# THE MOON JELLYFISH (*AURELIA AURITA*): A PROSPECTIVE PELAGIC AND ESTUARINE BIOINDICATOR

by

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A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Biology

Charlotte

2016

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# ABSTRACT

MATTHEW THOMAS LOWDER. The moon jellyfish (*Aurelia aurita*): a prospective pelagic and estuarine bioindicator (under the direction of DR. AMY H. RINGWOOD)

Due in part to the complexity of scyphozoan life histories, slight changes in environmental factors can have an effect on fecundity, growth & development and population fluctuations. Anthropogenic activities have been suggested as a major cause of increases in gelatinous communities. In addition, jellyfish, like other aquatic organisms, are exposed to a host of chemical contaminants in the ambient environment. Heavy metals, such as copper, are a major anthropogenic pollutant found throughout aquatic ecosystems. However, there are few accounts of copper contaminant effects on scyphozoans in terms of biochemical markers, or reported differences of responses between polyps, larvae, and adult medusa. Additionally, there are few accounts of contaminant effects coupled with environmental changes, such as temperature, salinity or pH fluctuations. We explored a variety of biochemical markers and behavioral endpoints to evaluate *Aurelia aurita* as a novel bioindicator species when exposed to a range of copper concentrations (5-50 ppb), for 48 hrs, temperature increases (18 to 24.5°C over two hours) for 48 hrs., Cu concentrations of 10 ppb coupled with temperature increase (18 to 24.5°C over two hours) for 48 hrs and differences between different life history stages (polyps, ephyrae and medusae) exposed to a range of copper concentrations (5-50 ppb). Acute toxicity (mortality) effects were observed at 50 ppb Cu in medusae but no significant mortalities were observed in animals exposed to lower Cu concentrations. *Aurelia aurita* was very sensitive to Cu. Lysosomal destabilization was observed at 5 ppb Cu; and high mortalities, 100%, were observed after only 48-hour exposures to 50

ppb Cu. Significant changes in behavior responses were also observed, and there was a significant correlation between behavior and lysosomal damage. Low baseline levels of GSH, an important antioxidant, were found in both oral arm and bell tissues; low GSH levels could contribute to their high sensitivity. The greatest effects of temperature were reduced GSH levels, which in and of itself is problematic, but can also increase susceptibility to pollutants and other stressors. Relative sensitivities of life history stages showed clear differences between polyps, ephyrae and medusae. *A. aurita* medusae were actually more sensitive than the ephyrae or polyp stages, further highlighting this species' high sensitivity to low levels of a common anthropogenic contaminant and establishes a framework of knowledge for using cellular biomarkers for jellyfish.

# ACKNOWLEDGMENTS

Firstly, I would like to thank the University of North Carolina at Charlotte, the Graduate School and the Department of Biological Sciences for accepting me into a challenging graduate program and allowing me the chance to earn a Masters of Science degree. This includes professors, administrative staff, fellow graduate students and fellow lab members. I would also like to thank my committee members, Dr. Stan Schneider and Dr. Adam Reitzel, for their continued knowledge and support for this project.

I would like to express my gratitude to my advisor, Dr. Amy Ringwood, as well. Without her knowledge, guidance and confidence in my abilities in research and writing, I would not have had the opportunity to pursue, or succeed, in this chapter of my life. I will always be thankful for this opportunity, the knowledge and skills that I have acquired, and for the passion for marine life, restoration and ecotoxicology that she has instilled in me.

I also want to thank my family and friends for their continued support and encouragement. You have seen me at my worst and been there for times when I didn't think I could finish this project. Your stability, reassurance, laughter and love helped make this possible.

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# CHAPTER 1: INTRODUCTION

Estuarine and marine ecosystems are continually threatened by anthropogenic modifications and inputs. Benthic bioindicator species such as bivalves are well-established models for characterizing ecosystem health, but there are few invertebrate bioindicator species for characterizing potential impacts in pelagic realms. Gelatinous zooplankton constitute a significant portion of biomass in many of these systems, and are represented by many phyla, including cnidarians (Schneider, 1989, Romeo et al, 1992). Cnidarians, such as the scyphozoans (jellyfish), are found in estuarine and marine systems in the free-swimming medusa form as well as sessile polyp forms in benthic habitats (Arai, 1997). The polymorphism (Hickman, 1967) observed in jellyfish species is also of importance. A sessile polyp and free-swimming medusa form in one species may behave and respond to environmental stressors and contaminants quite differently, which may have implications for the individual as well as populations. The polyp form has potential value as a traditional benthic, sessile bioindicator while the free-swimming medusa can serve as a valuable pelagic bioindicator. Thus, scyphozoans of the medusa form may prove to be valuable bioindicator for characterizing the effects of anthropogenic stressors in pelagic estuarine and marine systems. Furthermore, a cosmopolitan scyphozoan, such as *Aurelia aurita*, should serve as a valuable model organism for establishing a framework of knowledge that can be applied to other scyphozoans.

The class Scyphozoa (phylum Cnidaria) includes are traditionally referred to as the "true jellyfish." Both medusa and polyp forms exhibit radial symmetry around an oral-aboral axis, with tentacles on the oral side, surrounding the mouth. In medusae, oral arms are also present. The life history of these organisms is complex and involves multiple discreet stages (Figure 1). Adults exist as separate sexes and males release sperm into the water column whereas females release eggs or take up sperm so that fertilization occurs in the gastric pouches and young are brooded for a period of time in brood pouches located on the oral arms (Glichrist, 1937). Planula larvae spend a short period of time in the water column until finding a suitable substrate on which to attach, which can include artificial substrates, such as plastics and glass (Holst and Jarms, 2007). Once attached, the larvae metamorphose into polyps. The polyp form may asexually reproduce through budding, stolon formation, longitudinal fission and pedal laceration (formation of polyps from the pedal disc) (Kakinuma, 1975). Strobilation, the formation and release of ephyra from the polyp, is not a well-understood phenomenon. However, most studies suggest strobilation is induced under certain environmental conditions, such as temperature and salinity (Lucas, 2001) and food availability (Lucas, 2001), which induce gene expression responses involved in the initiation of strobilation, including retinoic acid pathways (Fuchs et al, 2014).

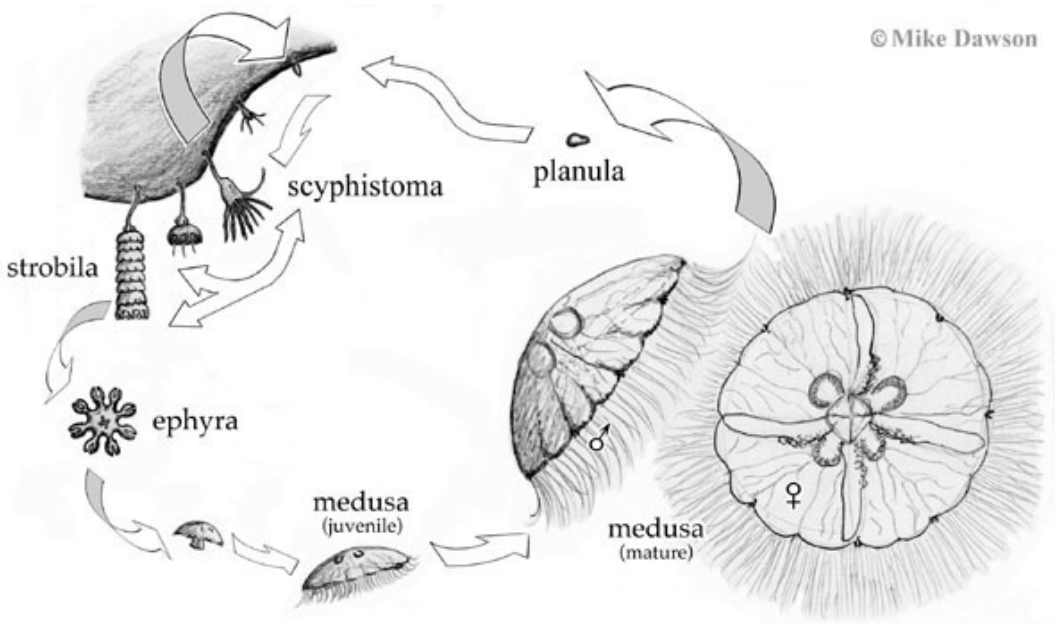


Figure 1.1: Schematic of the scyphozoan life-cycle, specifically *Aurelia aurita*. thescyphozoan.ucmerced.edu/Biol/Ecol/LifeHistory/ScyphozoaLH.html

*Aurelia aurita* is a cosmopolitan scyphozoan of the order Semaeostomae (Arai, 1997) with a global distribution (Arai, 1997; Lucas, 2001). *Aurelia* sp. are considered both eurythermal and euryhaline (Lucas, 2001). Figure 2 is taken from Lucas (2001) and illustrates their broad distribution to a variety of salinity and temperatures (e.g. common in salinities ranging from 18-34 ppt and temperatures ranging from 0-30 °C.

*Aurelia aurita* has been used as a model in studies to understand population dynamics (Lo and Chen, 2008), nutrient cycling (Schneider, 1989; Miller and Graham, 2012), larval substratum selection (Holst and Jarms, 2007; Webster and Lucas, 2012) and genetic controls of strobilation (Fuchs et al, 2014). *Aurelia aurita* has also been used in studies concerning cnidarian toxins and toxic impacts on human health and cnidarian physiology (Shaposhnikova et al, 2005; Seipel and Schmid, 2005) and more recently, to understand increasing anthropogenic concerns of jellyfish ‘blooms’ (Purcell et al, 2007; Dong et al, 2010; Richardson et al, 2009). Despite its widespread use for a variety of

biological studies, *A. aurita* has only recently been considered for ecotoxicological studies (Faimali et al, 2014; Costa et al, 2015; Echols et al, 2016).

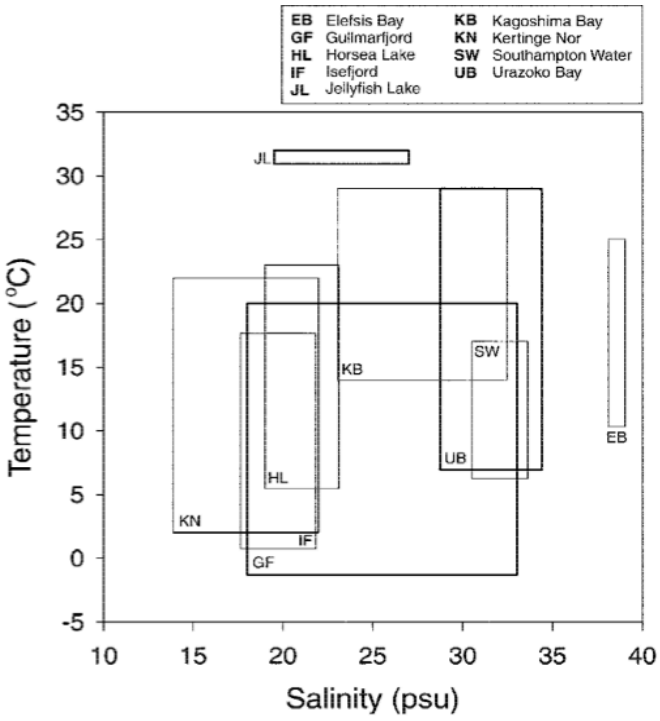


Figure 1.2: Graphical representation of environmental ranges (temperature and salinity) of various *Aurelia aurita* populations. Taken from Figure 1, Lucas, 2001. Locations represent marine habitats from Sweden (GF), England (HL, SW), Denmark (IF, KN), Greece (EB), Palau (JL), and Japan (KB, UB).

Bioindicator species are important tools utilized in ecotoxicology for determining the behavioral, physiological and biochemical responses to potential contaminants and pollutants that organisms are exposed to in the environment. However, commonly used species may not be present in some locales or habitats, so approaches that utilize other species that are present in an area of concern are needed. For example, oysters and mussels are valuable species to evaluate near-shore oil spills associated with benthic habitats, but these species might not be particularly useful for assessing potential impacts of an off-shore spill. While it may be difficult to make comparisons in some cases, due to different mechanisms of contaminant exposure, uptake, accumulation or sequestration,

using various bioindicator species gives more comprehensive analyses of contaminant effects on different species and populations. Interspecies comparisons also enable consideration of relative sensitivity. Populations can also be utilized to make comparisons of contaminant effects in varying geographical areas, as intraspecies comparisons (Rainbow, 1995). For ecotoxicology, it is very important to identify informative model organisms that can be used as biological indicators and enable site-specific assessments in a more robust way (Faimali et al, 2014). Model organisms can be analyzed with a variety of bio-monitoring assays that can give insight into the health of the organism, and ultimately the population and ecosystem (Ringwood et al, 1999). *Aurelia aurita* could potentially serve as a valuable model organism because of their life history strategy, presence in estuarine and pelagic environments and predator/prey trophic interactions (Faimali et al, 2014). *Aurelia aurita* exhibits many characteristics that make it a potentially valuable bioindicator species: abundance, ease of identification and collection, large enough for individual analysis, tolerant of transplantation and handling stress, and somewhat mobile but restricted locally by currents.

Heavy metal pollutants are widely present in aquatic systems due to geochemical and anthropogenic processes. However, anthropogenic activities pose a much greater source for many of these metals, especially at some of the toxic concentrations observed in contaminated environments. Metals in biological systems are classified as essential and nonessential. Essential metals include those that are needed in trace amounts for normal biological functions such as iron (Fe), copper (Cu) and zinc (Zn) (Mason & Jenkins, 1995; Ballatori, 2002). These metals are components of enzymes, storage proteins and transcription factors. Without these essential metals, normal cellular functions are

disrupted which can have dire implications. Other metals are classified as non-essential (Ballatori, 2002). These are metals that, to date, prove to have little or no biological functions, such as cadmium (Cd), lead (Pb) and mercury (Hg). Both essential and non-essential metals can have a wide range of toxic effects. Essential metals can cause toxic effects when concentrations are above or below the biologically critical level. When there is a deficiency of required metals, normal cellular function is inhibited. At higher concentrations, both essential and non-essential metals can interact with cellular components by changing conformation or displacing other metals with similar reactivity and disrupt function (Mason and Jenkins, 1995). Generally toxicity occurs when metal sequestration mechanisms such as GSH and metallothioneins (MT) are impaired or overwhelmed, and when the metals contribute to increased production of reactive oxygen species (Mason and Jenkins, 1995; Deb and Fukushima, 1997; Ringwood et al, 2004). Many transition metals can be considered radicals themselves, due to unpaired electrons in their various forms and they can continue to catalyze other free radicals (Halliwell & Gutteridge, 2007). For example, iron (Fe) and copper (Cu) lead to reactive oxygen species (ROS) via redox-reactions (i.e. Fenton reactions) that can cause sub-lethal cellular damage, such as lipid peroxidation, protein oxidation and DNA damage (Appenroth, 2010; Jomova & Valko, 2011). The following equation shows the generation of ROS via Fenton chemistry with iron and copper:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$
$$(Cu^{+)} \quad (Cu^{2+})$$

Excess ROS can lead to many cellular impairments including damage of DNA, lipids and proteins, senescence and death (Halliwell & Gutteridge, 2007). Subsequent diseases and pathological conditions are dependent on metal concentrations and the type

of tissues in which the cellular damage is located. Ultimately, cell damage is caused by oxidative stress due to oxyradicals (ROS) that have not been removed or chemically inactivated (Kelly, 1998).

Copper (Cu) is a heavy metal that is essential biologically as a cofactor in many enzymes that undergo redox-chemistry (Koch et al, 1997; Ringwood, Conners & DiNovo, 1998; Krupanidhi et al, 2008; Jomova and Valko, 2011) and in the electron transport chain (Valko et al, 2005). Despite its essential nature, Cu can elicit toxicity responses at levels as low as 1ug/L (Levy et al, 2007) and it is one of the most common metal contaminants. Natural concentrations of dissolved copper in open ocean seawater range from 0.03-0.39 ug/L or part per billion (ppb) (Bruland, 1983; Donat & Bruland, 1995) but much higher concentrations are found in polluted waters (2-150 nM; 11.7-877.2 ug/L) (Donat et al, 1994; Moffett et al, 1997). Deposits of copper ores can contribute trace amounts, but most copper in marine systems can be attributed to anthropogenic activities such as smelters, electric-plating facilities, fertilizers, herbicides, pesticides, anti-fouling paint, industrial runoff and mining activities (Stauber and Davies, 2000; Levy et al, 2007; Namiesnik and Rabajczyk, 2010).

Copper toxicity is dependent upon how much copper is available to the organism for uptake (bioavailability) and speciation of the metal. For copper, $Cu^{2++}$, the inorganic species, acts as the toxic species and one mode of toxicity is to high-jack uptake mechanisms for other essential metals like $Mn^{2++}$ and $Zn^{2++}$ (Sunda and Huntsman, 1983, 1998; Buck et al, 2006). Speciation depends on many physicochemical parameters including dissolved oxygen, pH, salinity, and dissolved organics (Deb & Fukushima, 1997). Bioavailability is also affected by the affinity of metals to interact with dissolved

organics, inorganics, and adsorption and redox changes in sediments (Burland, 1983; Donat & Burland, 1995). Therefore, organisms may experience different toxicity responses at the same copper concentration if environmental parameters vary.

Copper toxicity has been studied in many organisms, including fish, molluscs, crustaceans and other invertebrates (Ringwood et al, 1998; Krumschnabel et al, 2005; Valavanidis et al, 2006; Rhee et al, 2013). Many invertebrate species have been used in copper studies and exhibit variation in their sensitivities. For example, Johnston & Keough (2000) used $CuSO_4$-coated blocks to simulate Cu polluted waters. Timing and frequency of Cu release were manipulated around artificial settlement plates to see the effects on larval settlement and development for various invertebrate species. They found that barnacle species recruitment densities decreased by one-third, scyphozoan and sponge species were reduced by 50% and certain polycheate species were insensitive to the above-normal Cu concentrations. In a similar study from Brown et al (2004), a variety of biomarker assays (lysosomal stability, neurotoxicity, and metabolic impairment) were used to compare copper toxicity between commonly used bioindicator species: a shore crab (*Carcinus maenas*), common limpet (*Patella vulgata*) and blue mussel (*Mytilus edulis*). This study showed *P. vulgata* was highly sensitive to copper, with significant changes in biomarker responses at low concentrations of 6 ppb Cu and no survival at 40 and 70 ppb Cu. *C. maenas* showed intermediate sensitivity to Cu with dose-dependent response in three of the five biomarkers used at the highest concentration (70 ppb Cu). *C. maenas* showed no mortality. Compared to the other organisms in this study, *M. edulis* was insensitive to Cu. There was no mortality, and only the lysosomal destabilization showed significant results at 70 pbb Cu, with previous work citing lysosomal

destabilization occurring in *M. edulis* at concentrations as low as 40 ppb Cu (Viarengo et al, 2000).

There are numerous copper studies and toxicity data based on laboratory investigations, in which one stressor is applied in isolation; exposure to copper can be simulated while all other factors (physicochemical factors, feeding rates, etc.) are tightly controlled. But organisms rarely experience a single stressor in nature (Holmstrup et al, 2010) and *in situ* responses are a product of the combination of stressors from the environment. Given that physicochemical factors can influence the toxicity of a metal, and environmental factors, such as temperature, can also act as stressors, it is important to incorporate the potential effects of physicochemical stressors when characterizing metal toxicity responses for a more comprehensive view of how multiple stressors affect the organisms.

Natural environmental factors, both biotic and abiotic, can greatly influence growth and development, fecundity, reproduction and mortality of marine populations. Specifically, abiotic factors such as pH, salinity, temperature and dissolved oxygen can affect suspension and behavior in the water column, as well as immobilization, settlement and recruitment to substrates in cnidarians (Lucas, 2001). A recent study (Conley & Uye, 2015) showed that *A. aurita* planulae were able to delay metamorphosis and remain in the water column as swimming larvae in hyposaline conditions, potentially effecting polyp population distributions. This study, as do many others, show the importance of physicochemical parameters and highlights that marine organisms experience a combination of anthropogenic and natural environmental stressors (Rhee et al, 2013; Gomiero & Viarengo, 2014; Makabe et al, 2015; Lewis et al, 2016; Tills et al, 2016).

Fluctuating temperature is an environmental stress that is experienced, to some degree, by all marine organisms, especially in temperate habitats. Because temperature changes affect many physiological and biochemical processes, interactions between temperature and contaminants are to be expected (Heugens et al, 2001). Holmstrup et al (2010) reviewed existing literature of interactions between anthropogenic contaminants and environmental stressors, including copper, for freshwater organisms. Table 1 is derived from this review and shows various copper studies and it's interaction with temperature stress, specifically heat shock. It should be noted that while this table comprises studies of freshwater invertebrates, it illustrates the variation in copper-heat shock interactions experienced across many phyla. Coupled Cu toxicity and temperature fluctuation work has been done in marine invertebrates as well (Khan et al, 2006; Gomiero and Viarengo, 2014; Lewis et al, 2016).

Table 1.1: Summary of studies (derived from Holmstrup et al 2010 Table 1) showing results from experiments performed with the toxicant copper and the interactive effects of heat shock. The outcomes were no changes in Cu toxicity, synergistic effects or antagonistic effects of Cu and temperature.

| Toxicant | Test Organism | Species | Life Stage | Interaction | Reference |
|---|---|---|---|---|---|
| Copper | Snail | *Viviparus bengalensis* | Adult | Synergistic | Gupta & Khangarot (1981) |
| Copper | Amphipod | *Gammarus pulex* | Adult | Synergistic | Bat et al (200) |
| Copper | Mussel | *Dreissena polymorpha* | Adult | Synergistic | Rao & Khan (2000) |
| Copper | Copepod | *Diaptomus clavipes* | Adult | Synergistic | Boeckman & Bidweel (2006) |
| Copper | Water Flea | *Daphnia pulex* | Adult | None | Boeckman & Bidweel (2006) |
| Copper | Fish | *Ictalurus punctatus* | Adult | Antagonistic | Perschbacker (2005) |
| Copper | Crayfish | *Orconectes immunis* | Adult | Synergistic | Khan et al (2006) |
| Copper | Fish | *Pagrus major* | Adult | None | Furuta et al (2008) |
| Copper | Fish | *Paralichys olivaceus* | Adult | None | Furuta et al (2008) |

While there is some literature on heavy metal effects in cnidarians, most studies have focused on anthozoans (Brock and Bielmyer, 2013; Mitchelmore et al, 2003a; Mitchelmore et al, 2003b; Reichelt-Brushett and McOrist, 2003; Bielmyer et al, 2003; Anjos et al, 2014) and hydrozoans (Grant et al, 2010), with little information available on heavy metal effects in scyphozoans (Faimali, 2014; Templemann and Kingsford, 2010; Romeo et al, 1989). A recent study by Gambardella et al (2015) found *A. aurita* ephyrae to be the most sensitive species when compared to various other invertebrate species exposed to silver nanoparticles. However, this particular study focused solely on behavioral response endpoints and other available literature focuses on metal accumulation and behavioral endpoints. Thus far, cellular biomarkers have not been used to evaluate sub-lethal stress responses in *A. aurita*.

Cellular biomarkers are conducted using a variety of cellular and molecular assays to characterize quantitative and qualitative cellular response data to contaminants and toxicants (Moore et al, 2004). These tests are often used with bioindicator organisms and in environmental management programs because they provide health status on populations and ecosystems through the use of resident individuals, wide ranges of applicability, predictive value and fast and cost-effective results (Moore et al, 2004; Dallas & Jha, 2015). They can also be used to assess the health of an ecosystem under chronic stress and the effectiveness of remediation programs (Connors & Ringwood, 2000; Ringwood et al, 2003; Colin et al, 2016).

The overall goal of this study was to evaluate *A. aurita* medusae as a potential bioindicator species for pelagic systems using a suite of biomarker assays, including tissue damage (lipid peroxidation and lysosomal destabilization) and antioxidants

(glutathione) as well as tissue accumulation of the environmental contaminant copper and behavioral analyses. This work provides essential new knowledge regarding *A. aurita*'s sensitivity and suitability for pelagic biomonitoring and remediation programs. *Aurelia aurita* is an easily identifiable species, cosmopolitan and abundant in many pelagic systems, large enough for multiple analyses and fairly resistant to sampling stress. Some analyses of the sessile polyps were conducted that increases the existing body of knowledge for sedentary cnidarian bioindicators, but, the emphasis of this work has been with the pelagic life stages.

Invertebrates are highly underrepresented as bioindicators in pelagic systems. Fish often dominate as bioindicators in these environments. *Aurelia aurita* could provide information on water column exposures that may be applicable to other invertebrate species. Because the polyps are generally attached to substrata on or near the benthos, they may be more susceptible to contaminants that settle out of the water column and are adsorbed by benthic sediments. They would also be susceptible to contaminants that leech from the sediments induced by environmental changes (temperature, pH, salinity, etc.). The medusae would be more susceptible to contaminants that remain suspended in the water column as well as those that flux out of the sediment.

To evaluate *Aurelia aurita* medusae as a model bioindicator species, three main objectives were addressed:

**Objective 1**-To evaluate copper accumulation, tissue damage and glutathione levels when exposed to a range of copper concentrations in the tissues of *A. aurita* medusae.

*Hypothesis 1*-Medusae will show a dose-dependent response to copper concentrations. As concentration of copper increases, accumulation of copper deposits in tissue will increase. As concentration of the contaminant increase, lysosomal

destabilization and lipid peroxidation will increase and glutathione levels (GSH) will decrease.

**Objective 2**-To evaluate the effects of copper exposures, with simultaneous exposure to increasing temperatures, on tissue damage, glutathione levels, and copper tissue concentrations in *A. aurita* medusae.

*Hypothesis 2*- Temperature will exacerbate copper toxicity in medusae. Temperature in combination with copper exposures will increase tissue damage (lysosomal destabilization and lipid peroxidation) and reduced glutathione compared to copper alone; copper concentrations in tissues will be greater when temperatures are elevated.

**Objective 3**-To compare the relative sensitivities of different life history stages (polyps, ephyrae and adult medusae) of *A. aurita* when exposed to a range of copper concentrations.

*Hypothesis 3*- Polyps and ephyrae will be more sensitive to copper and have higher levels of tissue damage and glutathione when compared to adult medusae due to the sessile nature of polyps and the sensitivity during development of the ephyrae.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Copper Exposures

*In vivo* exposures were conducted with animals donated from Discovery Place Inc., a science museum in Charlotte, NC that propogates *A. aurita*. For each exposure (four total), various Cu solutions were prepared from a 500 ppm stock solution of cupric chloride. Serial dilutions of 50, 25, 10 and 5 ppb were used for the first two exposures and 25, 10 and 5 ppb solutions were used for the final two exposures. All copper solutions were prepared in Instant Ocean saltwater with 25-30 ppt salinity and 7.95-8.05 pH, at room temperature. Four to five adult jellyfish were placed in 2 gallon buckets with 7 L of 50, 25, 10, 5 ppb and 0 ppb (controls) Cu-saltwater solutions. During the 48-hour exposures, jellyfish were fed brine shrimp nauplii (*Artemia salina*) at approximately the 24-hour time point. Exposures were performed at room temperature (18-20°C) with constant aeration. Salinity and pH were monitored and recorded daily for each bucket. Additionally, behavioral observations were monitored and recorded at the beginning, during, and at the end of each exposure.

At the end of each exposure, animals were dissected and tissues were separated for biomarker and tissue Cu analyses. Freshly dissected living tissues, including bell, oral arm and tentacle were used for lysosomal destabilization assays. All remaining tissues were separated into bell and oral arm fractions and frozen at -80°C for total protein analyses, lipid peroxidation assays, total glutathione assays and for the determination of tissue Cu concentrations using atomic absorption spectrometry. Tissue processing and

analyses were performed in this manner for temperature exposures and Cu/temperature exposures.

2.2 Temperature Exposures

As with the copper exposures, animals were donated from Discovery Place, Inc. for temperature exposures. For each of the two 48-hour exposures, animals were placed in 2 gallon buckets with 7 L Instant Ocean saltwater with 25-30 ppt salinity, 7.95-8.05 pH, at room temperature. Temperature was closely monitored throughout both exposures, with starting temperatures at 18°C. Exposure temperatures were raised over a 2-hour period to 24.5°C. To do this, two 2-gallon buckets were placed in an insulated water bath and the temperature of the bath was raised with an Aqueon Pro50 50 W aquarium heater. Gradually raising the temperature, even over a short period of time, helped to ensure that results did not reflect shock induced by abrupt and immediate temperature changes. During the 48-hour exposures, jellyfish were fed brine shrimp nauplii (*Artemia salina*) at approximately the 24-hour time point and aeration was constant. Salinity, pH and temperature were monitored and recorded daily for each bucket. Additionally, behavioral observations were monitored and recorded at the beginning, during and at the end of each exposure, including pulsation rate (pulse/minute) and feeding behavior. Bell diameter was recorded initially and at the end of each exposure for each animal. At the end of each exposure, animals were dissected and tissues were separated for further biomarker analyses.

2.3 Copper and Temperature Exposures

Combined Cu and temperature exposures were performed similarly to temperature-only exposures, with the addition of a single Cu concentration. A

concentration of 10ppb Cu was used for all combined exposures. This concentration was chosen because this treatment group showed modest changes in behavioral and biomarker endpoints in Cu-only exposures. When combined with elevated temperatures, it is predicted that more pronounced changes would occur. Two 48-hour exposures were conducted. Water quality was measured throughout the exposure, as in the temperature exposures (25-30 ppt salinity, 7.95-8.05 pH). Starting temperatures of 18°C were increased over a 2-hour period to 24.5°C using an Aqueon Pro50 50 W aquarium heater to heat a water bath in which exposure buckets were located. Jellyfish were fed brine shrimp nauplii (*Artemia salina*) at approximately the 24-hour time point and aeration was constant. Salinity, pH and temperature were monitored and recorded daily for each bucket. Behavioral observations were monitored and recorded at the beginning, during and at the end of each exposure, including pulsation rate (pulse/minute) and feeding behavior. Bell diameter was recorded initially and at the end of each exposure for each animal. At the end of each exposure, animals were dissected and tissues were separated for further biomarker analyses.

2.3 Life History Stages Exposures

Different life history stages, including polyps, ephyrae and medusae were compared to evaluate differences and results of biomarker endpoints. As with copper exposures previously described, polyps and ephyrae were exposed to various copper concentrations (5, 10, 25, 50 ppb). All copper solutions were prepared in Instant Ocean saltwater with 25-30 ppt salinity and 7.95-8.05 pH, at room temperature. Fifteen to 20 poylps were placed in Pyrex dishes with 200 ml of Cu-saltwater and ephyrae were place in glass beakers with 500 ml of Cu saltwater. During the 48-hour exposures, polyps and

ephyrae were fed brine shrimp nauplii (*Artemia salina*) at approximately the 24-hour time point. Exposures were performed at room temperature (18-20°C). Glass beakers containing ephyrae were under constant aeration for oxygen and perturbation water to keep ephyrae suspended in the water column. Pyrex dishes containing polyps were not under aeration, as these dishes were shallow enough for oxygen exchange at the air-water surface. Salinity and pH were monitored and recorded daily for each dish and beaker. Behavioral observations were not monitored other than feeding behavior.

At the end of each exposure, polyps and ephyrae were pooled into three samples, consisting of 15-20 individuals, for biomarker analyses. Fresh, living polyps and ephyrae were used for lysosomal destabilization assays. All remaining polyps and ephyrae were separated into pooled samples and frozen at -80°C for lipid peroxidation assays and total glutathione assays. Due to these life history stages being less available for exposures, only biomarker analyses were performed after Cu exposures. Temperature and temperature-Cu exposures were not performed. Additionally, total protein and accumulation data were not performed because of the small masses of these life history stages and the masses needed to perform multiple analyses. To perform all analyses, tissue would need to be tripled or quadrupled for adequate amounts of tissue (i.e. 60-80 individuals would have been needed for each treatment group). Therefore, polyps, ephyrae and medusae were compared with lysosomal destabilization, lipid peroxidation and total glutathione assays after being exposed to various Cu concentrations.

2.5 Metal Analyses

Copper tissue concentrations were analyzed using atomic absorption spectrometry. Bell and oral arm tissues (0.5-1.0 g and 0.3-0.5 g, respectively), previously

frozen at -80°C for each exposure, were weighed (wet tissue weight), and lyophilized using a VirTis benchtop K Lyophilizer for 24 hours. Each sample was then weighed (dry tissue weight), homogenized and placed in glass vials with 2 ml of 50% ultra pure nitric acid. Glass vials were then placed into a vented glass box and microwaved five times for 30 seconds at 50% power, inoculated for 2-3 days at room temperature and the fully digested samples were then diluted with ultra-pure water to finalize sample preparations. Samples, standards and oyster standard reference tissues were then analyzed using a Perkin Elmer AAnalyst 200 (Waltham, MA, USA) atomic absorption spectrometer using flame and Deuterium background correction. Results were expressed as µg Cu per gram of dry tissue weight (ppm).

## 2.6 Toxicity Endpoints

### 2.6.1 Total Protein Analyses

The Bio-Rad protein assay (based on the Bradford method) was used to analyze tissue protein concentrations in both the oral arm and the bell of all jellyfish. The Bradford assay utilizes Coomassie Brilliant Blue G250 dye to tag proteins and allows for quick processing time and small sample volumes to detect stable protein conjugates (Bradford, 1976). Tissues were weighed and homogenized in 5 times volume of 8 mM sodium phosphate buffer at 7.4 pH, containing 0.2% Triton X-100 and 0.0174% phenylmethanesulfonyl fluoride (PMSF) then centrifuged (10,000 g for 15 minutes at 4°C). Subsamples of 100 µl of the supernatant were then diluted with 200 µl of sodium phosphate buffer not containing Triton X-100 or PMSF. Standards of known protein concentrations (Bovine Albumin Serum) were prepared (5, 2.5, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/mL) from a 10 mg/mL stock solution. Twenty- µl of samples and

standards were combined with 180 µl of Bio-Rad Coomassie Brilliant Blue Dye in a 96-well plate and analyzed at 595 nm with a MultiSkan Go 3.2 spectrophotometer (Thermo Fisher Scientific) after 5 minutes of incubation. Results were expressed in mg/g tissue wet weight.

2.6.2 Total Glutathione

Glutathione is a tripeptide (Glycine-Glutamate-Cysteine) that is the most abundant antioxidant in biological systems (Kosower & Kosower, 1978; Mason & Jenkins, 1995; Ringwood et al, 2003). During stress, levels of GSH may increase as a compensatory measure, but GSH levels may be depleted during prolonged periods of more severe stress (Ringwood et al, 1999), which makes cells more susceptible to additional stressors (eg, potentiation) (Viarengo et al, 1990; Regoli & Principato, 1995; Doyette et al, 1997; Connors & Ringwood, 2000; Ringwood & Connors, 2000).

To determine glutathione levels in tissues, a DTNB-GSSG Reductase Recycling assay for glutathione was used. Tissues were weighed and homogenized in 4 volumes of 5% sulfosalicylic acid (SSA) and centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (100µl) were further diluted with 200 µl of SSA. Standards of known glutathione concentrations were prepared (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 µM) from a 1 mM oxidized glutathione stock solution (Sigma-Aldrich, MO). Samples and standards (30µl) were added to 96-well plates with 150 µl of nicotinamide adenine dinucleotide phosphate (NADPH), 40 µl of 5,5'-dithiobis(2-nitrobenzoic acid), 23 µl of water. Just prior to reading the place, 7 µl of glutathione reductase from baker's yeast (GSSG-reductase, Sigma-Aldrich) was added to each well. A kinetic analysis was performed using a MultiSkan Go 3.2 spectrophotometer (Thermo Fisher Scientific) at

412 nanometers every 30 seconds for 2 minutes to determine the rate of conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by NADPH-dependent reductase (Figure 3). Results were expressed as nmol/g wet tissue.

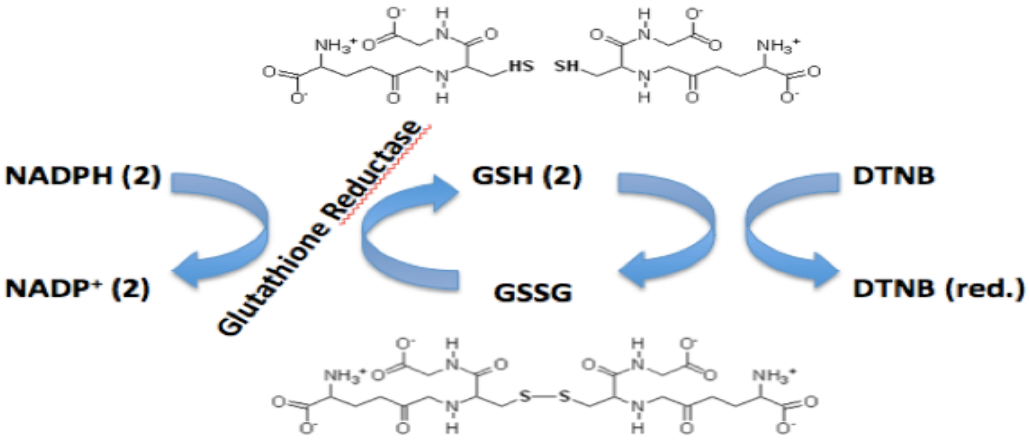


Figure 2.1: Diagrammatical representation of the GSH-recycling assay. All glutathione (oxidized and reduced) is converted and stabilized in the oxidized form; then the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) is caused by reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH).

### 2.6.3 Lysosomal Stability

A neutral red lysosomal destabilization assay was used to assess the integrity of lysosomal membranes (Ringwood et al, 2003; Moore, 2004; Moore, 2006; Canesi et al, 2010). Due to their functions in cellular detoxification and sequestration, lysosomes serve as primary target organelles for contaminants. Inhibition of the ATP-ase proton pump leads to degradation of the lysosomal membrane or direct membrane damage by oxyradicals con occur and, thus, leakage of lysosomal contents (proteases, lipases, etc.) into the cytosol. In damaged cells, neutral red dye is used to assess leakage of contents from the lysosome to the cytosol, which will ultimately lead to cell death. Lysosomal stability in hepatopancreatic (digestive gland) tissues of oysters is a well-established marker of cellular damage (Ringwood *et al.* 2005). Therefore, this assay was

incorporated into the analysis of tissue damage for *A. aurita*. Minor changes to the established protocol were used due to differences in oyster and jellyfish tissues. Pie-slice shaped tissue samples (including bell and oral arm tissues), approximately 150-250mg, were processed into primary cell preparations with Ca/Mg-free-saline (CMFS) and trypsin, sheared to break apart clumps of cells and filtered through 41μm nylon screen. Cells were rinsed and re-suspended in CMFS and a stock solution ($1/5^{th}$ dilution) of neutral red (NR) (0.04mg/mL) was added at a 1:1 ratio of NR to cell preparation volume. After a 60-minute incubation period cells were scored as either stable (NR contained within the lysosomes) or destabilized (NR diffusing into the cytoplasm from damaged lysosomes) at 400x magnification, with at least 50 cells scored from each preparation (Figure 4).

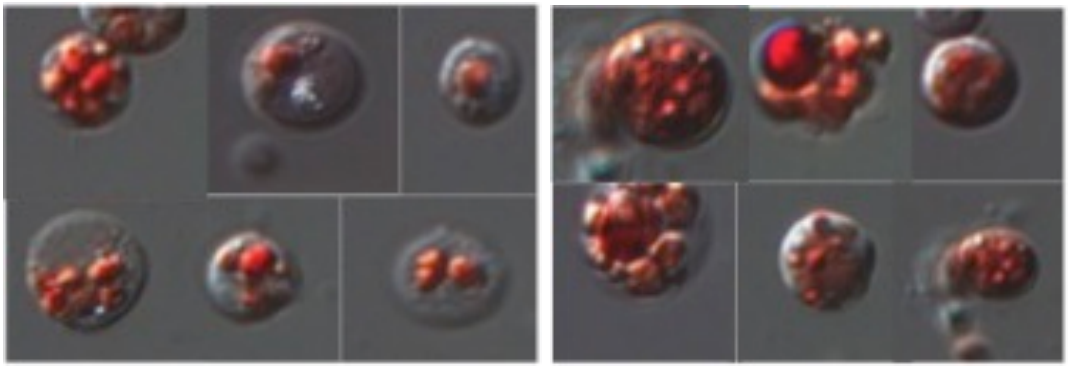

Figure 2.2: Phase contrast images of medusa cells that have taken up dye from the lysosomal assay. Stable (left) and unstable (right).

2.6.4 Lipid Peroxidation

Lipid peroxidation occurs when free radicals react with membrane lipids resulting in products, such as reactive aldehydes, that damage proteins, membranes and DNA (Kelly et al, 1998; Ringwood et al, 2003). Malondialdehyde (MDA) is the most abundant reactive aldehyde produced from the breakdown of polyunsaturated fatty acids of membrane phospholipids by oxygen free radicals, and is often used a general indicator of

oxidative damage (Moore 2006). Therefore, a MDA assay was used to measure the levels of oxidative damage (Ringwood *et al.* 2003). Tissues were weighed and homogenized in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0), then centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (50µl) were combined with 700 µl of 15% trichloroacetic acid containing (0.375%) thiobarbituric acid and 7 µl 2% butylated hydroxytoluene. Standards of known MDA concentrations were prepared (800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM) from a 10mM malondialdehyde tetraethylacetal stock solution (Acros organics, NJ), as previously described (Buege & Aust, 1978; Gray, 1978). Samples and standards were boiled in a water bath for 15 minutes, and then centrifuged (13000 RCF, 5 minutes, room temperature). The subsamples of each supernatant were then pipetted into a 96-well plate (200-µl in each well), and MDA levels were measured at 532 nm using a MultiSkan Go 3.2 spectrophotometer (Thermo Fisher Scientific). The results were then expressed as nmol/g wet weight tissue (Figure 5).

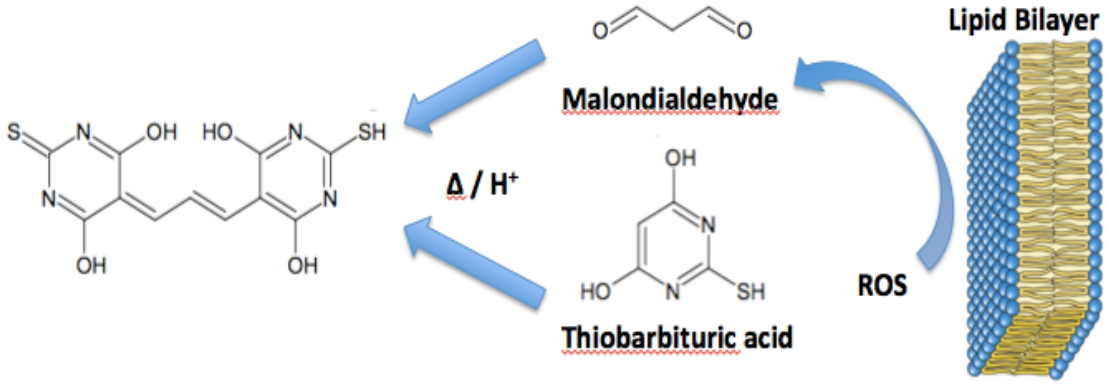


Figure 2.3: Diagrammatical representation of reactive oxygen species (ROS) reacting with phosolipids to produce the reactive aldehyde, malondialdehyde (MDA). MDA reacts with thiobarbituric acid in the MDA assay to form a colored adduct for lipid peroxidation analyses.

## 2.7 Behavioral Analyses

For all exposures, animal behavior was monitored. This included initial and final bell diameter, pulsation rate (pulses/minute) and feeding behavior. Bell diameter was recorded initially and at the end of each exposure for each animal as an observable measure of increased or decreased somatic growth. Pulsation rates were monitored for measures of individual activity in response to copper, temperature or both. Additionally, positions of the jellyfish (whether or not animals remained at the bottom of each bucket or if they remained in the water column) were recorded. Finally, feeding behavior was observed. This included whether or not individuals actively took in food and if it was ingested or not.

## 2.8 Statistical Methods

Data were analyzed using Sigma Stat 2.0 and Graphpad Prism 6. Data were pooled from different exposures for statistical analysis when significant differences between experiments were not detected. Analysis of Variance (ANOVA) was used to compare Cu treatments for the Cu-only exposures. When significant differences were detected ($p \leq 0.05$), a one-way ANOVA was used to analyze differences between treatment groups within each copper-only exposure, temperature-only exposures and copper-temperature exposures. Pairwise comparisons using the Student-Newman-Keuls methods were further used to determine significant differences between treatment groups and controls. When normality or equal variance tests failed, a one-way ANOVA on ranks was performed and pairwise comparisons were made using Dunn's methods to determine significant difference in copper-exposed groups to controls. Regression analyses were used to identify relationships between tissue Cu accumulation and lysosomal

stabilization, tissue Cu accumulation and pulsation rate, and lysosomal stabilization and pulsation rate. Additionally, regression analyses were used to determine possible dose-dependent responses for lysosomal stabilization and glutathione.

# CHAPTER 3: THE EFFECTS OF COPPER ON *AURELIA AURITA* AS A PROSPECTIVE BIOINDICATOR FOR MARINE SYSTEMS

## Introduction

Jellyfish are ubiquitous in marine systems, ranging from polar to tropical oceans and from shallow bays and estuaries to great depths of pelagic areas. Due in part to the complexity of scyphozoan life histories, even slight changes in environmental factors can have an effect on fecundity, growth and development and population fluctuations. Like other aquatic organisms, jellyfish are exposed to a host of chemical contaminants in the ambient environment. Therefore, a cosmopolitan scyphozoan, such as *Aurelia aurita*, could serve as a valuable model organism for environmental monitoring in pelagic ecosystems.

Heavy metals, such as copper (Cu), are major anthropogenic pollutants found throughout aquatic ecosystems. Anthropogenic sources of Cu pollution in marine systems include anti-fouling paint, fertilizers, herbicides, pesticides and industrial runoff (Stauber and Davies, 2000; Levy et al, 2007; Namiesnik and Rabajczyk, 2010). Despite its role as a pollutant in the environment, Cu is also biologically essential as a cofactor in many enzymes that undergo redox-chemistry (Koch et al, 1997; Mason and Jenkins, 1998; Ringwood et al, 1998; Krupanidhi et al, 2008; Jomova and Valko, 2011) and in the electron transport chain (Valko et al, 2005). Despite its essential nature, Cu can elicit

toxicity responses at levels as low as 1ug/L (Levy et al, 2007). In polluted waters, Cu concentrations can be much higher, 12-877 ug/L (Donat et al, 1994; Moffett et al, 1997).

While there is some literature on heavy metal effects in cnidarians, most studies have focused on anthozoans (Brock and Bielmyer, 2013; Mitchelmore et al, 2003a; Mitchelmore et al, 2003b; Reichelt-Brushett and McOrist, 2003; Bielmyer et al, 2003; Anjos et al, 2014) and hydrozoans (Grant et al, 2010), with little information available on heavy metal effects in scyphozoans (Lucas and Horton, 2014; Faimali, 2014; Templemann and Kingsford, 2010; Romeo et al, 1987). Thus far, cellular biomarkers have not been used to evaluate sub-lethal stress responses in *A. aurita*.

Cellular markers are often used with bioindicator organisms and in environmental management programs because they provide health status on populations and ecosystems through the use of resident individuals as well as wide ranges of application, predictive value and fast and cost-effective results (Moore et al, 2004; Dallas & Jha, 2015). They can also be used to assess the health of an ecosystem under chronic stress and the effectiveness of remediation programs (Connors & Ringwood, 2000; Ringwood et al, 2003; Colin et al, 2016). Benthic bioindicator species such as bivalves are well-established models for characterizing ecosystem health, but there are fewer invertebrate bioindicator species for characterizing potential impacts in pelagic realms. Therefore, *A. aurita* could serve as a novel bioindicator in these systems.

A variety of cellular markers, Cu tissue accumulation and behavioral responses were used to evaluate sub-lethal effects in *Aurelia aurita* when exposed to a range of environmentally realistic Cu concentrations. Two distinct tissues, oral arm and bell, were also used to determine tissues-specific differences in responses. This work serves to

establish a framework of knowledge that can be applied to other scyphozoans and compared to other cnidarian species.

## Methods

### *Cu Exposures*

*In vivo* exposures were conducted with animals donated from Discovery Place Inc., a science museum in Charlotte, NC that cultures *A. aurita*. For each exposure (four total), a range of Cu solutions were prepared from a 500 ppm stock solution of cupric chloride. Exposure concentrations of 5, 10, 25 and 50 ppb were used for the first two exposures and 5, 10 and 25 ppb solutions were used for the final two exposures. All Cu exposures were prepared in Instant Ocean saltwater with 25-30 ppt salinity and 7.95-8.05 pH. Four to five jellyfish were placed in 2 gallon buckets with 7 L of control or Cu treatments. During the 48-hour exposures, jellyfish were fed brine shrimp nauplii (*Artemia salina*) at the 24-hour and 48-hour time points. Exposures were performed at room temperature (18-20°C) with constant aeration. Salinity and pH were monitored and recorded daily for each bucket. Additionally, behavioral observations were monitored and recorded at the beginning, during, and at the end of each exposure.

At the end of each exposure, animals were dissected and tissues were separated for biomarker and tissue Cu analyses. Freshly dissected living tissues, including bell, oral arm and tentacle were used for lysosomal destabilization assays. All remaining tissues were separated into bell and oral arm fractions and frozen at -80°C for total protein analyses, lipid peroxidation assays, total glutathione assays and for the determination of tissue Cu concentrations using atomic absorption spectrometry.

### *Cu Accumulation*

Copper tissue concentrations were analyzed using atomic absorption spectrometry. Bell and oral arm tissues (0.5-1.0 g and 0.3-0.5 g, respectively), previously frozen at -80°C for each exposure, were weighed (wet tissue weight), and lyophilized using a VirTis benchtop K Lyophilizer for 24 hours. Each sample was then weighed (dry tissue weight), homogenized and microwave digested in ultra pure nitric acid. Fully digested samples were then diluted with ultra pure water. Samples, standards and oyster standard reference tissues were then analyzed using a Perkin Elmer AAnalyst 200 (Waltham, MA, USA) atomic absorption spectrometer using furnace mode and Deuterium background correction. Results were expressed as µg Cu per gram of dry tissue weight (ppm).

*Total Glutathione*

To determine glutathione (GSH) levels in tissues, a DTNB-GSSG Reductase Recycling assay for glutathione was used. Tissues were weighed and homogenized in 4 volumes of 5% sulfosalicylic acid (SSA) and centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (100µl) were further diluted with 200 µl of SSA. Standards of known glutathione concentrations were prepared (1.5-200 µM) from a 1 mM GSH stock solution (Sigma-Aldrich, MO). Samples and standards (30µl) were added to 96-well plates with 150 µl of nicotinamide adenine dinucleotide phosphate (NADPH), 40 µl of 5,5'-dithiobis(2-nitrobenzoic acid), 23 µl of water. Just prior to reading the plate, 7 µl of glutathione reductase from baker's yeast (GSSG-reductase, Sigma-Aldrich) was added to each well. A kinetic analysis was performed using a MultiSkan Go 3.2 UV/Vis spectrophotometer (Thermo Fisher Scientific) at 412 nanometers, with reads every 30 seconds for 2 minutes, and results were expressed as nmol/g wet tissue.

*Lysosomal Stability*

A neutral red lysosomal destabilization assay was used to assess the integrity of lysosomal membranes (Ringwood et al, 2005; Moore, 2004; Moore, 2006; Canesi et al, 2010). Pie-slice shaped tissue samples of bell and oral arm tissues, approximately 150-250mg, were processed into primary cell preparations with Ca/Mg-free-saline (CMFS) and trypsin, sheared to break apart clumps of cells and filtered through 41μm nylon screens. Cells were rinsed and re-suspended in CMFS and a stock solution of neutral red (NR) (0.04mg/mL) was added at a 1:1 ratio of NR to cell preparation volume. After a 60-minute incubation period cells were scored as either stable (NR contained within the lysosomes) or destabilized (NR diffusing into the cytoplasm from damaged lysosomes) at 400x magnification, with at least 50 cells scored from each preparation.

*Lipid Peroxidation*

Tissues were weighed and homogenized in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0), then centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (50μl) were combined with 700 μl of 15% trichloroacetic acid containing (0.375%) thiobarbituric acid and 7 μl 2% butylated hydroxytoluene. Standards of known MDA concentrations were prepared (6.25-800 μM) from a 10mM malondialdehyde tetraethylacetal stock solution (Acros organics, NJ), as previously described (Buege & Aust, 1978; Gray, 1978). Samples and standards were boiled in a water bath for 15 minutes, and then centrifuged (13000 RCF, 5 minutes, room temperature). The subsamples of each supernatant were then pipetted into a 96-well plate

(200-µl in each well), and MDA levels were measured at 532 nm using a MultiSkan Go 3.2 UV/Vis spectrophotometer (Thermo Fisher Scientific) and results were expressed as nmol/g wet weight tissue.

*Behavioral Responses*

For all exposures, animal behavior was monitored. This included initial and final bell diameter, pulsation rate (pulses/minute) and feeding behavior. Bell diameter was recorded initially and at the end of each exposure for each animal as an observable measure of increased or decreased somatic growth. Pulsation rates were monitored for measures of individual activity in response to Cu. Additionally, positions of the jellyfish (whether or not animals remained at the bottom of each bucket or if they remained in the water column) were recorded. Finally, feeding behavior was observed. This included whether or not individuals actively captured food and ingested it, captured food and egested it, or did not exhibit any feeding behavior.

*Statistical Methods*

Data were analyzed using Sigma Stat 2.0 and Graphpad Prism 6. Data were pooled from different exposures for statistical analysis when significant differences between experiments were not detected. Typically a one-way analysis of variance (ANOVA) was used to compare Cu treatments for the Cu-only exposures. When significant differences were detected ($p \leq 0.05$), *a posteriori* comparisons (Student-Newman-Keuls method) were used to identify differences between treatment groups and controls. When normality or equal variance tests failed, a transformation or a one-way ANOVA on ranks was performed and pairwise comparisons were made using Dunn's methods to determine significant differences in Cu-exposed groups and controls.

Regression analyses were used to identify relationships between tissue Cu accumulation and lysosomal destabilization, tissue Cu accumulation and pulsation rate, lysosomal destabilization and pulsation rates, and dose-dependent responses for lysosomal destabilization and glutathione.

## Results

### *Tissue Copper*

After jellyfish were exposed to increasing concentrations of Cu for 48 hr. exposures, tissue Cu concentrations significantly increased in the oral arms ($F=15.7$, $p<0.001$, Figure 1). Regression analysis indicated a significant, dose-dependent relationship between Cu exposure concentration and tissue accumulation, in oral arm tissues ($r^2=0.85$, $p<0.001$ Figure 3.2). A significant difference was detected for bell tissues in the 25 ppb Cu treatment group, compared to other treatment groups. ($F=8.0$, $p<0.001$, Figure 3.1). These data show that there was significant accumulation of Cu in *A. aurita* tissues, especially at the highest exposures were levels increased three- to fourfold.

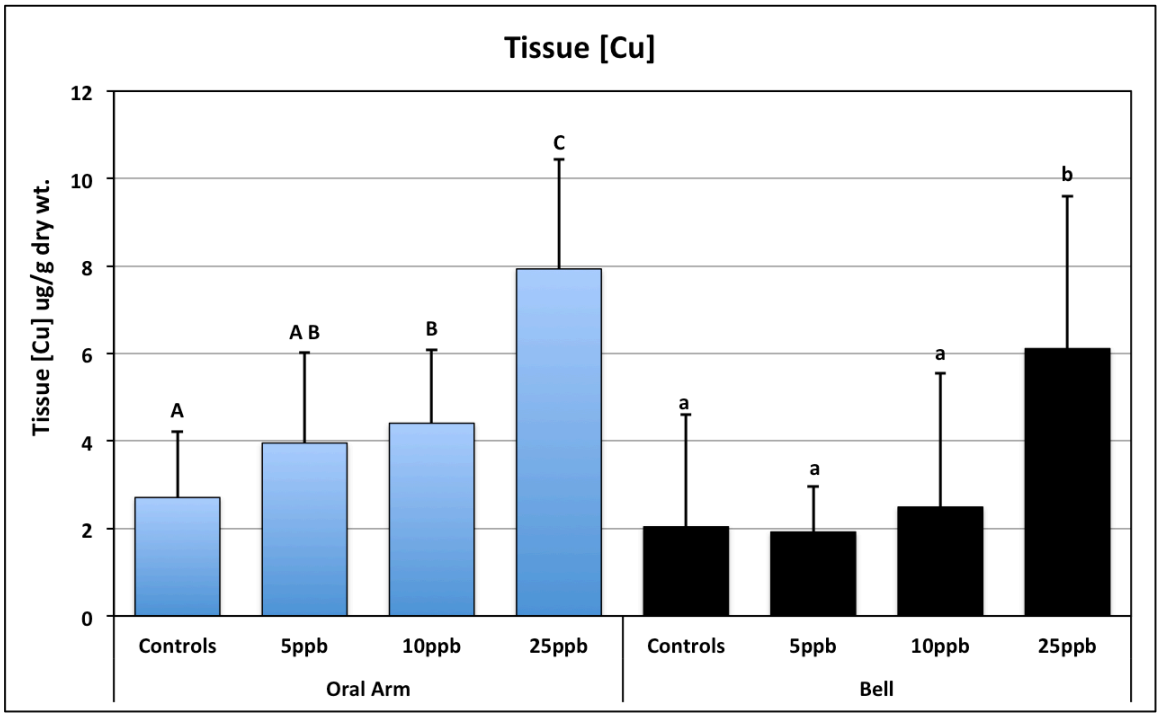


Figure 3.1: Tissue metal accumulation in oral arm and bell tissues of whole adult jellyfish exposed to a range of copper concentrations. Data are expressed as means + SD (oral arm, control n=12, 5ppb n=14, 10ppb n=17, 25ppb n=10; bell, control n=14, 5ppb n=19, 10ppb n=18, 25ppb n=18); results are the combined data from replicated experiments. Different letters indicate significant differences between treatment groups ($p<0.05$).

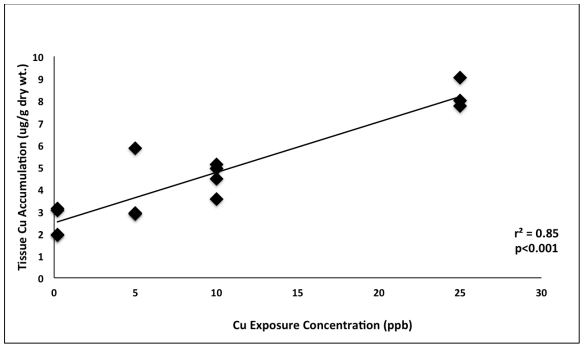


Figure 3.2: Tissue Cu accumulation as a function of Cu exposure concentration of medusae (n=14).

*Glutathione*

The GSH levels of the control groups were not significantly different across all exposures. Glutathione concentrations of jellyfish exposed to increasing concentrations of Cu showed some variation between exposures, but overall there was a trend of increasing GSH levels as Cu exposure concentrations increased. In the oral arm tissues,

GSH levels from controls ranged from approximately 35 – 60 nmol/ g wet weight. These may represent baseline levels for oral arm tissues in these animals. In the oral arm tissues, no significant differences between treatment groups were found in the July exposure ($p=0.104$, $F=2.6$, Figure 3.3), but significant differences were found between treatments for the exposures conducted during March, May, and September ($p=0.009$, $F=5.9$; $p=0.02$, $F=9.6$; $p=0.004$, $F=7.0$, respectively, Figure 3.3). There was evidence of GSH depletion in the July exposure and for the 10ppb treatment in the May exposure.

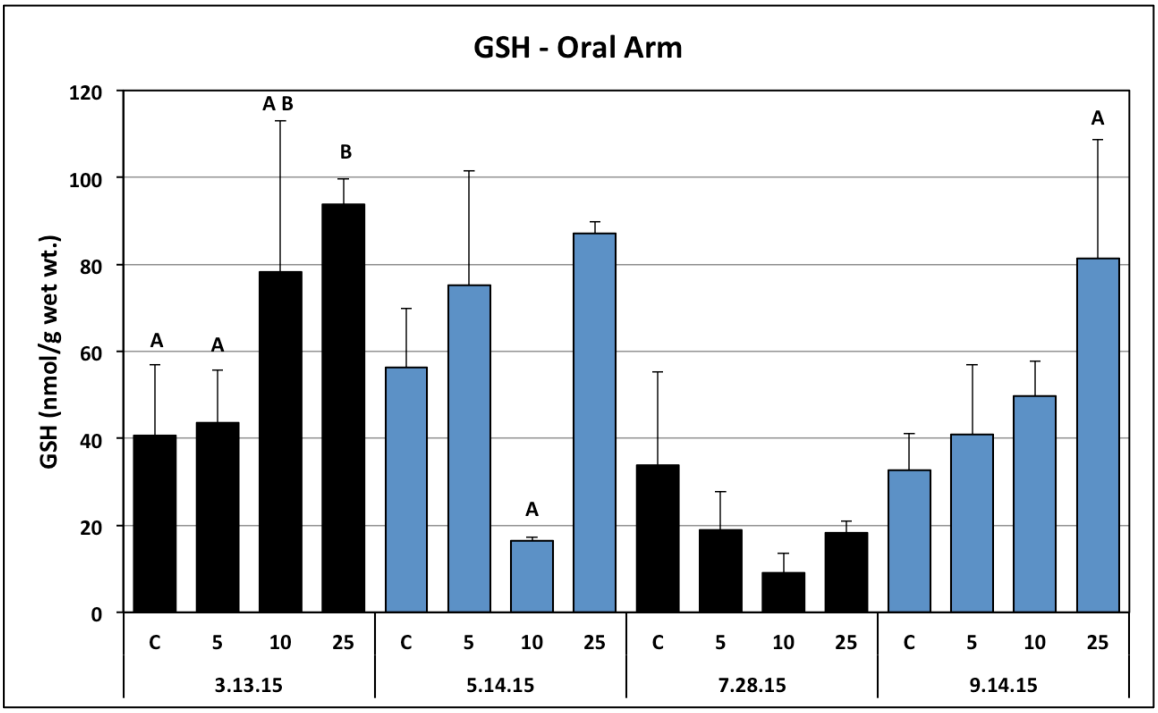


Figure 3.3: Total glutathione (GSH) concentrations in oral arm tissues of whole adult jellyfish exposed to a range of Cu concentrations from four individual exposures. Data are expressed as means + SD (control n=4-5, 5ppb n=4-5, 10ppb n=4-5, 25ppb n=4-5); Different letters indicate significant differences between treatment groups for each experiment ($p<0.05$).

Overall, the total GSH levels in bell tissues tended to be lower than those measured in oral arm tissues. The baseline GSH levels of bell tissues from controls ranged from 5-30 nmol/g wet weight, and there were no significant differences between exposure groups. When there were significant differences, Cu exposures were associated

with increased GSH. Bell tissues showed no significant differences between treatment groups for the May and July exposures (p=0.258, F=1.5; p=0.38, F=1.1, respectively, Figure 3.4), but significantly higher GSH levels were found for the March and September exposures (p=0.05, F=3.6; p=0.015, F=5.0, respectively, Figure 3.4).

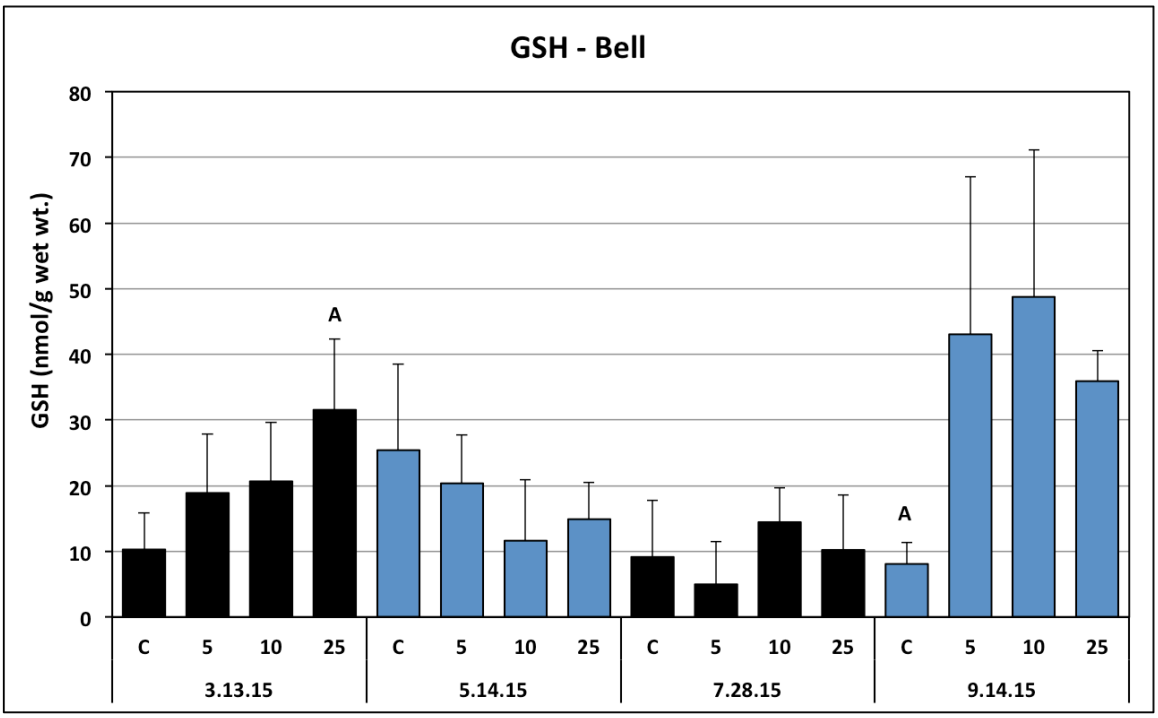


Figure 3.4: Total glutathione (GSH) concentrations in bell tissues of whole adult jellyfish exposed to a range of copper concentrations from four individual exposures. Data are expressed as means + SD (control n=4-5, 5ppb n=4-5, 10ppb n=4-5, 25ppb n=4-5); different letters indicate significant differences between treatment groups for each experiment ($p<0.05$).

*Lysosomal Stability*

Significant increases in lysosomal destabilization were found between treatment groups when exposed to increasing Cu concentrations (F=37.195, $p<0.001$, Figure 3.5). All treatment groups were significantly different and there was an overall destabilization increase of 16%. There were exposure-dependent increases in lysosomal damage as Cu exposure concentrations increased (Figure 3.8a), which were also highly correlated with Cu tissue concentrations (Figure 3.8b). Therefore, the lysosomal assay was a highly

sensitive biomarker for Cu exposures in *A. aurita* and was associated with bioaccumulation.

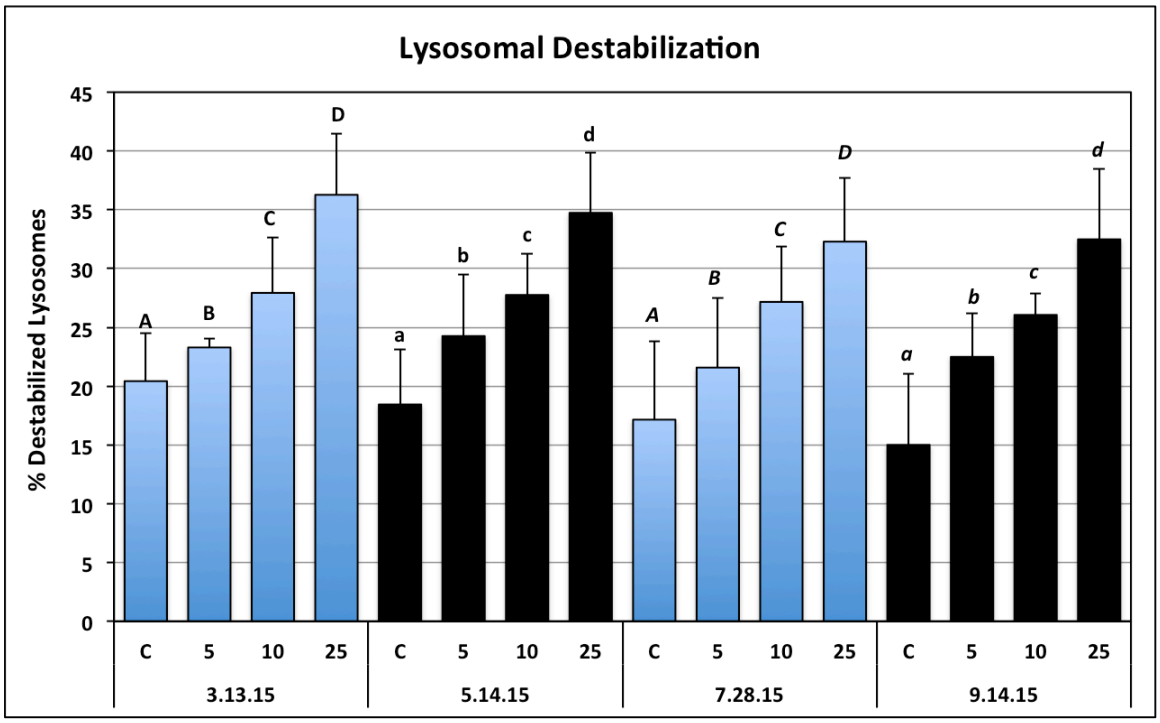


Figure 3.5: Percentages of lysosomal destabilization in tissues of whole adult jellyfish (oral arm and bell) exposed to a range of Cu concentrations. Data are expressed as means + SD (control n=16, 5ppb n=19, 10ppb n=20, 25ppb n=17). Different letters indicate significant differences between treatment groups ($p<0.05$).

*Lipid Peroxidation*

Lipid peroxidation was another measure of tissue damage used in this study. In the oral arm tissues, significant differences were not found between treatment groups for the March and May exposures ($p=0.331$, $F=1.246$; $p=0.889$, $F=0.208$, respectively, Figure 3.6). Significant increases were only observed for the 25 ppb treatment in the July exposure, but this could be due to extremely low control levels ($p=0.025$, $F=2.52$, Figure 3.6). A significant decrease was found in the 5ppb treatment in the September exposure ($p=0.027$, $F=9.184$, Figure 3.6). Overall, there was no consistent pattern of increased lipid peroxidation levels due to short-term Cu exposures. Control levels from the different experiments ranged from 15 – 75 nmol / g wet weight, so the starting levels and results

varied from exposure to exposure, making it more difficult to define consistent baseline MDA concentrations in oral arm tissues.

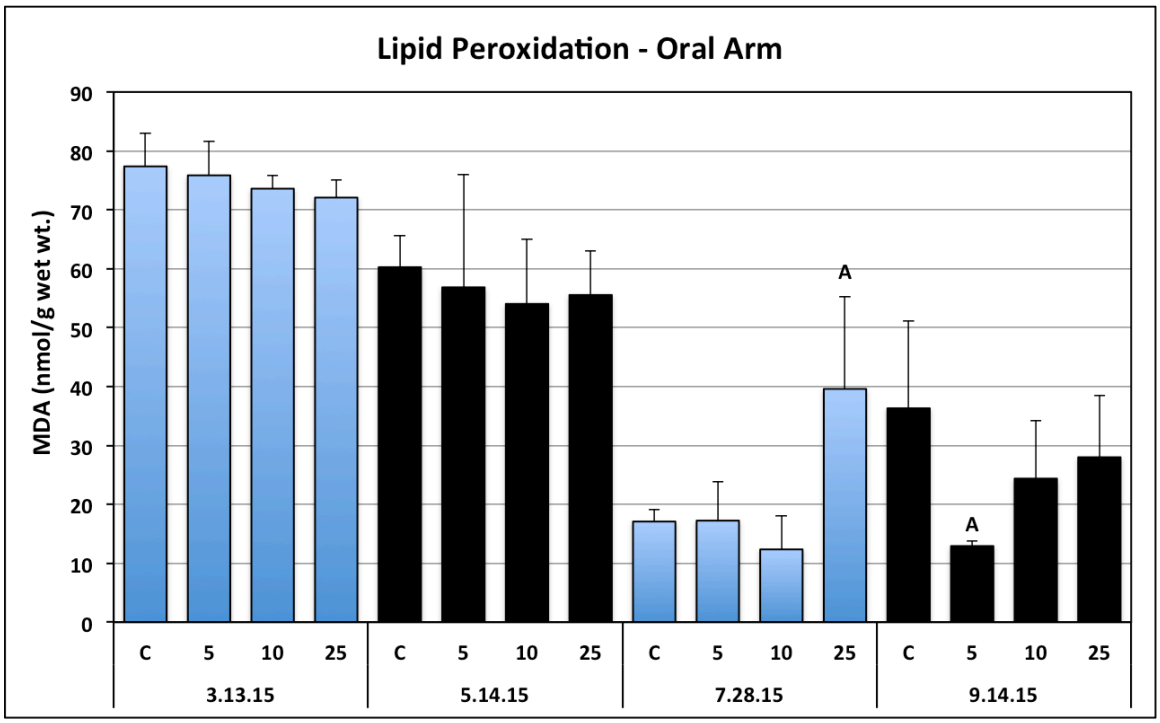


Figure 3.6: Lipid peroxidation (based on MDA concentrations) in oral arm tissues of whole adult jellyfish exposed to a range of Cu concentrations from four individual exposures. Data are expressed as means + SD (control n=4-5, 5ppb n=4-5, 10ppb n=4-5, 25ppb n=4-5); Different letters indicate significant differences between treatment groups for each experiment ($p<0.05$).

Bell tissues showed no significant increases in lipid peroxidation for Cu-treated jellyfish for the March, May, or September exposures (Figure 3.7). A significant decrease was observed in the 25ppb treatment group for the July exposure ($p=0.003$, $F=7.322$, Figure 3.7). As with the oral arm tissues, short-term Cu exposures did not result in increases in lipid peroxidation. Control levels varied significantly from exposure to exposure and baseline lipid peroxidation levels in bell tissues of control animals ranged from 15-70 nmol/g wet weight, similar to MDA concentrations of the oral arm tissues.

Regression analyses were used to identify significant relationships between the cellular biomarkers. The GSH levels tended to increase as lysosomal destabilization levels, although the p-value was slightly greater than 0.05 criterion ($p=0.062$, $F=4.143$,

Figure 3.9a). There was no significant correlation between total GSH and lipid peroxidation (Figure 3.9b).

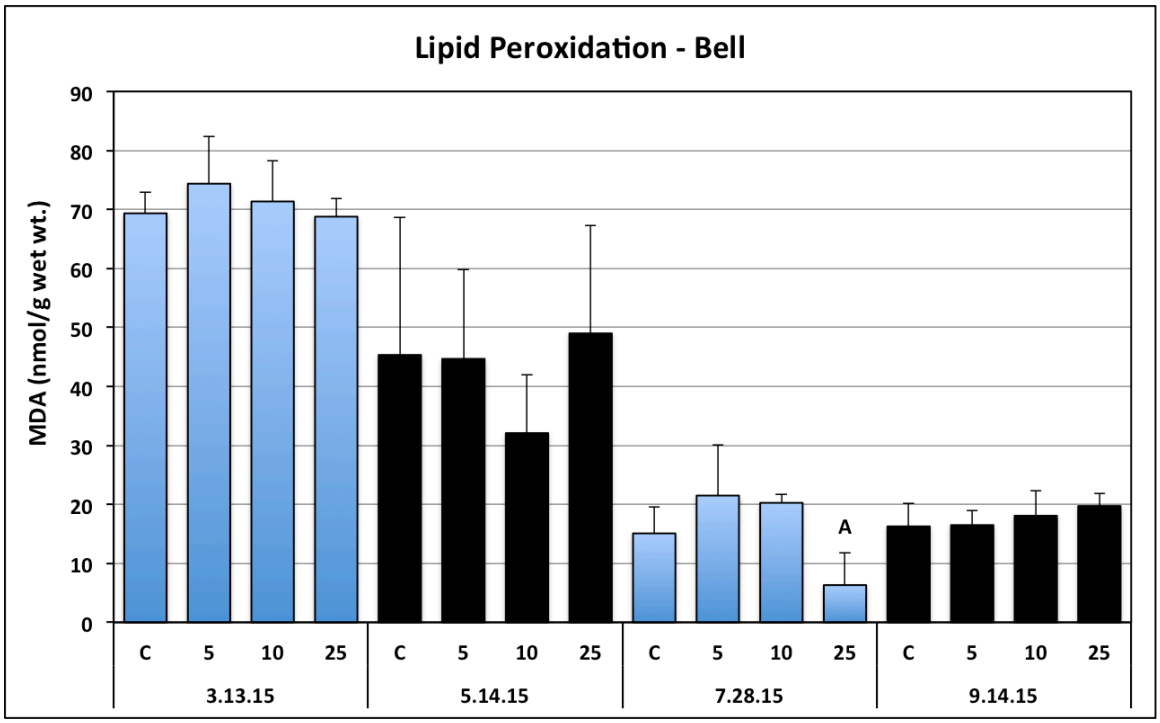


Figure 3.7: Lipid peroxidation (based on MDA concentrations) in bell tissues of whole adult jellyfish exposed to a range of Cu concentrations from four individual exposures. Data are expressed as means + SD (control n=4-5, 5ppb n=4-5, 10ppb n=4-5, 25ppb n=4-5); Different letters indicate significant differences between treatment groups for each experiment ($p<0.05$).

*Behavioral and Morphological Responses*

Behavioral observations and morphological changes (e.g. bell diameter) were quantified for all exposures. These data are summarized in Table 3.1.

Table 3.1: Behavioral endpoints: pulsation rate (pulses/min.), feeding behavior (✔=feeding occurred, ✗=no feeding), swimming behavior (✔=swimming throughout water column, ✗=clustered at bottom, no active swimming) and bell diameter (mm) for jellyfish exposed to Cu for 48 hrs. All data were recorded at 0, 24 and 48 hr. time points, except for bell diameter (0 and 48 hr. time points only). Data are expressed as means and standard deviations. Superscript letters indicate significant differences for treatment groups at each time point.

| **Behavioral Analyses** | | | |
|---|---|---|---|
| | **0 hrs.** | **24 hrs.** | **48 hrs.** |
| **Pulsation rate (pulse/min.)** | | | |
| **Controls** | 41.7 ± 19.17 | 38.2 ± 11.01$^{A}$ | 35.3 ± 9.59$^{A}$ |
| **5 ppb** | 36.5 ± 5.73 | 31.5 ± 4.94$^{AB}$ | 30 ± 7.65$^{B}$ |
| **10 ppb** | 31.6 ± 5.17 | 26 ± 8.18$^{B}$ | 26.1 ± 7.72$^{B}$ |
| **25 ppb** | 32.8 ± 5.99 | 16.3 ± 7.79$^{C}$ | 10.7 ± 7.79$^{C}$ |
| **50 ppb** | 32 ± 6.73 | 100% Mortality | 100% Mortality |
| **Feeding Behavior** | | | |
| **Controls** | ✔ | ✔ | ✔ |
| **5 ppb** | ✔ | ✔ | ✔ |
| **10 ppb** | ✔ | ✔ | ✔ |
| **25 ppb** | ✔ | ✔ | ✗ |
| **50 ppb** | ✔ | ✗ | ✗ |
| **Swimming Behavior** | | | |
| **Controls** | ✔ | ✔ | ✔ |
| **5 ppb** | ✔ | ✔ | ✔ |
| **10 ppb** | ✔ | ✔ | ✔ |
| **25 ppb** | ✔ | ✗ | ✗ |
| **50 ppb** | ✔ | ✗ | ✗ |
| **Bell Diameter (mm)** | | | |
| **Controls** | 87.26 ± 15.52 | | 82.34 ± 14.9 |
| **5 ppb** | 89.45 ± 25.02 | | 85.37 ± 29.12 |
| **10 ppb** | 86 ± 18 | | 82.4 ± 18.2 |
| **25 ppb** | 84 ± 15.51 | | 74.12 ± 12.26 |
| **50 ppb** | 93.6 ± 18.49 | | 100% Mortality |

At the beginning of each exposure (Time 0), pulsation rates and bell diameters were not significantly different ($p=0.083$, $F=6.679$; $p=0.832$, $F=0.290$, respectively, Table 3.1). At the 24 hr. and 48 hr. time points, significant differences in pulsation rate were found between treatment groups ($p<0.001$, $F=38.6$; $p<0.001$, $F=33.1$, respectively, Table 3.1) and pulsation rates significantly declined in a dose-dependent fashion as Cu

concentration increased. Furthermore, pulsation rate decreased as exposure time increased, especially in the 10 and 25 ppb Cu treatment groups. Decreases in final bell diameters were observed in all treatment groups with the most pronounced decline in the 25 ppb Cu treatment group. Swimming and feeding in the water column were observed throughout the duration of each exposure for controls, 5 and 10 ppb Cu treatment groups. Individuals in the 25 ppb Cu treatment group of all four exposures did not feed at the 48 hr. time point, and to cluster at the bottom of exposure containers with little to no active swimming. For the 50 ppb Cu treatment groups, the jellyfish showed no feeding and no active swimming at the 24 hr time point, with 100% mortality at the 48 hr. time point. These results indicate significant changes in behavioral responses with increasing Cu concentrations. Pulsation rates were significantly correlated to Cu exposure concentrations (Figure 3.8c) and also to tissue Cu levels (Figure 3.8d). Furthermore pulsations rates were also significantly related to lysosomal destabilization (Figure 3.8e).

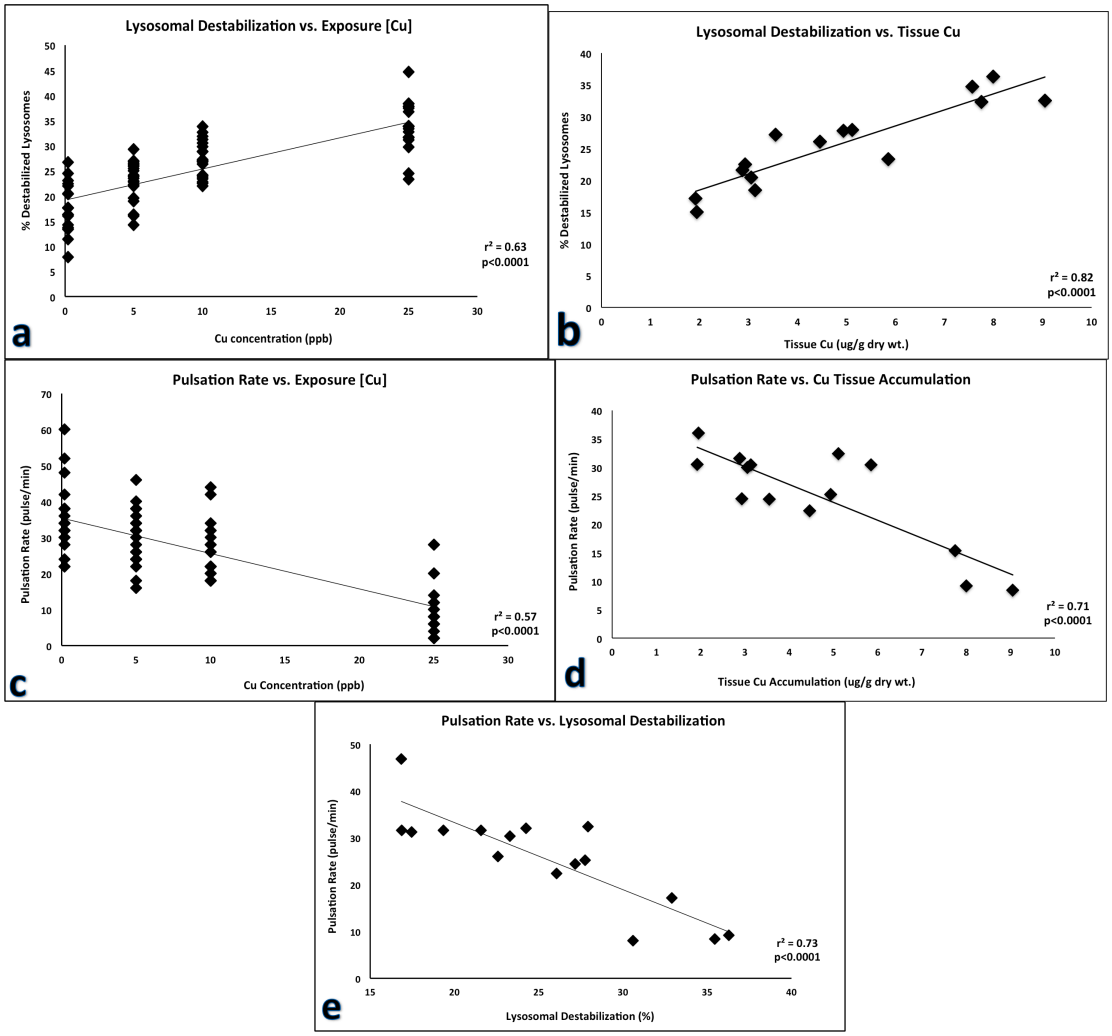


Figure 3.8: Regression analyses for a) lysosomal destabilization and Cu exposure concentration; b) lysosomal destabilization and tissue Cu accumulation: c) pulsation rate and Cu exposure concentration; d) pulsation rate and tissue Cu accumulation and e) lysosomal destabilization and pulsation rate (n=14-16).

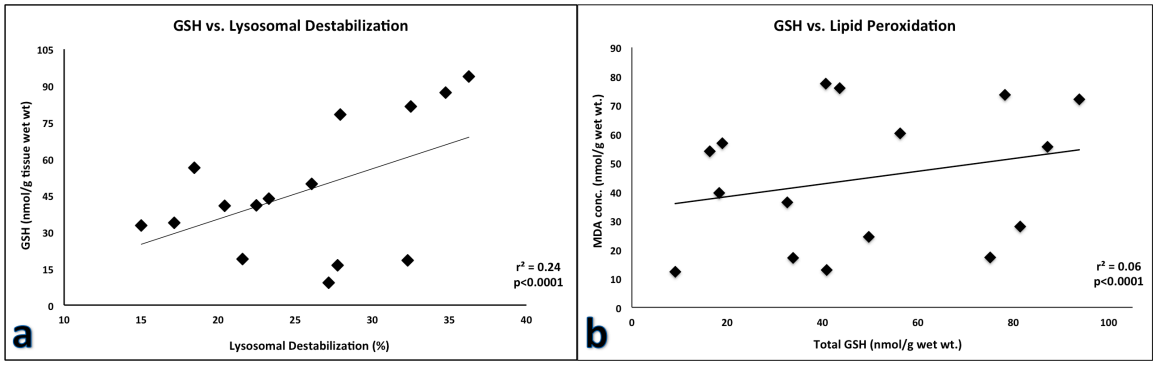


Figure 3.9: Regression analyses for a) total GSH and lysosomal destabilization and b) total GSH and lipid peroxidation (n=14-16).

## Discussion

Overall, these studies demonstrate that short-term exposures of a common marine pollutant, Cu, adversely affected lysosomal function and significantly affected swimming and behavioral responses of jellyfish. There was also evidence of oxidative stress, indicated by increases in GSH antioxidant responses. The increases in GSH probably served to minimize oxidative damage, so no significant increases in lipid peroxidation were observed. There were differences between oral arm and bell tissues for the oxidative stress biomarkers, but similar patterns of increasing GSH levels with increasing Cu exposures were observed between the two tissues. Copper accumulation, GSH, and lipid peroxidation were generally higher in the oral arm tissues than levels found in the bell tissues.

The lysosomal biomarker showed consistent results for all exposures, and indicated dose-dependent responses as Cu increased. Furthermore, lysosomal data showed significant correlations with behavioral endpoints as well as accumulation data, indicating linkages between this biomarker assay and higher order behavioral responses. While the lysosomal assay has not been previously used with jellyfish or other cnidarians, these results are comparable to those reported for other invertebrates (Ringwood et al, 2006, 2009, 2013; Edge et al, 2013). This further reinforces the value of this assay as a sensitive indicator of metal exposures that has broad applicability, and that can be used to compare relative sensitivities between species.

Behavioral responses also emerged as valuable indicators, and were related to tissue Cu as well as Cu exposure levels, and lysosomal responses. Pulsation rates decreased as Cu concentration increased for all exposures. Swimming behavior declined

and feeding ceased by 24 hrs. at 50 ppb and 48 hrs. at 25 ppb Cu. Behavioral endpoints have long been used to assess responses to contaminants due to their ease and non-destructive nature in invertebrates (Gerhardt, 1995; Gerhardt et al, 1998; Macedo-Sousa et al, 2008; Amiard-Triquet, 2009; Buffet et al, 2011) and vertebrates (Machado et al, 2013; Acosta et al, 2016). Like lysosomal assays, pulsation rates showed strong relationships with Cu exposure and tissue accumulation.

Baseline GSH levels were consistent across all exposures in both oral arm and bell tissues. Control GSH levels ranged from 35-55 nmol/g in oral arm tissues and 8-10 nmol/g in bell tissues. After 48 hr. exposures, a general trend of increased glutathione levels occurred when comparing different treatment groups. This trend was most pronounced in the oral arm tissues when compared to bell tissues. Depletion of total GSH pools related to metal and organic contaminants has also been reported, especially when exposures are more chronic (Regoli and Principato, 1995; Doyette et al, 1997; Ringwood et al., 1999). Chronic exposures could eventually lead to depletion of GSH as oxyradicals increase due to Cu -Fenton chemistry and increased GSH oxidation when conjugated to Cu (Mason and Jenkins, 1995; Deb and Fukushima, 1997; Kelly, 1998; Halliwell and Gutteridge, 2007; Jomova and Valko, 2011).

Despite increases in GSH levels during these Cu exposures, baseline GSH levels were very low when compared to other invertebrates, such as bivalves, as well as other cnidarians (Conners and Ringwood, 2000; Downs et al, 2000; Mitchelmore et al, 2003; Almeida et al, 2004; Abujamara et al, 2015). The highest GSH levels of *A. aurita* observed in these studies were approx. 40 – 60 nmol / g , much lower than those reported in anthozoans, that have approximately 10 times higher levels, or bivalves, that have 20

times higher levels. Low levels of such an important antioxidant could contribute to higher sensitivities of *A. aurita* and other Cnidarians to Cu and other pollutants.

Lipid peroxidation based on MDA levels remained unchanged across treatment groups in both tissues. Lipid peroxidation reflects oxidative damage caused by oxyradicals, when antioxidant responses are overwhelmed (Kelly et al, 1998; Halliwell and Gutteridge, 2007). This general trend of unchanged MDA levels in Cu-exposed jellyfish could be attributed to the increased GSH levels that were observed over these short-term exposures. Increases in GSH and other antioxidants are early signals of oxidative stress that can provide at least temporary protection against lipid peroxidation and other forms of oxidative damage (protein oxidation and DNA damage).

Dose-dependent tissue accumulation of Cu was observed, especially in the oral arm tissues, where levels increased up to fourfold. In bell tissues Cu levels increased up to threefold. The literature for tissue metal levels or accumulation in scyphozoans is very limited. A 2010 study by Templman and Kingsford investigated trace metal accumulation of the jellyfish *Cassiopea* sp. from various sites off the north and eastern coasts of Australia. Tissue concentrations of various trace metals (including Cu) in resident individuals did vary between the five sites used, which may indicate different trace metal concentrations in ambient waters. Furthermore, this study found mean tissue specific differences for oral arm (0.146-2.61 ug/g dry wt.) and bell (0.077-1.41 ug/g dry wt.), similar to findings in this study. This may be attributable to species-specific differences between *Cassiopea* and *Aurelia* species. An earlier study (Siddiqui et al, 1988) found much higher levels of Cu (16.1 ug/g dry wt.) in *A. aurita*. However, this study did not distinguish differences between tissues, and used whole animal fractions. A

more recent study (Munoz-Vera et al, 2016) evaluated bioaccumulation patterns of trace metals in the jellyfish *Rhizostoma pulmo* from various sites off the coast of SE Spain. Eight sampling stations were investigated in the Mar Menor lagoon and showed extremely high levels of metal accumulation in tissues when compared to seawater concentrations. Additionally, their results for Cu encompassed results found in these studies and showed clear tissue-specific accumulation differences for oral arm (1.15-3.54 mg/kg dry wt.) and bell tissues (0.85-1.69 mg/kg dry wt.).

This work provides essential new data regarding Cu accumulation and cellular responses in jellyfish exposed to short-term (48 hr), environmentally relevant concentrations of Cu. *Aurelia aurita* was very sensitive to Cu. Lysosomal destabilization was observed at 5 ppb Cu; and high mortalities, 100%, were observed after only 48-hour exposures to 50 ppb Cu. Significant changes in behavior responses were also observed, and there was a significant correlation between behavior and lysosomal damage. Low baseline levels of GSH, an important antioxidant, were found in both oral arm and bell tissues; low GSH levels could contribute to their high sensitivity. These results establish a valuable framework for using cellular biomarkers and behavioral assays for *A. aurita;* and illustrate their potential value as a bioindicator species and environmental monitoring tool for pelagic systems.

# CHAPTER 4: THE COMBINED EFFECTS OF TEMPERATURE AND COPPER ON JELLYFISH, *AURELIA AURITA*

## Introduction

Jellyfish are ubiquitous in marine systems, ranging from polar to tropical oceans and from shallow bays and estuaries to great depths of pelagic areas. Due in part to the complexity of scyphozoan life histories, even slight changes in environmental factors can have an effect on fecundity, development, growth and population fluctuations. *Aurelia aurita*, the common moon jellyfish, has been used as a model in studies to understand population dynamics (Lo and Chen, 2008), nutrient cycling (Schneider, 1989; Miller and Graham, 2012), larval substratum selection (Holst and Jarms, 2007; Webster and Lucas, 2012), strobilation and genetic controls (Fuchs et al, 2014), and increasing anthropogenic concerns of jellyfish 'blooms' (Purcell et al, 2007; Dong et al, 2010; Richardson et al, 2009). Despite its widespread use for a variety of biological studies, *A. aurita* has only recently been considered for ecotoxicological studies (Faimali et al, 2014; Costa et al, 2015; Echols et al, 2016).

Bioindicator species are important tools utilized in ecotoxicology for determining the behavioral, physiological and biochemical responses to potential contaminants and stressors that organisms are exposed to in the environment. However, commonly used species may not be present in some locales or habitats, so approaches that utilize other species that are present in an area of concern are needed. *Aurelia aurita* has many attributes that make it a potentially valuable bioindicator species for marine habitats:

abundance, ease of culturing and collection, life history strategy, presence in estuarine and pelagic environments and predator/prey trophic interactions (Faimali et al, 2014).

Fluctuating temperature is an environmental stressor that is experienced, to some degree, by all marine organisms, especially in temperate habitats. Global warming is likely to increase overall temperatures as well as cause greater fluctuations. Ocean temperatures have been estimated to rise by 0.2°C or more in the next 20 years (IPCC, 2007; Negri et al, 2013; IPCC, 2013; Digilio et al, 2016). Because temperature changes affect many physiological and biochemical processes, interactions between temperature and contaminants would be expected (Heugens et al, 2001).

Like other aquatic organisms, *A. aurita* is exposed to a multitude of chemical contaminants in the ambient environment, including heavy metals, such as copper (Cu). Copper is a major anthropogenic pollutant found throughout aquatic ecosystems from anti-fouling paints, fertilizers, herbicides, pesticide, industrial runoff and mining activities (Stauber and Davies, 2000; Levy et al, 2007; Namiesnik and Rabajczyk, 2010). However, Cu is essential biologically as a cofactor in many enzymes that undergo redox-chemistry (Koch et al, 1997; Ringwood et al, 1998; Krupanidhi et al, 2008; Jomova and Valko, 2011) and in the electron transport chain (Valko et al, 2005). Despite its essential nature, Cu can elicit toxicity responses at levels as low as 1ug/L (Levy et al, 2007) and it is one of the most common metal contaminants. While there is some literature on heavy metal effects in cnidarians, most studies have focused on anthozoans (Brock and Bielmyer, 2013; Mitchelmore et al, 2003a; Mitchelmore et al, 2003b; Reichelt-Brushett and McOrist, 2003; Bielmyer et al, 2003; Anjos et al, 2014) and hydrozoans (Grant et al,

2010), with little information available on heavy metal effects in scyphozoans (Lucas and Horton, 2014; Faimali, 2014; Templemann and Kingsford, 2010; Romeo et al, 1987).

Most laboratory temperature or Cu studies are conducted as single-stressor experiments. But organisms rarely experience a single stressor in nature (Holmstrup et al, 2010) and *in situ* responses are a product of the combination of environmental stressors. Physicochemical factors (temperature, pH, salinity, etc.) can influence the toxicity of a metal, as well as act as stressors. Therefore, it is important to incorporate the potential effects of physicochemical stressors when characterizing metal toxicity responses to consider how multiple stressors affect the organisms. To date, sub-lethal stress responses based on cellular biomarkers have not been used to evaluate toxicity in *A. aurita* nor have the effects of heavy metals in combination with fluctuating temperature been explored.

The purpose of this study was to use a variety of cellular biomarkers and behavioral responses to evaluate sub-lethal effects in *A. aurita* when exposed to a nominal Cu concentration (10 ppb), elevated temperature and a combination of Cu and elevated temperature. These studies are essential for understanding the potential impacts of combinations of anthropogenic and natural environmental stressors. (Rhee et al, 2013; Gomiero & Viarengo, 2014; Makabe et al, 2015; Lewis et al, 2016; Tills et al, 2016). These studies will also provide essential new data for *A. aurita* that will further show the value of this species as a bioindicator for environmental management programs.

## Methods

### *Exposure Studies*

Three treatments were performed in these studies: elevated temperature (TEMP-only), 10 ppb Cu concentration (Cu-only) and a combination of elevated temperature and 10 ppb Cu concentration (TEMP-Cu), with two exposures for each treatment. For each of the two 48-hour exposures, 4-5 adult jellyfish were placed in 2 gallon buckets with 7 L of treatment water (25-30 ppt salinity, 7.95-8.05 pH). Temperature was closely monitored throughout all exposures, with starting ambient temperatures at 18°C. This temperature was maintained throughout the duration of the Cu-only exposure and temperatures were raised over a 2-hour period to 25°C via a water bath with an Aqueon Pro50 50 W aquarium heater for TEMP-only and TEMP-Cu exposures. During the 48-hour exposures, jellyfish were fed brine shrimp nauplii (*Artemia salina*) at approximately the 24-hour and 48-hour time points and aeration was constant. Salinity, pH and temperature were monitored and recorded daily for each bucket.

### *Behavioral Responses*

Behavioral observations were monitored and recorded at the beginning, during and end of each exposure. Pulsation rate (pulse/minute) and swimming behavior (suspended in the water column or clustered at the bottom of container) were monitored daily. Feeding behavior (actively capturing food and ingesting it, capturing food and egesting it or no observable feeding behavior) was recorded at the 24- and 48-hour time points. Bell diameter was also recorded initially and at the end of each exposure for each individual.

*Tissue Processing*

At the end of each exposure, animals were dissected and tissues were separated for further analysis. Fresh, living tissue, including oral arm and bell, were used for lysosomal destabilization assays. All remaining tissues were separated into bell and oral arm fractions, and frozen at -80°C for lipid peroxidation and total glutathione (GSH) assays, as well as for the tissue Cu accumulation analysis using atomic absorption spectrometry (AAS).

*Cellular Biomarkers*

*Lysosomal Stability*

A neutral red lysosomal destabilization assay was used to assess the integrity of lysosomal membranes (Ringwood et al, 2003; Moore et al, 2004; Moore, 2006; Canesi et al, 2010). Pie-slice shaped tissue samples of bell and oral arm tissues, approximately 150-250mg, were processed into primary cell preparations with Ca/Mg-free-saline (CMFS) and trypsin, sheared to break apart clumps of cells and filtered through 41μm nylon screen. Cells were rinsed and re-suspended in CMFS and a stock solution of neutral red (NR) (0.04mg/mL) was added at a 1:1 ratio of NR to cell preparation volume. After a 60-minute incubation period cells were scored as either stable (NR contained within the lysosomes) or destabilized (NR diffusing into the cytoplasm from damaged lysosomes) at 400x magnification, with at least 50 cells scored from each preparation.

*Lipid Peroxidation*

Tissues were weighed and homogenized in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0), then centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (50μl) were combined with 700 μl of 15% trichloroacetic acid

containing (0.375%) thiobarbituric acid and 7 µl 2% butylated hydroxytoluene. Standards of known MDA concentrations were prepared (6.25-800 µM) from a 10mM malondialdehyde tetraethylacetal stock solution (Acros organics, NJ), as previously described (Buege & Aust, 1978; Gray, 1978). Samples and standards were boiled in a water bath for 15 minutes, and then centrifuged (13000 RCF, 5 minutes, room temperature). The subsamples of each supernatant were then pipetted into a 96-well plate (200-µl in each well), and MDA levels were measured at 532 nm using a MultiSkan Go 3.2 spectrophotometer (Thermo Fisher Scientific) and results were expressed as nmol/g wet weight tissue.

*Total Glutathione*

To determine glutathione (GSH) levels in tissues, a DTNB-GSSG Reductase Recycling assay for glutathione was used. Tissues were weighed and homogenized in 4 volumes of 5% sulfosalicylic acid (SSA) and centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (100µl) were further diluted with 200 µl of SSA. Standards of known glutathione concentrations were prepared (1.5-200 µM) from a 1 mM GSH stock solution (Sigma-Aldrich, MO). Samples and standards (30µl) were added to 96-well plates with 150 µl of nicotinamide adenine dinucleotide phosphate (NADPH), 40 µl of 5,5'-dithiobis(2-nitrobenzoic acid), 23 µl of water. Just prior to reading the place, 7 µl of glutathione reductase from baker's yeast (GSSG-reductase, Sigma-Aldrich) was added to each well. A kinetic analysis was performed using a MultiSkan Go 3.2 spectrophotometer (Thermo Fisher Scientific) at 412 nanometers every 30 seconds for 2 minutes and results were expressed as nmol/g wet tissue.

*Tissue Cu Analysis*

Copper tissue concentrations were analyzed using atomic absorption spectrometry. Bell and oral arm tissues (0.5-1.0 g and 0.3-0.5 g, respectively), previously frozen at -80°C for each exposure, were weighed (wet tissue weight), and lyophilized using a VirTis benchtop K Lyophilizer for 24 hours. Each sample was then weighed (dry tissue weight), homogenized, microwaved and digested. Fully digested samples were then diluted with ultra-pure water. Samples, standards and oyster standard reference tissues were then analyzed using a Perkin Elmer AAnalyst 200 (Waltham, MA, USA) atomic absorption spectrometer using furnace mode and Deuterium background correction. Results were expressed as μg Cu per gram of dry tissue weight (ppm).

*Statistical Methods*

Data were analyzed using Sigma Stat 2.0 and Graphpad Prism 6. Data were pooled from different exposures as no significant differences between experiments were detected. A one-way analysis of variance (ANOVA) was used to compare controls, temp.-only, Cu-only and temp.-Cu treatment groups. When significant differences were detected ($p \leq 0.05$), *a posteriori* comparisons (Student-Newman-Keuls method) were used to identify differences between treatment groups and controls. When normality or equal variance tests failed, a transformation or a one-way ANOVA on ranks was performed and pairwise comparisons were made using Dunn's methods to determine significant differences in treatment groups and controls.

Results

*Tissue Cu*

TEMP-only, Cu-only and TEMP-Cu exposed animals were compared to controls to determine if there were differences in Cu accumulation between groups. The TEMP-Cu exposed group had significantly higher Cu accumulation in the oral arm and bell tissues when compared to the other three groups ($p<0.001$, $F=8.8$; $p<0.001$, $F=17.5$, respectively, Figure 4.1).

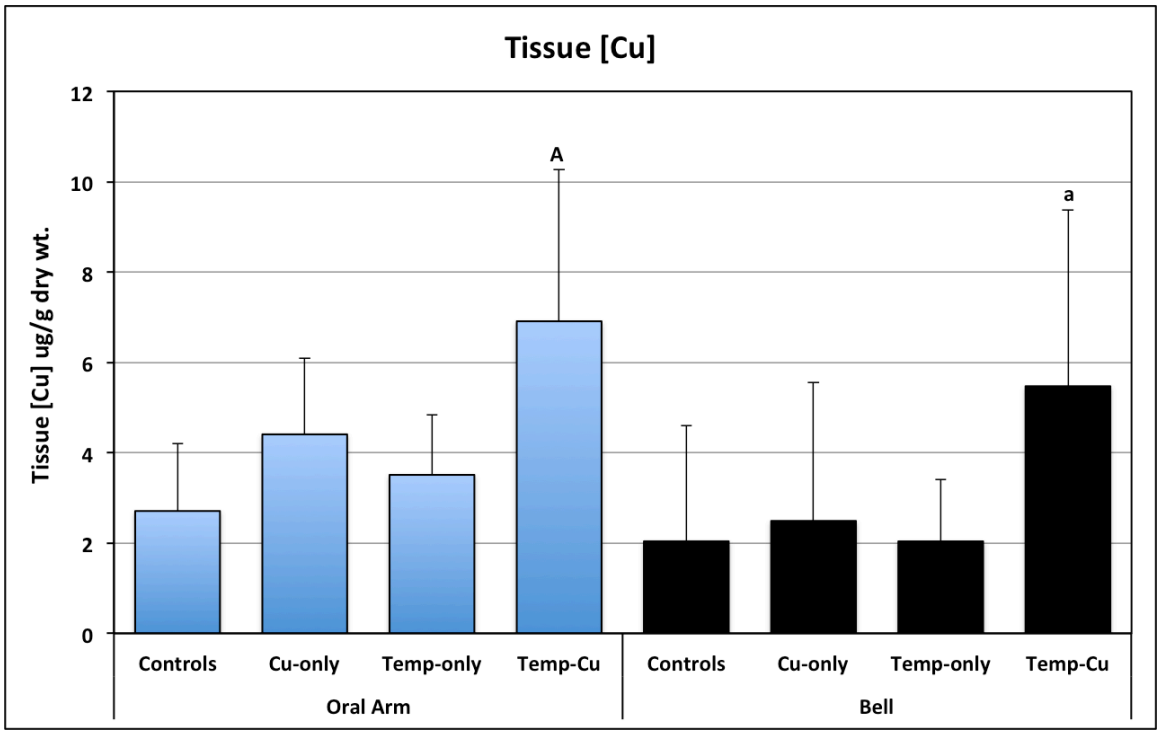


Figure 4.1: Tissue metal accumulation in oral arm and bell tissues of controls, Cu-only temp.-only and temp.-Cu treatment groups. Data are expressed as means + SD (oral arm, control n=12, 10ppb=17, temp=10, temp-Cu=12; bell, control n=14, 10ppb=18, temp=13, temp-Cu=12); results are the combined data from replicated experiments. Like letters indicate no significant differences among treatment groups and different letters indicate significant differences ($p<0.05$).

Temperature increase alone had the smallest effect on metal accumulation, not significantly different from controls ($p=0.38$). There was an increase in Cu accumulation when animals were exposed to 10 ppb Cu; however, only in the oral arm tissues and that was not significantly higher ($p=0.09$). But animals exposed to 10 ppb Cu and elevated

temperatures, TEMP-Cu, showed significantly higher accumulation in both tissues ($p<0.001$). The increase was approximately three times higher than control values in both tissues. TEMP-Cu exposures appeared to have a synergistic effect on Cu accumulation in these animals.

*Behavioral Responses*

Pulsation rate showed significant differences between treatment groups at all three time points (0 hrs., $p<0.001$, $F=10.8$); 24 hrs., $p<0.001$, $F=11.0$; 48 hrs., $p<0.001$, $F=11.9$, Table 4.1) and within the treatment groups (Cu-only, $p=0.02$, $F=4.0$; TEMP-Cu, $p<0.05$, $F=5.3$ Table 1) over time. The results were somewhat confounded by differences in pulsation rates between treatments at Time 0. However, overall the results showed no changes in pulsation rates over time for Controls or TEMP-only treatments, decreased pulsation rates in Cu-only treatment groups by the 24 hr. time point and increased pulsation rates in TEMP-Cu treatment groups by the 48 hr. time point, although these rates were only slightly significantly different from Controls at 48 hr. No significant differences were observed in feeding behavior (all individuals ate at the 24- and 48-hr. time points) or swimming behavior (all individuals remained suspended in the water column throughout each exposure). Finally, no significant differences in initial and final bell diameters were observed for controls or any treatment groups.

Table 4.1: Pulsation rate (pulse/minute) All data recorded at 0, 24 and 48 hr. time points. Data expressed as means and standard deviations. Superscript letters indicate significant differences between treatment groups at each time point (columns) and * indicate significant differences between time points for each treatment group (rows).

| Behavioral Analyses | | | |
|---|---|---|---|
| | **0 hrs.** | **24 hrs.** | **48 hrs.** |
| **Pulsation rate (pulse/min.)** | | | |
| **Controls** | $41.7 \pm 19.17^{A}$ | $38.2 \pm 11.01^{A}$ | $35.3 \pm 9.59^{A}$ |
| **Cu-only** | $36.5 \pm 5.73^{B}$ | $31.5 \pm 4.94^{B\,*}$ | $30 \pm 7.65^{B\,*}$ |
| **TEMP-only** | $22.5 \pm 7.51^{C}$ | $26.5 \pm 5.74^{B}$ | $24.1 \pm 2.61^{B}$ |
| **TEMP-Cu** | $28.5 \pm 5.21^{B\,C}$ | $29.7 \pm 3.01^{B}$ | $33.7 \pm 6.82^{A\,*}$ |

*Glutathione*

Overall there was a pattern of lower GSH levels at elevated temperatures and higher GSH levels when exposed to Cu only. Significant differences between controls and Cu-only exposures were detected in the bell tissues ($p=0.004$, $F=13.1$, Figure 4.2) and results for the oral arm tissues were nearly significant ($p=0.06$, $F=2.6$, Figure 4.2). While elevated temperature did not significantly affect GSH, there was a consistent pattern of low GSH levels-25-50% lower than controls. The GSH levels for the TEMP-Cu exposures were more similar to TEMP-only exposures than either controls or Cu-only exposures.

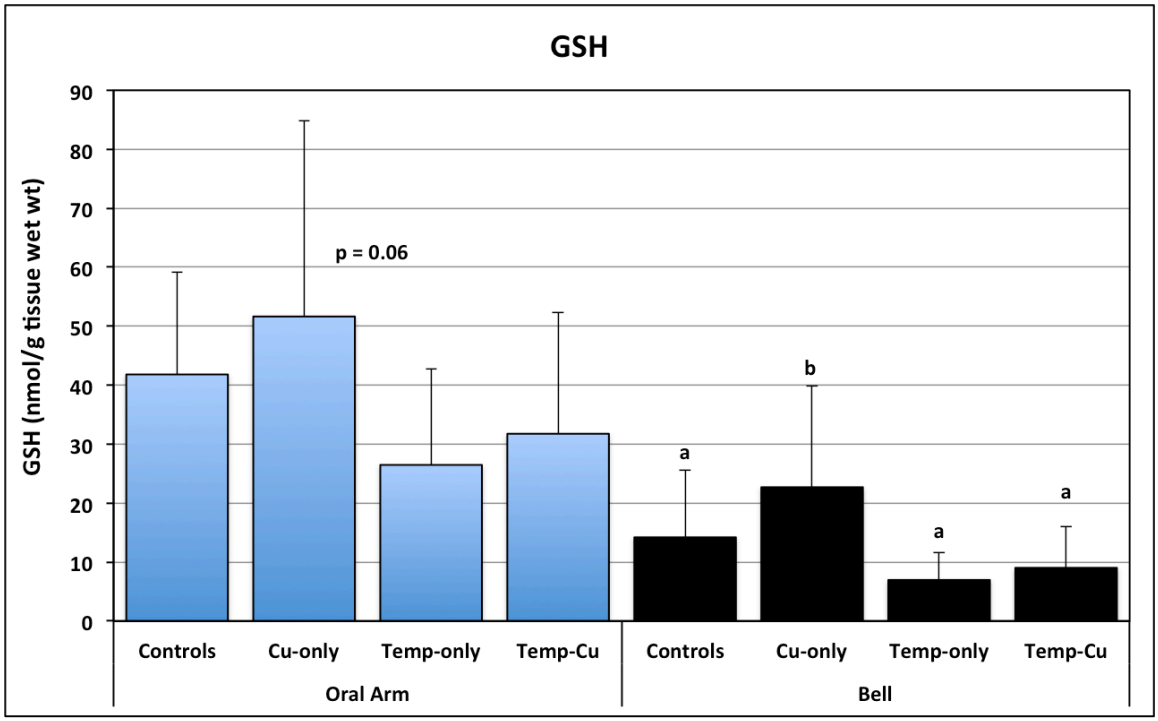


Figure 4.2: Total glutathione concentrations in oral arm and bell tissues of whole adult jellyfish controls and treatment groups (Cu-only, temp.-only and temp.-Cu). Data are expressed as means + SD (oral arm-control n=16, 10ppb=12, Temp=11, Temp + Cu=14; bell-control n=16, 10ppb=14, Temp=9, Temp + Cu=16); Like letters indicate no significant differences between treatment groups and different letters indicate significant differences ($p<0.05$).

*Lysosomal Stability*

The lysosomal destabilization assay was performed on tissue samples that represented whole animal, including both oral arm and bell tissues. Significant differences were found between controls and all other treatment groups ($p<0.001$, $F=16.9$, Figure 4.3). Temperature alone caused the least toxicity, followed by Cu-only. TEMP-Cu exposure caused the greatest toxicity, however, there was no significant difference between Cu-only and TEMP-Cu treatments. This biomarker was most affected by Cu.

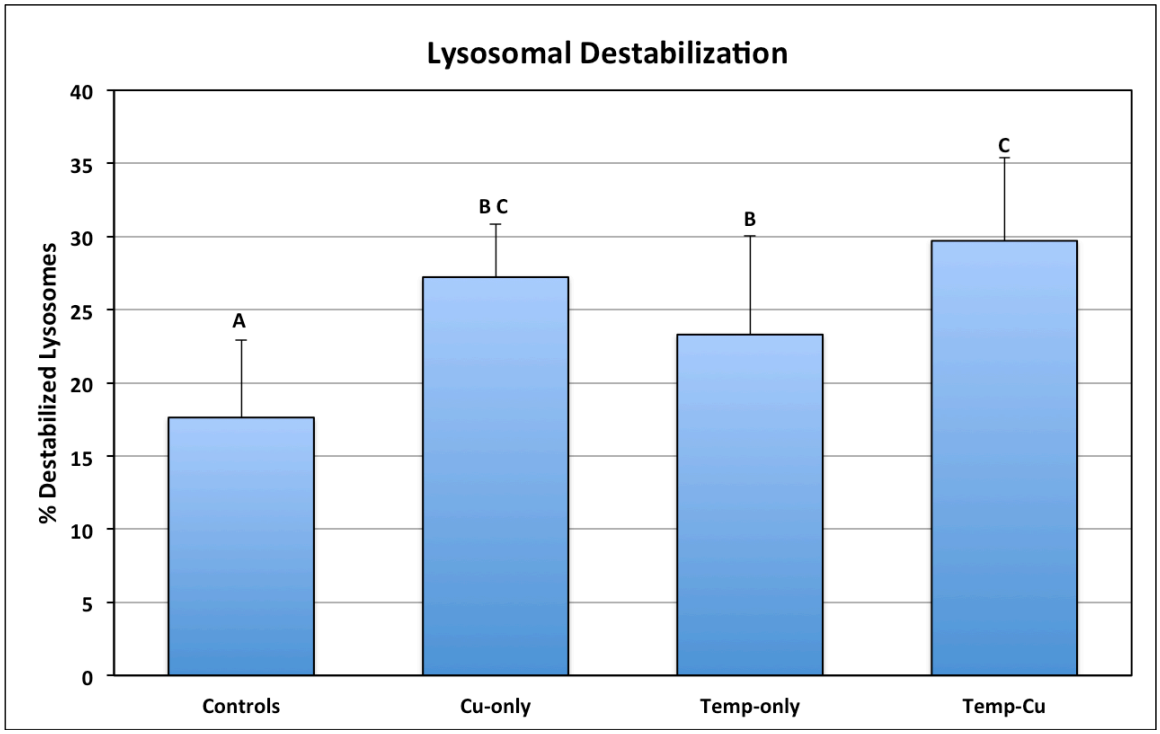


Figure 4.3: Percentages of lysosomal destabilization of whole adult jellyfish (oral arm and bell tissues) controls and treatment groups (Cu-only, temp.-only and temp.-Cu). Data are expressed as means + SD (control n=16, 10ppb=20, Temp=10, Temp + Cu=17); results are the combined data from replicated experiments. Different letters indicate significant differences between treatment groups ($p<0.05$).

*Lipid Peroxidation*

Another measure of tissue damage used for these studies was lipid peroxidation. This assay showed significant differences between treatment groups for both oral arm and bell tissues ($p<0.001$, $F=34.3$; $p<0.001$, $F=29.6$, respectively, Figure 4.4). In both tissues, elevated temperature was associated with lower lipid peroxidation. In the oral arm tissues, TEMP-Cu responses were not significantly different from controls but Cu-only responses were significantly different ($p=0.04$). However, in the bell tissues, the TEMP-Cu treatment caused a significant increase in lipid peroxidation that was not observed in either Cu-only or TEMP-only treatments.

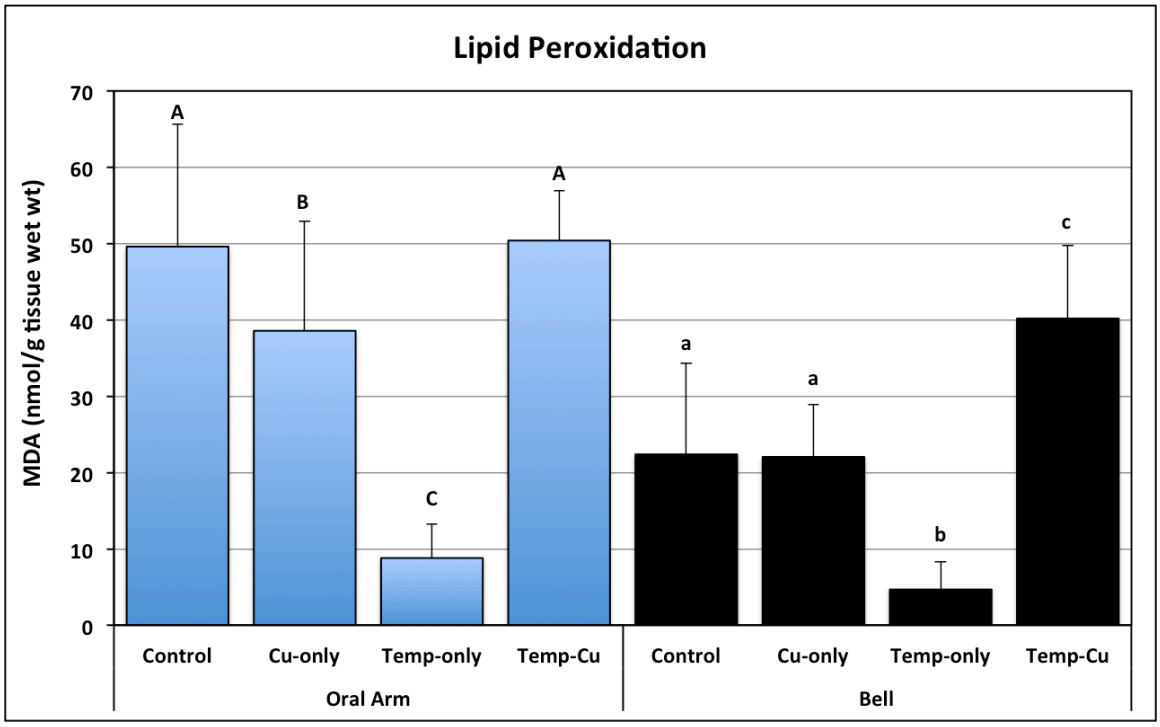


Figure 4.4: MDA concentrations in oral arm and bell tissues of whole adult jellyfish controls and treatment groups (Cu-only, temp.-only and temp.-Cu). Data are expressed as means + SD (oral arm-control n=9, 10ppb=8, Temp=9, Temp + Cu=17; bell-control n=12, 10ppb=14, Temp=6, Temp + Cu=17); Like letters indicate no significant differences between treatment groups and different letters indicate significant differences ($p<0.05$).

## Discussion

Our current findings show that *A. aurita*, and potentially other jellyfish, could face important impacts of increasing temperatures, especially when they encounter even low levels of pollutants. In this study of elevated temperatures, Cu, and a combination of both, there were significant effects on biomarker and behavioral responses of *A. aurita*. Increasing sea surface temperatures is an inevitable challenge that many organisms will face as climate change continues (Doney et al, 2012; Byrne et al, 2013). Temperature stress has been shown to have strong impacts on fundamental physiological functions that can ultimately lead to changes in abundance and distribution of species and alter pelagic, benthic, and estuarine communities (Harley et al, 2006; Rosenzweig et al, 2008; Pansch et al, 2012; Byrne et al, 2013). Studies have shown that increased temperature leads to

increased metabolic rate (Robertson et al, 2001), and thus may affect higher-order behaviors as well as fundamental cellular responses. In our studies, temperature alone tended to cause modest increases in pulsation rates and cellular damage, but caused significant decreases in antioxidant cellular responses. Antioxidant capacity was analyzed by examining GSH, one of the most prominent tri-peptide antioxidant in biological systems. Due to its importance, baseline levels must be maintained to ensure cellular homeostasis and resistance to increased oxidative stress (McCarthy et al, 2013). Decreased GSH levels were observed when jellyfish were exposed to elevated temperatures in both oral arm and bell tissues. Reductions in antioxidant responses during periods of increased temperatures could lead to temperature-induced toxicity as well as vulnerability to other stressors such as pollutants.

While modest, but significant increases in lysosomal destabilization were observed, levels of MDA were actually lower for the TEMP-only exposures, which could reflect collateral effects of increases in other compensatory mechanisms that are sensitive to temperatures, such as heat shock proteins (Hsps), to maintain homeostasis and combat cellular damage (Stefan et al, 1991; Parsell et al, 1993; Mayer et al, 2005; Chaurasia et al, 2016). Heat shock proteins are among the most conserved proteins known, induced by fluctuating temperatures as well as other stressors, such as hypoxia and metal pollutants (Lindquist and Craig, 1988; Sanders et al, 1991; Gupta et al, 2010). Although Hsps were not the focus of this study, these proteins are likely instrumental as compensatory mechanisms. Studies have shown increased gene expression for Hsps in *A. aurita* under similar stress conditions (Schroth et al, 2005) as well as Hsps induction and responses to

various environmental responses in other cnidarians (Downs et al, 2002; Kingsley et al, 2003; Reitzel et al, 2008; Seveso et al, 2016).

Copper tissue levels increased slightly in both oral arm and bell tissues of animals exposed to these low levels (10 ppb) of Cu alone. While there were no increases in lipid peroxidation, increases in GSH levels were observed for Cu only exposures. Generally, increases in GSH suggest compensatory responses to some type of cellular stress. Decreased lipid peroxidation data in oral arm tissues and unchanged MDA levels in bell tissues in Cu-only exposures suggests that increased GSH reduced the potential for oxidative stress, further reinforcing the importance of this compensatory antioxidant mechanism. However, significant increases in lysosomal destabilization in Cu-only exposed animals, when compared to the controls, suggest that cellular damage was occurring during short-term exposures to low levels of Cu. The lysosomal biomarker showed increased destabilization compared to controls, comparable to results with other invertebrates under similar metal exposures (Ringwood et al, 2006, 2009, 2013; Edge et al, 2013; Cain et al., 2015). This reinforces the use of lysosomal assays as an early indicator of sub-lethal stress over a broad range of species.

While behavioral responses showed that Cu-only exposures elicited decreased pulsation rate over time, there was a significant increase in pulsation rate in TEMP-Cu exposures. Increases in cellular metabolic rates and changes in cellular responses related to temperature increases could increase the bioavailability and bioreactivity of Cu. During TEMP-Cu exposures, tissue Cu concentrations were significantly higher than controls or Cu only exposures in both bell and oral arm tissues. Metal tissue accumulation, as well as toxicity, has been shown to increase with elevated temperatures

in other marine invertebrates (Bat et al, 2000; Heugens et al, 2003; Khan et al, 2006; Lewis et al, 2016). Other studies have shown that metal exposures can increase basal cellular metabolic demands through upregulation of compensatory and protective mechanisms, such as Hsps (Calow, 1989, 1991; Ringwood et al, 1998; Ivanina et al, 2008; Sokolova and Lannig, 2008; Sokolova et al, 2012).

A significant increase in lipid peroxidation in bell tissues was observed in TEMP-Cu exposures, but no change in oral arm tissues was observed. Lysosomal damage also occurred, at a higher percentage during TEMP-Cu exposures than controls or other treatment groups. The reduced antioxidant capacity could have further exacerbated oxidative stress. Under longer, chronic TEMP-Cu exposures, exhaustion of antioxidants and protective mechanisms and increased tissue Cu accumulation, even greater increases in tissue damage and mortality would be expected.

Summary

These studies indicate significant changes in cellular biomarker and behavioral responses to temperature as well as temperature in combination with Cu, a common marine pollutant. The greatest effects of temperature were reduced GSH levels, which in and of itself is problematic, but can also increase susceptibility to pollutants and other stressors. The combination of biomarker and behavioral responses further highlights the potential value of *A. aurita* as a pelagic and estuarine bioindicator to metal as well as temperature stress.

# CHAPTER 5: THE RELATIVE SENSITIVITIES OF DIFFERENT LIFE HISTORY STAGES OF *AURELIA AURITA* TO COPPER

## Introduction

Estuarine and marine ecosystems are continually threatened by anthropogenic modifications and inputs. Benthic bioindicator species such as bivalves are well-established models for characterizing ecosystem health, but there are few invertebrate bioindicator species for characterizing potential impacts in pelagic realms. Gelatinous zooplankton constitute a significant portion of biomass in many of these systems, and are represented by many phyla, including cnidarians (Schneider, 1989, Romeo et al, 1992). Cnidarians, such as the scyphozoan, *Aurelia aurita*, are found in pelagic habitats of estuarine and marine systems in the free-swimming medusa form. As part of their life history they also have a sessile polyp form in benthic habitats (Arai, 1997).

The life history of these relatively primitive organisms is remarkably complex. Adults exist as separate sexes and males release sperm into the water column whereas females release eggs or take up sperm so that fertilization occurs in the gastric pouches and larvae are brooded for a period of time in brood pouches located on the oral arms (Glichrist, 1937). Planula larvae spend a short period of time in the water column until finding a suitable substrate on which to attach, which can include artificial substrates, such as plastics and glass (Holst and Jarms, 2007). Once attached, the larvae metamorphose into polyps. The polyp form may asexually reproduce through budding,

stolon formation, longitudinal fission and pedal laceration (formation of polyps from the pedal disc) (Kakinuma, 1975). Strobiliation, the formation and release of free-swimming ephyrae from the polyp, is not a well-understood phenomenon. However, most studies suggest strobilation is induced under certain environmental conditions (i.e. decreased temperature and salinity) and high food availability (Lucas, 2001) These environmental factors are believed to induce gene expression responses involved in the initiation of strobiliation, including retinoic acid pathways (Fuchs et al, 2014). Transformation into juvenile jellyfish occurs over a period of weeks, with the formation of oral arms and a well-developed bell with tentacles.

The life history polymorphisms (Hickman, 1967) observed in jellyfish species is of particular importance. A sessile polyp and free-swimming medusa form in one species may behave and respond to environmental stressors and contaminants quite differently, which can have significant implications for jellyfish populations. The polyp form has potential value as a traditional benthic, sessile bioindicator while the free-swimming medusa can serve as a valuable pelagic bioindicator. Additionally, ephyrae, the developmental stage produced by the polyp, can also offer insights as a bioindicator during such an important juvenile life stage. Thus, scyphozoans-polyp, ephyrae and adult forms-can be used as valuable bioindicators for characterizing the effects of anthropogenic stressors in pelagic and estuarine systems. Furthermore, a cosmopolitan scyphozoan, such as *Aurelia aurita*, may serve as a valuable cnidarian model organism for establishing a framework of knowledge that can be applied to other cnidarians.

*Aurelia aurita* has been used as a model in studies to understand population dynamics (Lo and Chen, 2008), nutrient cycling (Schneider, 1989; Miller and Graham,

2012), larval substratum selection (Holst and Jarms, 2007; Webster and Lucas, 2012) and strobilation and genetic controls (Fuchs et al, 2014). *Aurelia aurita* has also been used in studies concerning cnidarian toxins, impacts on human health and cnidarian physiology (Shaposhnikova et al, 2005; Seipel and Schmid, 2005). Despite its widespread use for a variety of biological studies, *A. aurita* has only recently been considered for ecotoxicological studies (Lucas and Horton, 2014; Faimali et al, 2014; Costa et al, 2015; Echols et al, 2016).

Heavy metals, such as copper (Cu), are a major anthropogenic pollutant found throughout aquatic ecosystems. Anthropogenic sources of Cu pollution in marine systems include anti-fouling paint, fertilizers, herbicides, pesticides, and industrial runoff (Stauber and Davies, 2000; Levy et al, 2007; Namiesnik and Rabajczyk, 2010). Despite its role as a pollutant in the environment, Cu is also biologically essential as a cofactor in many enzymes that undergo redox-chemistry (Koch et al, 1997; Ringwood et al, 1998; Krupanidhi et al, 2008; Jomova and Valko, 2011) and in the electron transport chain (Valko et al, 2005). Despite its essential nature, Cu can elicit toxicity responses at levels as low as 1ug/L (Levy et al, 2007). In polluted waters, Cu concentrations can be much higher, 12-877 ug/L (Donat et al, 1994; Moffett et al, 1997).

While there is some literature on heavy metal effects in cnidarians, most studies have focused on anthozoans (Brock and Bielmyer, 2013; Mitchelmore et al, 2003a; Mitchelmore et al, 2003b; Reichelt-Brushett and McOrist, 2003; Bielmeyer et al, 2010; Anjos et al, 2014) and hydrozoans (Grant et al, 2010), with little information available on heavy metal effects in scyphozoans (Lucas and Horton, 2014; Faimali, 2014;

Templemann and Kingsford, 2010; Romeo et al, 1987). Thus far, cellular biomarkers have not been used to evaluate sub-lethal stress responses in *A. aurita*.

Cellular markers are often used with bioindicator organisms and in environmental management programs because they provide health status on populations and ecosystems through the use of resident individuals serving as early-warning indicators. They can be used on a variety of species and can be fast and cost-effective (Moore et al, 2004; Dallas & Jha, 2015). They can also be used to assess the health of an ecosystem under chronic stress and assess the effectiveness of remediation programs (Ringwood et al, 1999; Connors & Ringwood, 2000; Ringwood et al, 2003; Colin et al, 2016).

A variety of cellular markers were used to evaluate sub-lethal effects and differences in polyps, ephyrae and medusae of *Aurelia aurita* when exposed to a range of environmentally realistic copper (Cu) concentrations. The relative sensitivities of different life history stages were compared. This work also serves to establish a framework of knowledge using a combination of biomarkers that can be applied to other scyphozoans and compared to other cnidarian species.

## Methods

### *Exposures*

Different life history stages, including polyps, ephyrae and medusae were compared to evaluate relative sensitivities between the life history stage using a suite of biomarker endpoints. Medusae, polyps and ephyrae were exposed to various Cu concentrations (5, 10, 25, 50 ppb). All Cu solutions were prepared in Instant Ocean saltwater with 25-30 ppt salinity and 7.95-8.05 pH, at room temperature. Four to five medusae were placed in 2 gallon buckets with 7 L of treatment water, 15-20 poylps were

placed in pyrex dishes with 200 ml of treatment water, and 15-20 ephyrae were placed in glass beakers with 500 ml of treatment water. During the 48-hour exposures, all life history stages were fed brine shrimp nauplii (*Artemia salina*) at the 24 and 48 hr. time points. Medusae were under constant aeration. Glass beakers containing ephyrae and polyps were under constant gentle aeration for oxygenation, and to keep ephyrae suspended in the water column. Salinity and pH were monitored and recorded daily for all treatments. Each exposure was repeated once, for a total of two replicate experiments.

At the end of each exposure, some individual polyps and ephyrae were processed for lysosomal assays and the remaining were pooled into two samples, consisting of 15-20 individuals, frozen at -80°C, for glutathione (GSH) and lipid peroxidation analyses. Individual medusae were used for each biomarker analysis. Oral arm and bell tissues were used for lysosomal destabilization assays and oral arm tissues were frozen at -80°C for lipid peroxidation and GSH assays.

*Total Glutathione*

To determine GSH levels in tissues, a DTNB-GSSG Reductase Recycling assay for glutathione was used. Tissues were weighed and homogenized in 4 volumes of 5% sulfosalicylic acid (SSA) and centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (100µl) were further diluted with 200 µl of SSA. Standards of known glutathione concentrations were prepared (1.5-200 µM) from a 1 mM GSH stock solution (Sigma-Aldrich, MO). Samples and standards (30µl) were added to 96-well plates with 150 µl of nicotinamide adenine dinucleotide phosphate (NADPH), 40 µl of 5,5'-dithiobis(2-nitrobenzoic acid), 23 µl of water. Just prior to reading the place, 7 µl of glutathione reductase from baker's yeast (GSSG-reductase, Sigma-Aldrich) was added to

each well. A kinetic analysis was performed using a MultiSkan Go 3.2 UV/Vis spectrophotometer (Thermo Fisher Scientific) at 412 nanometers every 30 seconds for 2 minutes and results were expressed as nmol/g wet tissue.

*Lysosomal Stability*

A neutral red lysosomal destabilization assay was used to assess the integrity of lysosomal membranes (Moore, 2004; Ringwood et al, 2005; Moore, 2006; Canesi et al, 2010). Pie-slice shaped tissue samples of bell and oral arm tissues, approximately 150-250mg, were processed into primary cell preparations with Ca/Mg-free-saline (CMFS) and trypsin, sheared to break apart clumps of cells and filtered through 41μm nylon screen. Cells were rinsed and re-suspended in CMFS and a stock solution of neutral red (NR) (0.04mg/mL) was added at a 1:1 ratio of NR to cell preparation volume. After a 60-minute incubation period cells were scored as either stable (NR contained within the lysosomes) or destabilized (NR diffusing into the cytoplasm from damaged lysosomes) at 400x magnification, with at least 50 cells scored from each preparation.

*Lipid Peroxidation*

Tissues were weighed and homogenized in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0), then centrifuged (13000 g at 4°C for 5 minutes). Subsamples of the supernatant (50μl) were combined with 700 μl of 15% trichloroacetic acid containing (0.375%) thiobarbituric acid and 7 μl 2% butylated hydroxytoluene. Standards of known MDA concentrations were prepared (6.25-800 μM) from a 10mM malondialdehyde tetraethylacetal stock solution (Acros organics, NJ), as previously described (Buege & Aust, 1978; Gray, 1978). Samples and standards were boiled in a water bath for 15 minutes, and then centrifuged (13000 RCF, 5 minutes, room

temperature). The subsamples of each supernatant were then pipetted into a 96-well plate (200-µl in each well), and MDA levels were measured at 532 nm using a MultiSkan Go 3.2 UV/Vis spectrophotometer (Thermo Fisher Scientific) and results were expressed as nmol/g wet weight tissue.

*Statistical Methods*

Data were analyzed using Sigma Stat 2.0 and Graphpad Prism 6. Data were pooled from different exposures for statistical analysis because there were no significant differences between experiments. A one-way analysis of variance (ANOVA) was used to compare Cu treatments. When significant differences were detected ($p \leq 0.05$), *a posteriori* comparisons (Student-Newman-Keuls method) were used to identify differences between treatment groups and controls. When normality or equal variance tests failed, a transformation or a one-way ANOVA on ranks was performed and pairwise comparisons were made using Dunn's methods to determine significant differences in copper-exposed groups and controls.

Results

*Total Glutathione*

Glutathione results varied significantly between the different life history stages. Polyps clearly showed much higher levels of GSH than either ephyrae or medusae. There were no significant differences for the 5 ppb treatment but at 10 ppb Cu, there was a significant increase in GSH concentration; then significant decreases in GSH concentrations were observed at 25 and 50 ppb Cu ($p<0.001$, $F=22.603$, Figure 5.1). For the ephyrae, GSH levels increased with increasing Cu exposure concentration. GSH levels of control ephyrae were $1/10^{th}$ that of the polyps and showed significant increases

in GSH, especially at the 25 and 50 ppb treatments ($p<0.001$, $F=9.219$, Figure 5.2). Medusae oral arm tissues had very low GSH levels compared to the polyps and ephyrae, approximately $1/10^{th}$ that of ephyrae and $1/100^{th}$ that of the polyps ($p=0.179$, $F=4.9$, Figure 5.3). Although medusae oral arm tissues did not show significant differences between treatment groups, it should be noted that there was 100% mortality at the 50 ppb treatment.

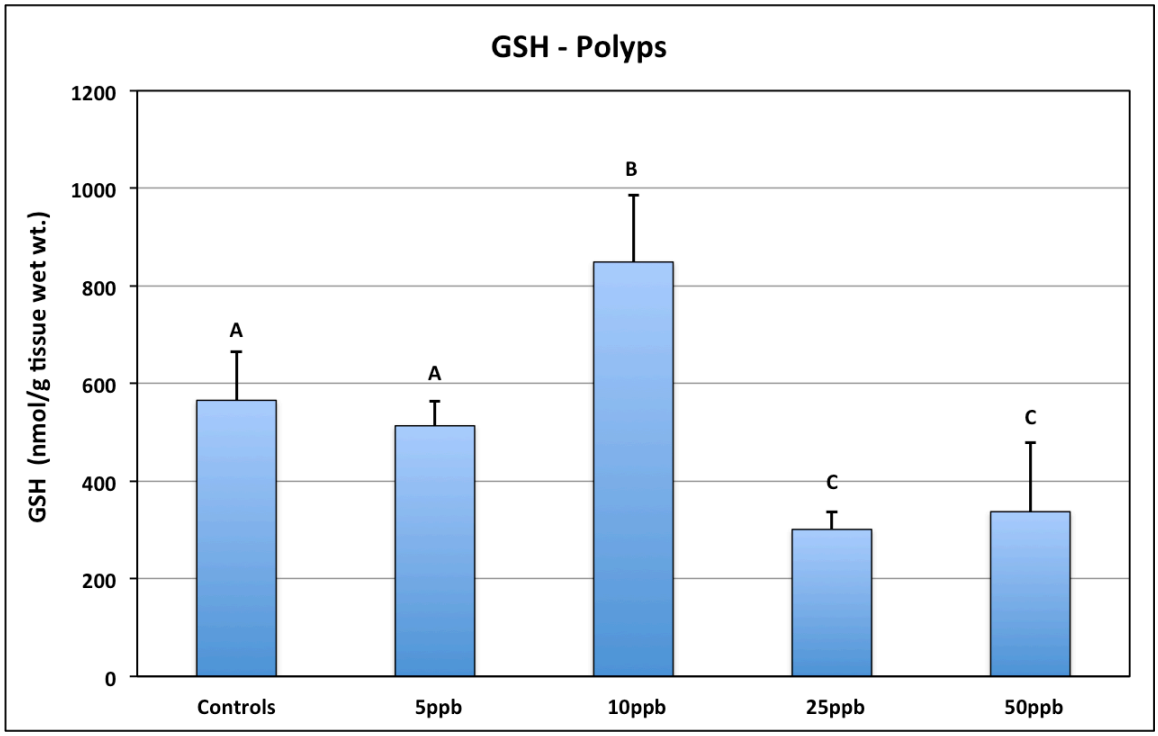


Figure 5.1: Total GSH concentrations in polyps controls and Cu treatment groups. Data are expressed as means + SD (n=5 pooled samples). Different letters indicate significant differences between treatment groups ($p<0.05$).

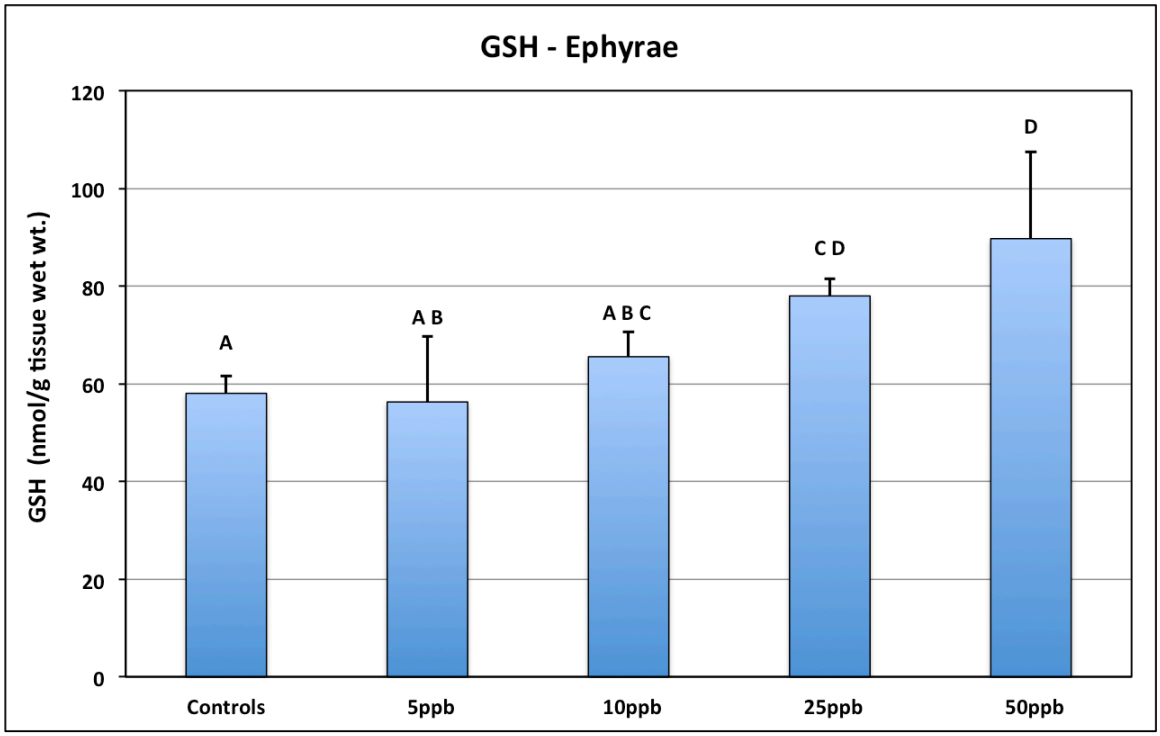


Figure 5.2: Total GSH concentrations in ephyrae controls and Cu treatment groups. Data are expressed as means + SD (n=5 pooled samples). Different letters indicate significant differences between treatment groups ($p<0.05$).

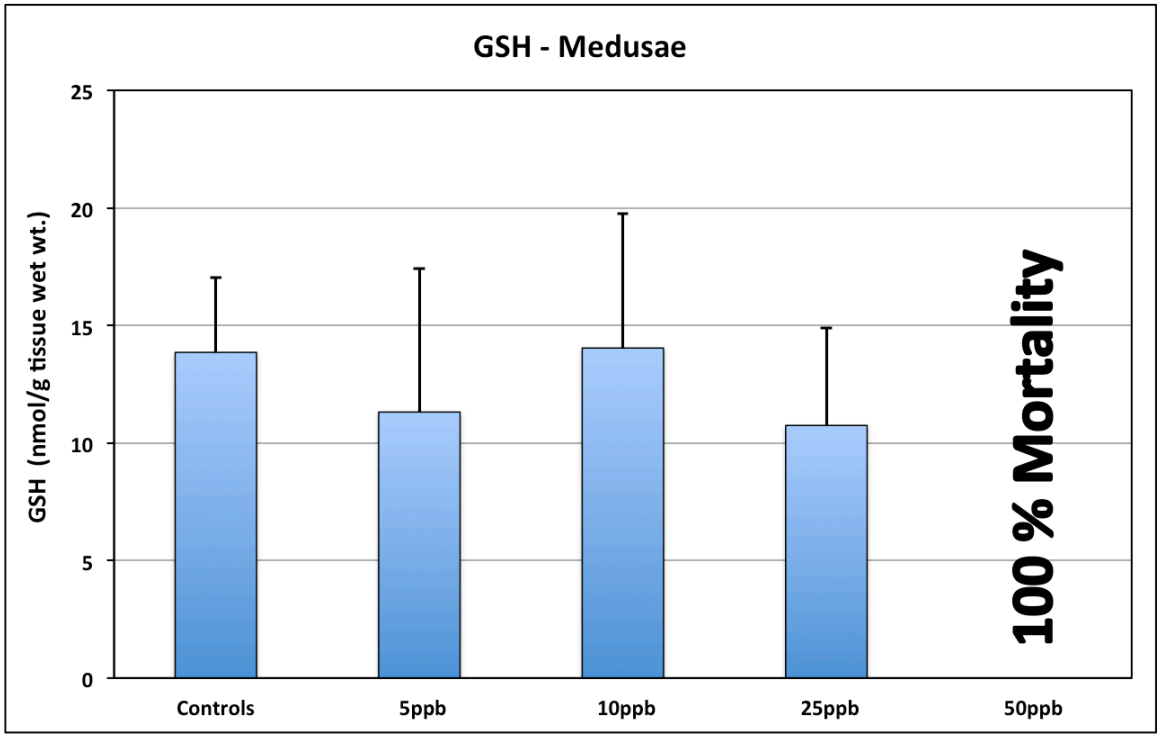


Figure 5.3: Total GSH concentrations in medusae (oral arm) controls and Cu treatment groups. Data are expressed as means + SD (medusae controls n=9, 5ppb n=14, 10ppb n=10, 25ppb n=10); There were no significant differences between treatment groups ($p<0.05$).

*Lysosomal Stability*

Lysosomal destabilization followed a similar pattern for all life history stages for *A. aurita*. Although there was not a significant difference in treatment groups for polyps, the data showed that there were increases in the number of destabilized lysosomes as Cu concentration increased, especially at 25 and 50 ppb Cu ($p=0.054$, $F=3.386$, Figure 5.4). The ephyrae showed a similar pattern, with little to no increases between control, 5 and 10 ppb Cu treatment groups and more pronounced increases at 25 ppb Cu and a significant increase from the control at 50 ppb Cu ($p=0.044$, $F=3.655$, Figure 5.4). There were significant increases in lysosomal destabilization between all treatment groups for the medusae ($p<0.001$, $F=37.195$, Figure 5.4), with adverse effects observed at concentrations as low as 5 ppb.

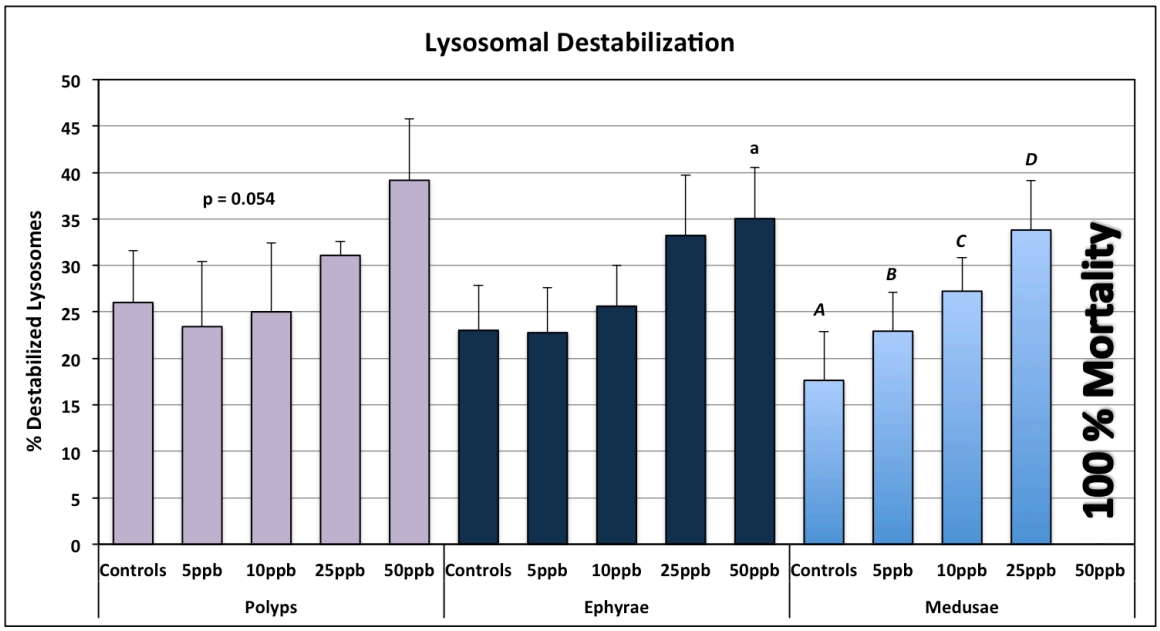


Figure 5.4: Percentages of destabilized lysosomes in polyps, ephyrae and medusae (oral arm), controls and Cu exposed. Data are expressed as means + SD (polyp and ephyrae control, 5, 10, 25, 50 ppb n=3; medusae controls n=16, 5ppb n=19, 10ppb n=20, 25ppb n=17); Different letters indicate significant differences between treatment groups ($p<0.05$).

*Lipid Peroxidation*

Generally, lipid peroxidation levels showed divergent responses to Cu dependent on the life history stage. For the polyps, there were no significant differences in lipid peroxidation between treatment groups ($p=0.501$, $F=0.866$, Figure 5.5). In the ephyrae stage, significant differences were found ($p<0.001$, $F=22.867$, Figure 5.5), and greater oxidative damage was observed in ephyrae exposed to 50 ppb Cu. At 5 and 10 ppb Cu, the MDA concentrations were lower than the 25 and 50 ppb exposures and almost significantly lower than the controls. Medusae showed no evidence of oxidative stress, as there were no significant differences between treatment groups ($p=0.359$, $F=1.104$, Figure 5.5), but they suffered 100% mortality after only 48 hr. exposures to 50 ppb Cu.

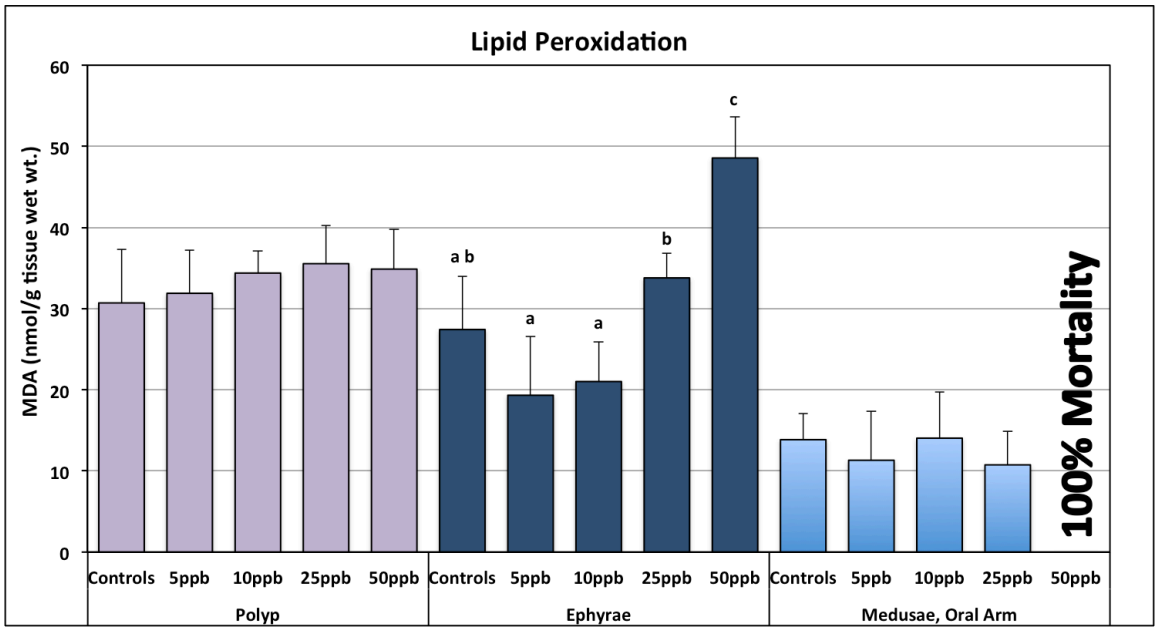


Figure 5.5: Total MDA concentrations in polyps, ephyrae and medusae (oral arm), controls and Cu exposed. Data are expressed as means + SD (polyp and ephyrae control, 5, 10, 25, 50 ppb n=5; medusae controls n=8, 5ppb n=14, 10ppb n=10, 25ppb n=10); Different letters indicate significant differences between treatment groups ($p<0.05$).

## Discussion

These studies indicate differential sensitivities in the different life history stages even after only short-term (48 hr.) exposures to environmentally relevant Cu concentrations. Adverse effects on cellular biomarker responses were observed at 25 ppb Cu for all life history stages, and evidence of perturbation was consistently observed at 10 ppb Cu. Mortality was only observed in medusae, with 100% mortality at concentrations of 50ppb Cu, but no mortality was observed in polyps or ephyrae. This indicates that the medusae may actually be more sensitive than polyp or ephyrae stages, and highly sensitive when compared to other invertebrate species under similar stress conditions (Downs et al, 2001; Mitchelmore et al, 2003; Almeida et al, 2004; Edge et al, 2013; Abujamara et al, 2014).

Glutathione levels were significantly higher in polyps when compared to ephyrae or medusae. Generally, increases in GSH indicate cellular stress but can also serve as compensatory responses, and GSH levels tended to increase at higher Cu exposures. Decreased GSH levels indicate that compensatory responses are overwhelmed and that there is an imbalance between antioxidants and oxyradicals, leading to increased oxidative stress (Halliwell & Gutteridge, 2007). Initially, the increases in GSH levels of the polyps at 10 ppb Cu functioned as a compensatory response, but at higher concentrations, 25ppb and 50ppb Cu, polyps showed significantly decreased GSH levels compared to controls.

Medusae had the lowest baseline GSH levels and polyps also showed evidence of GSH depletion at the higher Cu exposure concentrations. The results are consistent with the paradigm of increased sensitivity when antioxidant levels are low or depleted. This

further reinforces the importance of this assay by allowing comparisons of relative sensitivities to other invertebrates, as well as different life history stages, when exposed to similar stress conditions.

Lysosomal destabilization increased with increasing Cu concentrations in polyps, ephyrae and medusae, indicating that this is a valuable, sensitive biomarker of tissue damage in cnidarians, especially when exposed to metals. These results are comparable to other invertebrates (Ringwood et al, 2006, 2009; McCarthy et al, 2013; Edge et al, 2014; Cain et al 2015). While ephyrae and polyps were not affected until 25 ppb Cu, the medusae showed lysosomal toxicity at 5 ppb Cu and they were all dead after 48 hrs. at 50 ppb Cu.

Overall, lipid peroxidation results remained unchanged as Cu exposure concentration increased. These results indicate that jellyfish, especially the medusae, are not readily prone to oxidative damage. No significant increases in lipid peroxidation were observed in polyps or medusae, and only in ephyrae at 50 ppb Cu. Lipid peroxidation data for cnidarians are not extensive, however, the MDA levels in this study are low when compared to similar measurements in other invertebrates (Almeida et al, 2004; Edge et al, 2013; Abujamara et al, 2014). In fact, unpublished work from our lab with corals under similar acute Cu toxicity data showed 10-15 times greater MDA levels than did jellyfish in these studies.

Although few studies have been conducted to determine heavy metal effects in scyphozoans, our results indicated that polyps did not exhibit oxidative damage or significant mortality during these short-term exposures, consistent with work conducted by Lucas and Horton (2014). In their studies, *A. aurita* polyps were exposed to a range of

Cu concentrations (20-200 ppb) and overall polyp condition was assessed after 21 days. They found that the 200 ppb Cu exposures had the most severe responses with quickly developing deformities, reduction in asexual reproduction and 100% mortality at the end of the 21-day exposures. Our results are consistent with this finding as no mortality was found with any of our polyp exposures, even at the highest concentration, 50ppb Cu. There were, however, significant changes in tissue damage. There was also evidence of GSH perturbation. GSH levels were higher at 10 ppb Cu, suggesting a compensatory response at this concentration, but by 25 and 50 ppb GSH depletion was observed, setting the stage for oxidative damage. Therefore, the antioxidant GSH biomarker served as an early-warning indicator

Previous work in hydrozoans showed increased colonies and increased reproductive output when exposed to low (1-10 ppb) concentrations of Cu (Stebbing, 1981, 2002). In the current study, we also observed no toxicity at 10 ppb and indeed did observe increased GSH at this concentration in polyps. However, we then observed a significant increase in lysosomal destabilization and a significant decrease in GSH at 25 pbb Cu, indicating increased cellular damage and reduced antioxidant capacity. Both of these biomarkers have shown links to reproductive success (Ringwood et al, 2004; Moore et al, 2004) so we would predict adverse effects on reproductive success when Cu levels approach 25 ppb Cu. Significant impacts on marine systems where jellyfish and other species experience declines in reproductive success when exposed to even nominal levels of heavy metals have been shown in previous studies (Reichelt-Brushett and Harrison, 2005; Wijnhoven et al, 2009, Lucas and Horton, 2014).

Marine invertebrates experience a wide variety of anthropogenic contaminants at varying concentrations. Estuaries, where contaminant pollution is more prevalent, provide ideal substrate conditions for polyp attachment on natural surfaces, such as shell, but they also settle on wood, concrete, plastics, etc. (Hoover and Purcell, 2009) and, thus, may be exposed to pollution for chronic time periods. Species-specific sensitivities to acute and chronically exposed populations can lead to changes in biodiversity and community composition (Warwick, 2001). Additionally, chronically exposed polyps could cause changes in medusae populations through altered strobilation events. When strobilation does occur, newly developing ephyrae and medusae may also be affected by contaminants as currents move them from estuaries to pelagic systems.

Despite experiencing environmental and anthropogenic stress, jellyfish are known to tolerate wide ranges of environmental conditions (temperature, salinity, etc.) and anthropogenic disturbances such as hypoxia and eutrophication (Lucas et al, 2001; Ishii et al, 2008; Thein et al, 2012). Polyp survival is key to subsequent medusae populations; during unfavorable conditions, polyps employ protective strategies such as inhibition of strobilation and formation of podocysts (Arai, 2009; Di Camillo et al, 2010; Thein et al, 2012). These results suggest that compensatory mechanisms like GSH may also be significantly elevated in the polyp stage, when compared to ephyrae and medusae stages, to combat environmental and anthropogenic disturbances and ensure polyp survival. Interestingly, in addition to the extremely low levels of GSH in the medusae, they showed no evidence of upregulation of GSH (or increases in lipid peroxidation) in spite of the rapid increases in lysosomal destabilization and high mortalities.

Overall, this body of work provides essential new data regarding relative sensitivity of life history stages and establishes a framework of knowledge for using cellular biomarkers for jellyfish. *A. aurita* was very sensitive to environmentally relevant concentrations of Cu and the adult medusae were actually more sensitive than the ehpyrae or polyp stages, further highlighting this species' high sensitivity to low levels of a common anthropogenic contaminant. This work further demonstrates *A aurita*'s use as a bioindicator species for estuarine and pelagic, as well as benthic, systems as a tool for environmental monitoring.

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