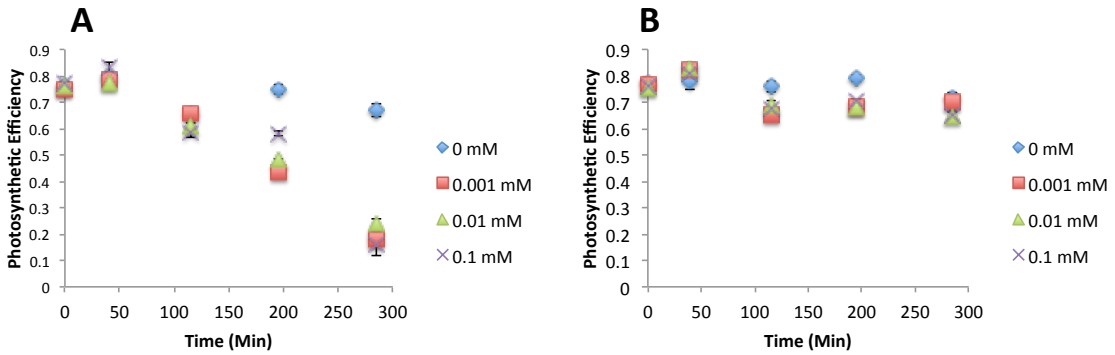

Figure 5. PAM Fluorometry titration of neocuproine concentrations* with C. reinhardtii *under heat shock, 42 °C (A) and control 25 °C (B). *Every hour, a white light XY diving PAM (Walz) provided saturated pulses (intensity 8, gain 1) to* C. reinhardtii *culture (9,180,000 cells/mL) in either 25 °C or 42 °C that had been dark adapted for 10 minutes. The* C. reinhardtii *contained 0.001, 0.01, 0.1 or 0 mM neocuproine in triplicate, added right before the first reading at time 0. The photosynthetic efficiency, YII in the PAM software, was plotted against time.*

C. reinhardtii culture with 0.01 mM neocuproine dissolved in 100% methanol, along with *C. reinhardtii* culture containing an equivalent amount of 100% methanol as control was incubated with DCF-DA which in the presence of ROS becomes oxidized to fluorescent DCF, measurable at wavelength 495 nm. Five time points were used throughout the experiment (control 25 °C 0', heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180') (Figure 6). All supernatant values for each time point were normalized to the baseline ROS values from TAP media (no cells) either with 0.01 mM neocuproine or methanol also containing DCF-DA. The fold change was calculated at each point between the culture containing neocuproine and that with only methanol

control before normalizing to the time 0 (no heat shock) value. The cell pellet values were normalized to a blank with TAP media and cell lysis buffer before calculating the fold change at each time point between the culture containing neocuproine vs. methanol control and normalizing to time 0. The normalization ensured that all effects seen were a result of the neocuproine in the presence of the heat shock.

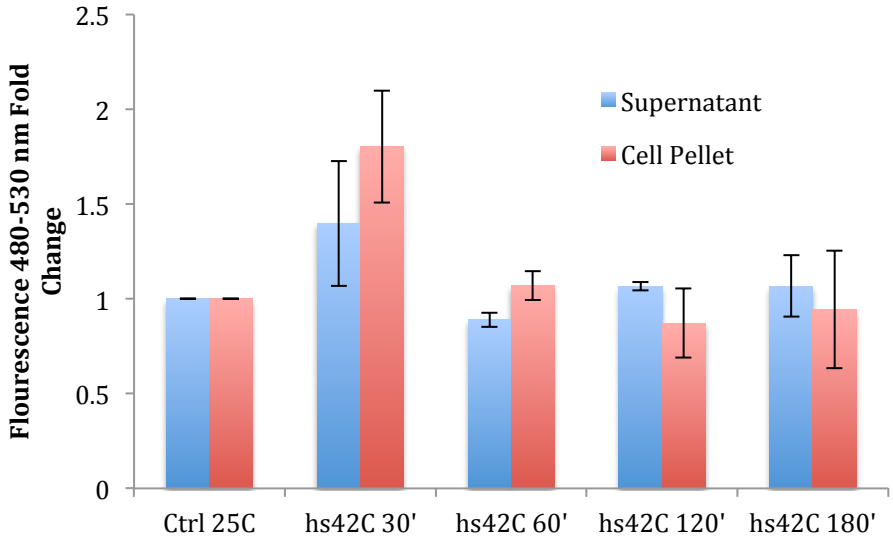

Figure 6. ROS levels of* C. reinhardtii *with 0.01 mM neocuproine Cu(I) chelator in response to heat shock (42 °C). *DCF-DA was added to* C. reinhardtii *culture. In the presence of ROS, DCF-DA is oxidized to fluorescent DCF, detected at wavelengths from 480 nm ex-530 nm em. The fluorescence measurements for the supernatant were normalized to those from the cell medium and the levels of the control at 25 °C. n=3 independent experiments.*

As a result of the heat shock (42 °C), there was an increase in the ROS levels in both the supernatant and the cell pellet at 30 minutes, but this increase was not statistically significant (Figure 6). In the presence of neocuproine in addition to heat stress, the supernatant fold change values after 30 minutes of heat shock were less than

those under heat shock alone (Figure 3). One possibility was that it took longer than 30 minutes for neocuproine to penetrate inside the cell wall, but that neocuproine was immediately able to bind copper in the supernatant allowing for a decrease in ROS supernatant values, but not in the cell pellet by 30 minutes of heat shock (42 °C). By 60, 120 and 180 minutes of heat shock (42 °C), the ROS levels in both the supernatant decreased back down to those of the control (Figure 6). These results suggest that the removal of copper inside the cell was sufficient to cancel both the extracellular and intracellular ROS changes produced by heat (Figure 3).

Initially the raw fluorescence cell pellet values in the presence of neocuproine were less than those in the presence of the methanol control, while the supernatant values were equivalent between the two cultures. Thus, the difference in the fold change between supernatant and the cell pellet of the culture containing neocuproine and the fold change of the culture with only a methanol control resulted in a slight increase (~3) over the first three heat shock time points (control 25 °C, heat 42 °C 30', heat 42 °C 60'). However, by 120 minutes of heat shock, the raw fluorescence values in the cell pellet from neocuproine dropped drastically compared to those from just the methanol control, so that this difference between the neocuproine fold change of the supernatant to the cell pellet increased to over 15.

	Ctrl 25C	hs42C 30'	hs42C 60'	hs42C 120'	hs42C 180'
Difference Between Neocuproine and Methanol Fold Change of Supernatant to Cell Pellet	2.679	1.880	3.032	15.835	24.234

Table 2. Raw fluorescence data demonstrates a decrease in intracellular ROS due to neocuproine based on the increased ratio of relative fluorescence of the supernatant compared to the cell pellet. *At each time point (control 25 °C 0', heat 42 °C 30', heat 42 °C 60', heat 42 °C 120', heat 42 °C 180'), the ratio of supernatant to cell pellet was calculated from the raw fluorescence values and averaged for each independent experiment before taking the difference between the culture exposed to 0.01 mM neocuproine and that exposed to the methanol control (n=3).*

On the other hand, the drop in ROS by 60 minutes may be a result of cell death due to the toxic additive effects of both the chelator and the presence of heat as the neocuproine chelator was titrated at 25 °C. Cell death may also explain the high levels of variability and less reproducibility between the independent experiments. One experiment had to be discarded because *C. reinhardtii* cultures with a higher OD of ~0.7, still in the linear growth phase, died in the presence of the neocuproine. From these results, we were unable to determine whether the ROS effect was due to the lack of copper or cell death. In order to distinguish between these two possibilities, it was necessary to test the effects of neocuproine alone without the additional stressor of heat shock.

The goal was to determine whether methanol, the solvent for neocuproine, was toxic or whether neocuproine itself could be toxic. The viability of *C. reinhardtii* culture (9,180,000 cells/mL) with 2 µL of 25% methanol, the minimum amount of solvent necessary for neocuproine to dissolve, 2 µL of 100% methanol, or with neocuproine in 2 µL 25% methanol was compared to that of *C. reinhardtii* culture alone by measuring

fluorescence, excitation at 480 nm, emission at 600 nm (Figure 7). Viability was monitored by fluorescence of the chlorophyll since *C. reinhardtii* are photosynthetic organisms.

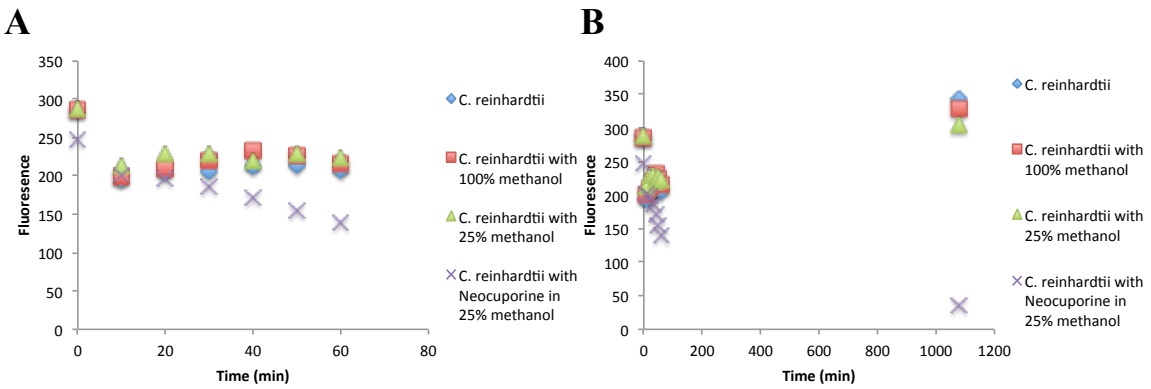

Figure 7. Only neocuproine but not methanol solvent was toxic to* C. reinhardtii *culture after 18 hours. *Every ten minutes for the first hour (A) and 18 hours later (B) fluorescence detected at wavelength from 480nm (excitation)-600 nm (emission) of C. reinhardtii culture (9,180,000 cells/mL). The* C. reinhardtii *contained no methanol, 2 µL of 100% methanol, 2 µL of 25% methanol or 2 µL of 25% methanol with 0.01 mM neocuproine added at time 0.*

After one hour at 25 °C (Figure 7) a decrease in fluorescence was detected in the *C. reinhardtii* culture containing 0.01 mM neocuproine in 25% methanol but not in the *C. reinhardtii* culture with 25% methanol. After 18 hours, all cultures, including the one containing 0.01 mM neocuproine in methanol, survived. However, in order to alleviate some of the harsh effects of methanol on the *C. reinhardtii* culture, neocuproine dissolved in 25% methanol was used to determine the effects of neocuproine without heat shock. The results indicates that neither methanol nor neocuproine, under 25 °C for long durations of time, were toxic to the *C. reinhardtii* cells. This result parallels the initial titration experiment, seen above in Figure 4, where *C. reinhardtii* culture survived 42 °C

with 0.01 mM neocuproine for over 180 minutes. Thus, the lack in change in ROS seen in Figure 6 was not due to the toxicity of neocuproine or methanol solvent. Rather, the removal of copper by neocuproine was sufficient to alleviate and prevent the heat induced increase in ROS both in the cell pellet and the supernatant. This illustrates a new specific role for copper in eliciting the ROS response during heat stress.

Neocuproine toxicity in the absence of heat stress

In order to determine the effect of neocuproine alone, *C. reinhardtii* culture with 0.01 mM neocuproine dissolved in 25% methanol, along with *C. reinhardtii* culture containing 25% methanol control was combined with DCF-DA which in the presence of ROS becomes oxidized to fluorescent DCF, measurable at wavelength 495 nm for all time points (25 °C 0', 25 °C 30', 25 °C 60', 25 °C 120') (Figure 8). The 180' time point was removed since this experiment was conducted after the gene expression result where RNA degradation seen by this time caused large variability and little reproducibility in expression levels. All supernatant values were normalized to the baseline ROS values from TAP media either with 0.01 mM neocuproine in 25% methanol or 25% methanol at each time point. The fold change was calculated at each point between the culture containing neocuproine and that with only methanol control before normalizing to the time 0, no neocuproine. The cell pellet values were normalized to a blank with TAP medium and cell lysis buffer before calculating the fold change at each time point between the culture containing neocuproine and that with only a methanol control and normalizing to time 0 of neocuproine.

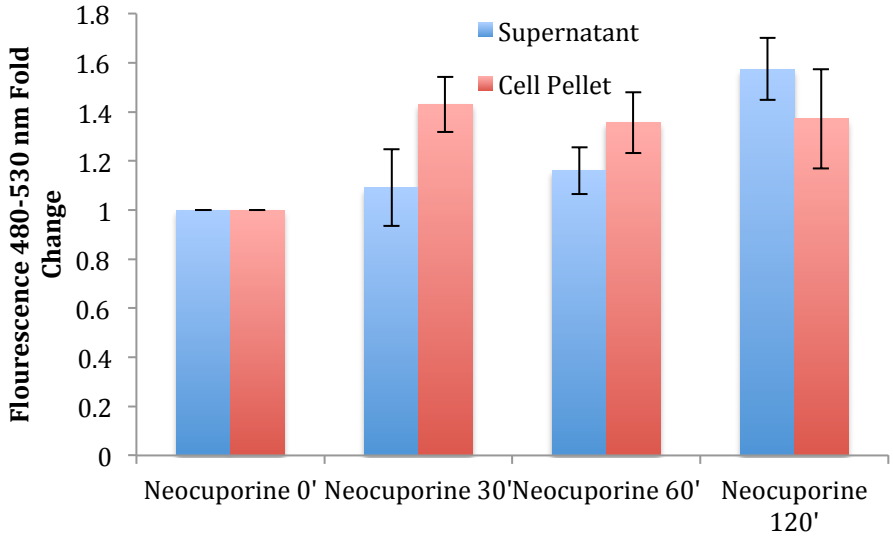

Figure 8. ***ROS levels of*** **C. reinhardtii** ***with 0.01 mM neocuproine Cu(I) chelator dissolved in 25% methanol at 25 °C.*** *DCF-DA was added to* C. reinhardtii *culture. In the presence of ROS, DCF-DA is oxidized to fluorescent DCF, detected at a wavelength from 480nm (excitation) -530 nm (emission). The fluorescence measurements for the supernatant were normalized to those from the media and the levels of the control containing 25% methanol. n=3 independent experiments*

Over the time course of neocuproine at 25 °C, there was a significant increase in the ROS levels in the supernatant by 120 minutes (Figure 8, p=0.019). For the cell pellet this jump in ROS levels was detectable by as early as 30 minutes (Figure 8, p=0.031). These results support the hypothesis that ROS is a universal first consequence to different environmental stressors in *C. reinhardtii*.

Superoxide Dismutase

Superoxide dismutase (SOD), glutathione peroxide and catalase enzymes break down ROS at high levels, since ROS can cause damage to the cell. SOD contains a Cu/Zn metal cofactor, whose activity may be changed in the presence of oxidative stress and

chelated Cu(I). Both during my summer at NASA Ames Research Center and at Wellesley, two methods were used to measure SOD activity in *C. reinhardtii* both in the presence and absence of heat shock as well as in the presence and absence of neocuproine.

During the summer at NASA Ames Research Center, the BioLabs assay kit containing xanthine/xanthine oxidase system to generate superoxide anions and a chromagen was used to produce a water-soluble dye upon reduction by superoxide anions. The superoxide dismutase activity was determined as the inhibition or reduction of chromagen and thus a color change. In the presence of more SOD, there are fewer superoxide anions and thus less fluorescence detected at 490 nm.

For the *C. reinhardtii* culture with 0.01 mM neocuproine as a Cu(I) chelator, there was a significant decrease in superoxide anions and thus an increase in SOD activity as a function of heat shock (42 °C) but that the increase was not detectable by the assay range (Figure 9). These results suggest that in response to heat shock (42 °C), *C. reinhardtii* uses existing Cu/Zn SOD to break down the ROS. However, it should be noted that this assay was not specific to SOD and that the increase in activity detected could only be due to the increase in ROS as it was not possible to separate the two (*Figure 3*).

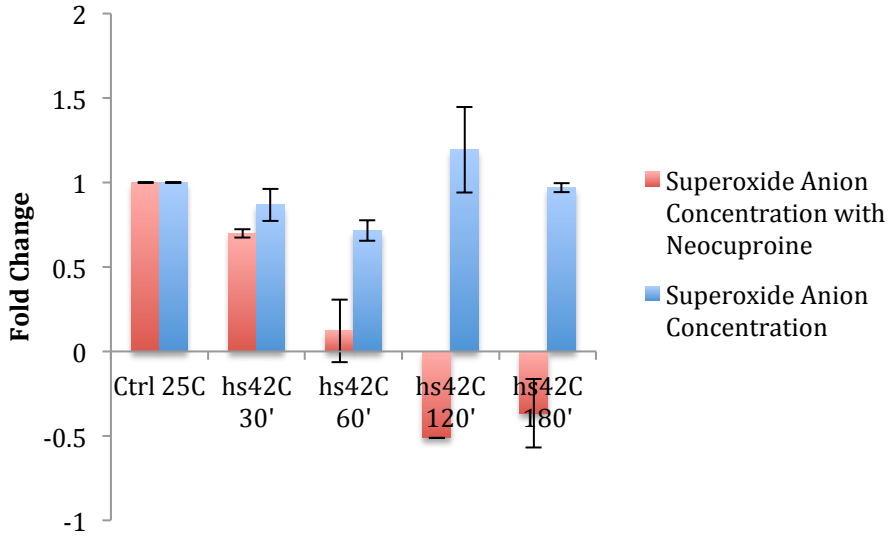

Figure 9. SOD activity of* C. reinhardtii *with 0.01 mM neocuproine Cu(I) chelator in response to heat shock (42 °C). *SOD anions remained relatively constant in the C. reinhardtii control containing only methanol, while decreasing in the* C. reinhardtii *culture containing 0.01 mM neocuporine. The cell pellet was lysed prior to combining with chromogen and SOD Assay buffer (Oxiselect Superoxide Dismutase Activity Assay). In the presence of increased SOD, there are fewer superoxide anions and thus less fluorescence detected at wavelength 490nm after a 60 min incubation at 37 °C. Fluorescence values were normalized to time 0 of the incubation period and the control at 25 °C. N=2 independent experiments.*

Thus, the goal was to use a different assay (Dojindo) to quantify only the SOD enzyme activity without ROS. This method or kit employs Dojindo's highly specific water-soluble tetrazolium salt, WST-1, which produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of WST-1 reduction by superoxide anion was linearly related to xanthine oxidase activity and inhibited by SOD. Surprisingly, SOD activity from *C. reinhardtii* without xanthine oxidase for all *C. reinhardtii* samples (C1 where OD_{750} was 0.67 C2 where OD_{750} was 0.14 and C3 where OD_{750} was 0.079, light gray) was higher than that of the blank containing no sample and

just xanthine oxidase (B1, dark gray) (Table 3). S1 was the coloring without inhibitor blank, S3 was the reagent blank SS was the *C. reinhardtii* sample with xanthine oxidase and S2 was the *C. reinhardtii* sample without xanthine oxidase.

This result indicates that the baseline levels of SOD activity in *C. reinhardtii* were higher than those generated upon the addition of the enzyme to stimulate SOD activity for inhibition. Thus, using this assay, there was not a way to accurately measure SOD inhibition and hence activity. One suggestion was to use more enzyme, but this alone would not solve the problem with the assay.

	C1 (OD = 0.67)	**C2 (OD = 0.14)**	**C3 (OD = 0.079)**	**B1**	**B2 C1 (OD = 0.67)**	**B2 C2 (OD = 0.14)**	**B2 C3 (OD = 0.079)**	**B3**	**B1-B3**
Slope	0.478	0.476	0.298	**0.173**	0.086	0.034	0.004	0.007	0.166

Table 3. SOD activity of* C. reinhardtii *at 25 °C. *Absorbance at 450 nm was higher in the* C. reinhardtii *culture (C1, light gray) without xanthine oxidase than the positive control (B1) such that it was not possible to determine inhibition and ultimately SOD activity. The cell pellet was lysed prior to combining with xanthine oxidase and WST-Buffer (Dojindo). Absorbance was measured over a 20 minute period at 450 nm.*

Unfortunately, it was not possible to measure SOD activity using the WST-1 salt in *C. reinhardtii*. It currently remains unclear whether in the presence of heat shock as a form of oxidative stress, the SOD enzymatic activity increases to break down ROS allowing the *C. reinhardtii* culture to survive. There was a measurable decrease in *crr1* and *ctr1* at the gene expression level, suggesting that copper was not being imported inside the cell (Figure 3). Additionally, copper removal by neocuproine prevented the increase in ROS seen during heat shock (Figure 3, Figure 6). These results indicate that

the mediation of ROS levels was a result of copper activity directly or by a mechanism separate from a copper-dependent ROS scavenging enzyme. For the copper dependent enzyme such as SOD, copper removal would stimulate a decrease in enzyme activity and thus increase the ROS levels while the opposite effect was observed (Figure 6). However, there may still be a role for SOD in the heat stress response.

Discussion

In order to determine the implications of copper transport in response to oxidative stress at the gene expression level as well as the biochemical level, ROS and gene expression were examined using heat shock (42 °C). ROS values were compared to those detected from *C. reinhardtii* culture in the presence of 0.01 mM neocuproine, a copper chelator, at room temperature (25 °C) as well as under the influence of heat shock (42 °C).

The results support the hypothesis that the mechanism of stress response in *C. reinhardtii* is an increase in the level of ROS. In response to different environmental stressors-namely heat shock and the removal of copper using the chelator neocuproine (25 °C)- both the supernatant and cell pellet showed significant detectable increases in ROS levels (*Figure 3*, Figure 8). One concern was the toxicity of the methanol solvent for neocuproine as a possible explanation for the decrease in ROS levels in the presence of both neocuproine and heat (42 °C) after an initial rise (Figure 6). However, both *C. reinhardtii* in 100% methanol and *C. reinhardtii* culture containing 0.01 mM neocuproine in 25% methanol, survived for eighteen hours at 25 °C (Figure 7). Thus, removal of copper by neocuproine was sufficient to alleviate and prevent the heat induced increase in ROS both in the cell pellet and the supernatant, illustrating a new specific role for copper in eliciting the ROS response during heat stress (Figure 6).

Interestingly, the raw fluorescence values of the supernatant were at least ten-fold greater than for the pellet, indicating that most of the ROS produced comes from outside the *C. reinheradtii* cell in the extracellular matrix (*Table 1*). This suggests a mechanism by which the extracellular matrix buffers the cells from ROS-induced stress, which may

be a result of evolutionary pressure. Evolutionarily, the unicellular alga *Chlamydmonas* is part of the order *Volvocales* that ends with the multicellular differentiated cells in the genus *Volvox*.[5, 33] The families of genes involved in developmental processes occurring in *Volvox,* but not *Chlamydomonas,* are overall very similar but with one important difference: *Volvox* has many more genes that encode cell wall and ECM proteins and these extra genes are quite distinct from those present in *Chlamydomonas*.[5] The individual cells of *Chlamydomonas* are surrounded by an ECM that is composed of hydroxyproline-rich glycoproteins.[34] These glycoproteins are present in both an inner layer as well as an outer layer, which is greatly expanded in *Volvox* that may help to cement the *Volvox* cells together.[22]

Over the twelve step program proposed to explain the development of multicellularity in the volvocine algae lineage, the cell walls first become an ECM before being followed by a large increase in the ratio of ECM volume to cell volume.[35] It has been suggested that an advantage for an evolutionary expansion in the ECM is that it allows the algae to compete for growth limiting nutrients.[36] From our experiments we propose a new role for ECM, as a sink for metals that modulate the ROS response, triggered by changes in the environment. This is supported by the observation that the raw fluorescence values were at least ten-fold greater in the supernatant than in the cell pellet (Table 1) so ROS is likely produced in the ECM and collected in the supernatant during heat stress. This ROS production is alleviated by the experimental chelation of copper (Figure 6). While the inactivation of copper can take place in the cell by down-regulating copper transporters in response to stress, the question remains whether copper inactivation in the ECM is also part of the response in the natural environment, or

whether the diffusion of ROS into the medium rather than into cells is sufficient to buffer the cells against toxic ROS. The mediation of the ROS response in the ECM and inside cells most likely protects *C. reinhardtii* and helps cells survive different environmental conditions. Thus the role of the ECM expansion in the evolution of multicellularity could be related to buffering multicellular organisms against changes in the environment. The hypothesis that ECM can be a sink for metals is also supported by the fact that EPS in cyanobacteria, even without containing a sheath, are able remove metals from the surrounding environment by association to the cell surface.[30, 37] Additionally one layer of *Chlamydomonas* has 20 proteins in non-covalent interactions, while a second layer has only a few proteins which may be able to associate with metals.[38]

Results from RNA extraction and RT-qPCR, indicated a decrease in expression of *crr1* compared to no heat shock (25 °C) detectable as early as 30 min, paralleled by a decrease in *ctr1* but not in *ctr2* suggesting that *ctr1* is a target of *crr1* signaling during heat shock (Figure 4)[27]. This result is new since no previous proteomic studies have focused on copper-related genes or proteins in response to heat shock in *Chlamydomonas*.[16, 19] The gene expression data indicate that there was selective regulation of copper transporters between *ctr1* and *ctr2* even though both *ctr* genes are known to be targets of *crr1* dependent signaling.[25] *Ctr1* was down-regulated during exposure to heat shock, possibly to block copper from entering the cell. It is still unclear whether *ctr2* was also down-regulated to stop copper transport from inside the *C. reinhardtii* cell, or that in response to heat shock, regulation of only *ctr1* was necessary to survive. In zinc-limited *C. reinhardtii* cells, the transcript abundance for all three CTR genes decreased.[39] One possible interpretation is that the levels of copper inside *C.*

reinhardtii in response to heat shock did not fluctuate greatly, thus not necessitating the down regulation of *ctr2* in addition to *ctr1* or that another factor induced by heat shock, other than *crr1* contributes to the differential regulation of the two transporters.

The reduced forms of transition metals such as copper act as catalysts and can react with hydrogen peroxide to produce hydroxyl radicals in the Fenton Reaction.[24] Thus, copper is tightly regulated since excess copper becomes toxic and can lead to lipid peroxidation.[40] Using Nanoscale Secondary Ion Mass Spectrometry (NANOSIMS) technology that is able to localize copper into intracellular compartments, *C. reinhardtii* was able to keep intracellular copper constant between approximately 1 x 10^7 and 2.5 x 10^7 atoms per cell.[39] Additionally, SOD, containing a copper metal cofactor, can react with hydrogen peroxide producing molecular oxygen, a hydroxide anion and a hydroxyl radical in the Haber Weiss Reaction.[40] The hydroxyl radical is very unstable, but the most dangerous ROS since even low concentrations can oxidize membrane lipids and cause the denaturation of proteins and nucleic acids.[24]

Under conditions of copper deficiency, the first line defense allows *crr1* to become up-regulated such that copper can assimilate into the cell through *ctr1* and *ctr2*.[41] Since after 30 minutes of heat shock *crr1* expression was significantly lower than that of the control at 25 °C, this suggests that in response to heat shock there was no lack of copper inside the *C. reinhardtii* cells. Our results support the hypothesis that the immediate response to stress in *C. reinhardtii* is to recruit copper regulation to mediate toxic levels of ROS, and allow ROS to act as signaling molecules for a specific response at the gene expression level.

Thus, it remains interesting to examine the gene expression levels in the presence of the Cu(I) chelator neocuproine and in the presence of neocuproine combined with heat shock. Such experiments were started but not fully completed since there was some difficulty extracting RNA from these samples. The working hypothesis is that in the presence of neocuproine which removes Cu(I) from the cell, *crr1* and *ctr1* gene expression levels will increase to import copper back into the cell. Additionally, since *ctr1* and *ctr2* were selectively regulated in response to heat shock, *ctr2* might be up-regulated in addition to *ctr1* or just *ctr2* instead of just *ctr1*.

Future studies should focus on examining on what is happening inside the *C. reinhardtii* cell. This can be done from both an enzymatic standpoint using SOD, as well as trying to determine whether ROS detected in the supernatant was not excreted from inside the cell, but rather was produced in the outer cell wall or extracellular matrix of the algae and diffused in the supernatant. Unfortunately, using DCFH-DA and fluorescence to measure ROS levels provided too much variation. DCFH-DA was unable to detect a significantly significant increase in cell pellet data by 180 minutes of heat shock. Thus, in order to detect changes at a microscopic level Inductively Coupled Mass Spectrometry (ICP-MS) would be a better choice to measure metal concentrations, specifically Cu, inside the cell. Such work has been done using the NANO-SIMS technology at Lawrence Livermore National Lab to spatially detect elements, including copper, simultaneously, based on their mass.[39] Although this technology has very low detection limits and allows for *in situ* experiments, it might be cumbersome when applying heat shock.[42] Thus, ICP-MS would be recommended for future studies, as demonstrated by being able to detect nanomolar concentrations of silver from silver nanoparticles in *C. reinhardtii*.[43]

Even though the current experiments involving SOD were inconclusive, the results from gene expression and ROS suggest the importance of copper and potentially other metals in the *C. reinhardtii* oxidative stress response (Figure 9, Table 3). There may still be increased SOD activity, an enzyme containing a copper cofactor responsible for breaking down ROS, despite the decrease in gene expression of copper transporters to bring copper back into the cell.[24, 44] These results provide new insights about the cellular response to stress and its possible mediation during long-term environmental pressure (including spaceflight), and contribute a new molecular and biochemical viewpoint to understanding the evolution of multicellularity.

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