Ecotoxicology of Natural and Anthropogenic Extreme Environments

by

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University Program in Ecology
Duke University

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program
in Ecology in the Graduate School
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ABSTRACT

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Abstract

Reactive oxygen species (ROS) are produced endogenously in all aerobes and are induced by environmental stressors. ROS oxidize and disable essential cellular components such as DNA, proteins, and lipid membranes. Exposure to metals, polycyclic aromatic hydrocarbons (PAHs), and some pesticides can induce oxidative stress in marine invertebrates. All aerobic organisms have a network of antioxidants and enzymes to quench ROS and prevent oxidative damage. This dissertation examines antioxidant and oxidative stress biomarkers in endemic molluscs and crabs from two natural extreme environments: deep-sea hydrothermal vents in the Lau and North Fiji Basin, and cold seeps in the Gulf of Mexico. In addition, the acute toxicity and sub-lethal effects of four insecticides and an herbicide are examined in the estuarine blue crab, *Callinectes sapidus*. Blue crabs are North Carolina's most important fishery species and are frequently found in agricultural drainage ditches, an example of an anthropogenic extreme environment.

Total glutathione, catalase, superoxide dismutase, and lipid peroxidation levels were of the same respective order of magnitude in the two vent gastropods, *Alviniconcha* sp. and *Ifremeria nautilei*, and vent mussel, *Bathymodiolus brevior*. These biomarkers activities were similar to those from previous reports on Mid-Atlantic Ridge mussels, except for ~100-fold higher lipid peroxidation levels among Lau molluscs. Principal

component analysis (PCA) of mollusc tissue-specific biomarker levels grouped individuals by species rather than by site.

Biomarker levels in the seep mussels *Bathymodiolus childressi, B. brooksi*, and *B. heckerae* were similar across species except for elevated foot and gill cytosolic SOD in mussels from MC-640 compared to those from AC-645. PCA of seep mussel biomarker levels differentiated by species with *B. childressi* isolated from *B. brooksi* and *B. heckerae*. The addition of *B. brevior* biomarker data to the PCA showed them grouping around *B. brooksi* and *B. heckerae*. *Bathymodiolus childressi* is ancestral to the other species and contains only methanotrophic endosymbionts. Whether symbionts play a role in alleviating possible toxic conditions remains unknown.

Pesticides were acutely toxic to blue crabs in the order of Lambda-cyhalothrin > imidacloprid ≈ aldicarb > acephate ≈ Roundup® (glyphosate). Megalopae were almost always more sensitive to pesticides than early stage juveniles. Commercial formations of pesticides generally showed similar toxicity to active ingredients alone. Exposure to LC_{20} levels of acephate, aldicarb, imidacloprid and Roundup significantly increased the frequency of juvenile mortality after molting. There was no significant change in total glutathione or lipid peroxidation of exposed megalopae. Lambda-cyhalothrin-, imidacloprid-, and aldicarb-based products have the potential to cause acute toxicity and molting-related mortality in shallow creeks and ditches.

Dedication

For everyone who ever believed in me and was patient enough to wait.

Contents

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List of Abbreviations

AC-645	Alaminos Canyon lease block 645, seep site
a.i.	Active Ingredient
ANOVA	Analysis of Variance statistical test
ASW	Aged Sea Water
CAT	Catalase
CH_4	Methane
CSOD	Cytosolic Superoxide Dismutase
DUML	Duke University Marine Laboratory, Beaufort, NC
EPR	East Pacific Rise
EST	Estuarine Sea Water (not aged)
GoM	Gulf of Mexico
GSH	reduced Glutathione
GSSG	oxidized Glutathione
H_2S	Hydrogen Sulfide
HH	Hine Hina vent site, Lau Basin
J1, J4	Juvenile crab molt stage. J1 is the first juvenile molt.
KM	Kilo Moana vent site, Lau Basin
λ-cyhalothrin	Lambda-cyhalothrin

LB	Lau Basin
LC_{20}	Lethal Concentration 20 (20% dead)
LC_{50}	Lethal Concentration 50 (50% dead)
LPO	Lipid Peroxidation
MAR	Mid-Atlantic Ridge
MC-640	Mississippi Canyon lease block 640, seep site
MDA	Malondialdehyde (for LPO)
MH	Mussel Hill vent site, North Fiji Basin
MSOD	Mitochondrial Superoxide Dismutase
NCDA&CS	North Carolina Department of Agriculture & Consumer Services
NFB	North Fiji Basin
OPP	Office of Pesticide Programs
PAH	Polycyclic aromatic hydrocarbons
SOD	Superoxide Dismutase
TBARS	Thiobarbituric acid reactive substances (for LPO)
TM	Tui Malila vent site, Lau Basin
TTM	Time to Metamorphosis

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Chapter 1. Introduction: Extreme Marine Environments

Overview

The title of "extreme environment" is an anthropocentric idea. For humans, the entire deep-sea is an extreme environment because we can not live there and find it hard to believe that others can. Likewise Beaufort, NC is an extreme environment from a deep-sea organism's point of view. Environments to which organisms have adapted and within which they have evolved are no longer "extreme" from their own perspective. Science is much more versed in the ways and means of organisms that share a "normal" with humans simply because they are more familiar, easier to work with, and often of economic importance. Much of what we do know has been learned by stressing organisms under artificial chemical or physical extremes and noting the changes in various processes. Additional, and occasionally unforeseen, insights can be gained by investigating the processes of interest in organisms that have evolved for tens of millions of years to live at what I call natural extreme environments.

The continued addition of synthetic chemicals to the world's oceans through agricultural and residential runoff, accidental spills, and intentional dumping has created anthropogenic extreme environments in coastal oceans and estuaries. Estuaries are critical ecosystems and serve as the nursery grounds for many of the commercially harvested fishery species. The coastal plain of the southeast United States is highly

infiltrated with rivers, creeks, and ditches that drain large agricultural fields. This intertwined juxtaposition increases the potential for lethal and sub-lethal impacts of pesticides and herbicides and their "inactive" ingredients on ecologically and economically important estuarine species.

Among the processes that maintain cellular homeostasis are those that maintain the proper balance of pro-oxidants and antioxidants in the cell. Exposure to chemicals that promote the creation of pro-oxidants or alternatively diminish antioxidant defenses can leave organisms open to cellular oxidative damage and induce oxidative stress. Metals, polycyclic aromatic hydrocarbons, and pesticides are a few of the chemical classes known to induce oxidative stress in marine invertebrates. Changes in levels of oxidative damage and concentrations of antioxidant peptides and enzymes have been used as biomarkers to gauge responses of organisms to chemical stressors. Few reports, however, have been published on those biomarkers in marine invertebrates endemic to extreme environments with naturally high concentrations of chemicals that normally induce oxidative stress. Studies of these biomarkers in marine invertebrates endemic to metal-rich deep-sea hydrothermal vents and hydrocarbon-rich cold seeps are presented in this dissertation.

In this dissertation I present ecotoxicological studies of two natural extreme environments and one anthropogenic extreme environment. I begin in Chapter 1 with

introductions to cellular oxidative processes as well as the settings of the three extreme environments studied in Chapters 2 – 4.

In Chapter 2, I examine antioxidant and oxidative stress biomarkers in three molluscan and one crab species endemic to metal-rich deep-sea hydrothermal vents in the Lau and North Fiji Basins, Southwest Pacific Ocean. Comparisons of biomarker levels are made among different tissues, among vent sites, and among species.

In Chapter 3, I examine antioxidant and oxidative stress biomarkers in three species of mussel endemic to cold seeps in the Gulf of Mexico. Comparisons of biomarker levels are made among different tissues, among seep sites, and among species. Additional comparisons are made to congeners from hydrothermal vents.

In Chapter 4, I present results of acute toxicity assays of five pesticides commonly applied to cotton in post-larval and juvenile blue crabs. Sub-lethal effects of pesticide exposure are presented for molting rate and success, and antioxidant and oxidative stress biomarkers. Additionally antioxidant and oxidative stress biomarkers are reported for blue crabs collected from an agricultural ditch and from Beaufort, NC.

In Chapter 5, I present a summary of my findings and conclusions.

Background

Antioxidants and Oxidative Stress

The stepwise reduction of molecular oxygen to water during cellular respiration creates highly reactive oxygen intermediates in all cells of aerobic organisms (Winston and Di Giulio, 1991; Fridovich, 1998; Halliwell and Gutteridge, 1999). Among these reactive oxygen species (ROS) are the superoxide ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$), and the non-radical hydrogen peroxide (H_2O_2). ROS can oxidize and disable essential cellular components like lipids, DNA, and proteins (Halliwell and Gutteridge, 1999). Excessive oxidative damage can lead to cellular apoptosis and/or tissue necrosis. Oxygen radicals can be particularly harmful because they can form chain reactions that create more radicals, thus multiplying their damaging potential (reviewed by Fridovich, 1998). Exposure of organisms to xenobiotics that either promote the production of ROS or alternatively hinder the removal of ROS can lead to an imbalance in pro-oxidants and antioxidants and result in oxidative damage.

A network of ubiquitous antioxidants and enzymes has evolved to protect cells from the harmful effects of ROS and maintain cellular homeostasis. Among the many defense mechanisms are the superoxide dismutases (SOD) and catalase enzymes, and the tripeptide antioxidant glutathione. SODs are all very efficient enzymes that operate at diffusion-limited rates (Fridovich, 1998). There are several different varieties of SODs, each with different metals in its active site, but all catalyze the reaction of $2O_2^- + 2H^+ \rightarrow$

$H_2O_2 + O_2$. SODs in the cytoplasm of eukaryotic cells have Cu and Zn in their active sites. Cu is believed to be the redox cycler while Zn is believed to be largely structural (Fridovich, 1998). Prokaryotes and eukaryotic mitochondria express a Mn-containing SOD that is unrelated (based on amino acid sequence) to CuZnSOD (Fridovich, 1998). SOD dismutates one ROS but it makes another in hydrogen peroxide. Peroxide can be destroyed within cells by catalase, a heme-containing enzyme that changes $2H_2O_2$ into $O_2 + 2H_2O$. Catalase is one of the most efficient enzymes known (Lledías et al., 1998; Matés, 2000).

The tripeptide glutathione (γ-glutamyl-cysteine-glycine) is one of the cell's major thiols, and serves to protect cells from endogenous and exogenous compounds (Meister and Anderson, 1983). Glutathione can donate an electron to ROS and cycle between its reduced (GSH) and oxidized forms (GSSG). Glutathione cycles back to its reactive GSH state with the help of enzyme glutathione reductase. Besides redox cycling, glutathione can directly bind and detoxify heavy metals (Singhal et al., 1987; Freedman et al., 1989; Viarengo and Nott, 1993) as well as be bound to organic xenobiotics by the enzyme glutathione S-transferase. When bound to metals or other xenobiotics, glutathione unable to redox cycle and quench ROS. Therefore accumulation of metals and other xenobiotics can result in the depletion of reduced and total glutathione (Canesi et al., 1999), reducing a cell's antioxidant defense.

Concentrations of antioxidants like glutathione, enzymes such as superoxide dismutase and catalase, together with oxidative damage are used as biomarkers of pollution in marine teleost fish and molluscs (reviewed by Winston and Di Giulio, 1991; Livingstone, 2001). Changes in oxidative stress biomarkers in coastal mussels have resulted from environmental and laboratory exposures to several different chemical classes including redox active metals (e.g. Viarengo et al., 1998; Canesi et al., 1999; Vlahogianni and Valavanidis, 2007; Giarratano et al., 2010) and polycyclic aromatic hydrocarbons (PAHs) (e.g. Livingstone et al., 1990; Solé et al., 2007; Bebianno and Barreira, 2009).

Deep-sea Hydrothermal Vents

Before the first hydrothermal vents were discovered in the late 1970s on the Galapagos Rift (Lonsdale, 1977), the circulation of seawater into oceanic crust was theorized to occur based on abundant metalliferous sediments in certain regions of the deep ocean (e.g. Bostrom and Peterson, 1969; Corliss, 1971) and missing mid-ocean ridge heat (Lister, 1972). What was not widely predicted, however, was the associated thriving community made up of mussels, clams, crabs, and tubeworms. A few years later scientists in the *Alvin* submersible at 21°N on the East Pacific Rise (EPR) came across "black smoker chimneys" with billowing black fluid exiting structures several meters tall. Attempts to measure the temperature of the effluent resulted in a melted

thermometer assembly (Broad, 1997). Vent fluid enriched in hydrogen sulfide, methane, and metals support high biomass, low diversity metazoan communities that feed on free-living or use endosymbiotic chemoautotrophic bacteria. Hydrothermal vents and their associated communities have been discovered on almost every mid-ocean ridge and spreading center thoroughly explored (Van Dover, 2000). Enormous lengths of ridge crest remain unexplored; mostly in the southern hemisphere, the Indian Ocean, Western Pacific, and Polar regions.

The geology and physical properties of a particular spreading center determines the chemistry of the vent fluid, which in turn influences the biology and ecology of the resident vent communities. As newly formed oceanic crust cools, it cracks and allows bottom water to percolate down to depths at which it is heated by shallow magna chambers. At elevated pressures and temperatures chemical reactions between seawater and ocean crust create an anoxic and acidic (pH = 2 – 4) fluid loaded with gases (H_2S, CH_4, H_2, CO_2) and metals (Fe, Mn, Zn, Pb, Cu, Co, Ag, Mg) (reviewed by Tivey, 2007). If reaction temperatures exceed the boiling point at that pressure, the vent fluid can boil and separate into a gas-enriched vapor phase and a metal and salt-enriched brine phase (Von Damm, 1995). Highly focused and un-diluted vent fluid is often released at black smoker chimneys. As 300 – 400 °C vent fluids are rapidly cooled by the 2 °C bottom water, dissolved metals form metal-sulfides and precipitate out of solution creating the namesake black smoke and chimney walls.

Areas of diffuse vent fluid flow can be extensive and are fueled by hydrothermal fluids that have been diluted with seawater before reaching the seafloor. These fluids are often associated with nearby black smokers and usually share a common original (end-member) vent fluid source. Geochemical and microbial processes can alter the chemical composition of low temperature fluids adding and removing biologically important chemicals (Von Damm and Lilley, 2004). Areas of diffuse flow <30 °C support most of the vent biomass. These highly variable areas alternatively provide sulfide, methane, and CO_2 from vent fluid and oxygen from ambient seawater to support chemosynthesis. Vent organisms must be well adapted to cope with wide and rapid fluctuations in concentration of essential and toxic compounds and physical conditions (Johnson et al., 1986; 1988).

Lau and North Fiji Basin

The hydrothermal vent organisms that are assayed in Chapter 2 were obtained from vents within the Lau and North Fiji Basins. The South Pacific islands of Fiji are bounded to the Southeast by the Lau Basin (LB) and to the west by the North Fiji Basin (NFB), two back-arc basins. Back-arc basins are formed by tensional forces created by the subduction of one oceanic plate beneath another. In this case the Pacific plate is being subducted beneath the Australian plate at the Tongan Trench, creating the volcanic Tofua volcanic arc and Lau Back-arc Basin behind it. Back-arc basins differ

slightly from mid-ocean ridges because of the influence the subducting plate has on the mantle and consequently the new crust and supported hydrothermal systems. Old subducting tectonic slabs can be overlain by thousands of meters of sediment and associated porewater and hydrated crust can alter the mantle wedge (Gaetani and Grove, 1998; Martinez et al., 2006), the source of the spreading center magma. Early in the formation of the basin, the new crust is heavily influenced by the melting crust, sediment, and water of the subducting plate. Spreading centers greater than ~150 – 200 km from the volcanic arc show increasingly mid-ocean-ridge-like geochemistry (basalt) and hydrothermal characteristics (Martinez et al., 2006).

The V-shaped Lau Basin began forming ~6 million years ago with progressive southward seafloor spreading (Taylor et al., 1996). The ~400 km long Eastern Lau Spreading Center (including the Valu Fa Ridge, *sensu* Martinez et al., 2006) crosses the melt area influenced by the subduction zone with the northern end ~100 km and the southern end ~40 km from the volcanic arc. Therefore the ELSC and its hydrothermal vents present a natural gradient in many physical characteristics including depth, spreading rate, bedrock type, and ultimately vent chemistry (Fouquet et al., 1991a, b; Martinez et al., 2006; Mottl et al., submitted). From north to south, ridge depth decreases from ~3000 to ~1700 m, full spreading rate decreases from 97 to 39 mm/yr, and crust changes from basalt to andesite (Taylor et al., 1996; Martinez et al., 2006; Escrig et al., 2009). Due to these geological changes, vent chemistry changes, with decreasing H_2S, Fe,

and Zn concentrations from north to south (Hsu-Kim et al., 2008). Overall LB vent fluids are more acidic and relatively enriched in dissolved Zn, Pb, As, Mn, and Cd (Fouquet et al., 1991), and depleted in H_2S compared to mid-ocean ridge vent fluids. Lau Basin vent sites sampled in this dissertation from North to South are Kilo Moana, ABE, Tui Malila, and Hine Hina.

The North Fiji Basin is a mature back-arc basin (Kim et al., 2006) that began to form ~12 million years ago and is spreading at a rate of ~60 mm/yr (Auxende et al., 1995). Several high and low temperature hydrothermal vents have been described (Grimaud et al., 1991; Desbruyères et al., 1994; Ishibashi et al., 1994, Koschinsky et al., 2002). The Mussel Hill vent is a low temperature (<13 °C) vent site located at 2000 m depth on the Central Fiji Ridge near a triple junction (Koschinsky et al., 2002). Sampling of diffuse flow around Mussel Hill in 1998 (Koschinsky et al., 2002) indicated phase separation (subsurface boiling) was taking place with the vapor phase (enriched in gases and depleted in metals) being released, similar to nearby high temperature vents sampled in 1989 and 1991 (Grimaud et al., 1991; Ishibashi et al., 1994). Average diffuse flow fluid showed neutral pH, 30 µmol/kg sulfide, 0.40 µmol/kg CH_4, 239 nmol/kg dissolved Mn, 137 nmol/kg Fe, and 473 nmol/kg Zn (Koschinsky et al., 2002). Calculation of end-member (undiluted vent fluid) composition of the Mussel Hill diffuse flow fluid indicated it came from the same source as low salinity, transparent (no precipitation) high temperature (265 °C) fluid from nearby anhydrite ($CaSO_4$) chimneys (e.g. White

Lady) (Ishibashi et al., 1994; Koschinsky et al., 2002). These results suggest minimal geochemical or microbial alteration of hydrothermal fluid is taking place after subsurface dilution. North Fiji Basin vent fluids have amongst the lowest end-member concentrations of Fe (0.7 – 8.8 μmol/kg) and Mn (26 – 40 μmol/kg) in hydrothermal samples (Koschinsky et al., 2002). Low metal concentrations and high sulfide concentrations make North Fiji Basin vents very suitable for vent invertebrates (Koschinsky et al., 2002).

Lau and North Fiji Basin Vent Megafauna

Lau and North Fiji Basin vent ecosystems are dominated by the two Provannid gastropods *Alviniconcha* spp. (Kojima et al., 2001) and *Ifremeria nautilei* (Bouchet and Waren, 1991) and by the mussel *Bathymodiolus brevior* (von Cosel et al., 1994; Desbruyères et al., 1994). All three molluscs contain endosymbiotic thiotrophic γ-proteobacteria within their hypertrophied gills (Endow and Ohta, 1989; Windoffer and Giere, 1997; Dubilier et al., 1998; Borowski et al., 2002; Suzuki et al., 2005, 2006a, b). This endosymbiosis necessitates that host molluscs live within the diffuse flow vent effluent with sufficient reduced sulfide to feed their symbionts. These three molluscs tend to live in zones around vents with *Alviniconcha* occupying the zone closest to the vent orifice and *I. nautilei* and *B. brevior* in successive zones further away (Desbruyères et al., 1994; Henry et al., 2008; Waite et al., 2008; Podowski et al., 2009). Henry et al. (2008) explained

this pattern as a consequence of the superior autotrophic potential and temperature tolerance of *Alviniconcha* compared to the lower sulfide tolerance of *I. nautilei* and the lower temperature tolerance of *B. brevior*. Consequently *Alviniconcha* lives in the zone with higher temperatures (9–20 °C) and higher concentrations of vent-derived chemicals than *I. nautilei* (6–14 °C) and *B. brevior* (3–6 °C) (temperatures from Waite et al., 2008). High concentrations of thiosulfate ($S_2O_3^{2-}$) were often found around only *B. brevior* suggesting it could be the reduced fuel of choice for its endosymbionts (Waite et al., 2008).

The top predators in these vent ecosystems are the brachyuran crab *Austinograea alayseaee* and the anomuran spider crab *Paralomis hirtella*. Neither of these species contains endosymbionts and both are obligate predators/scavengers primarily of vent invertebrates. *A. alayseaee* crabs roam freely but are often found in and around gastropod and mussel beds well within physical and chemical influence of the vent fluid. They experience similar chemical conditions, at least most of the time, as *Alvinichoncha*, *I. nautilei*, and *B. brevior*, but retain the ability to quickly move from adverse physical and chemical conditions. Confamilial brachyuran crabs along the EPR, *Bythograea thermydron*, travel away from the vent area to hatch their eggs and perhaps for other reasons (Perovich et al., 2003). It is not known whether *A. alayseaee* displays this behavior as well, but it likely can and does travel to non-vent areas, at least periodically.

Cold Seeps

Cold seeps have been described along active and passive continental margins around the world (Sibuet and Olu, 1998; Cordes et al., 2009). Paull et al. (1984) first described cold seep communities dominated by tubeworms and mussels at the base of the West Florida Escarpment in the Gulf of Mexico (GoM). These communities appeared strikingly similar to those at the recently discovered hydrothermal vents (Kennicutt et al., 1985) and are likewise supported by chemoautotrophic endosymbionts. Dozens of seep sites have been discovered within the GoM on the upper and lower Louisiana Slope ranging in depth from 400m to 2200m (Sibuet and Olu, 1998; Cordes et al., 2009). In contrast to most hydrothermal vents that have high and variable fluid flux, seeps tend to be sedimented and have low diffuse flux over long time spans.

GoM seeps of the Louisiana slope are driven by salt tectonics. The extensive Louann evaporate formation deposited during the Jurassic is overlain by layers of sandstone and shale impermeable to accumulated petroleum and hydrocarbons from deep Mesozoic source rock (Kennicutt et al., 1992). The additional weight of sediments deformed the weak salt layer into salt diapirs (pillars) that cracked and faulted the impermeable overlying layers. These faults provide conduits for the migration of hydrocarbons through the sediments to the seafloor.

Petroleum hydrocarbons can be heavily altered by microbial processes during their upward migration. Two of the most important microbial processes are anaerobic

hydrocarbon oxidation and sulfate reduction. Consortia of sulfate reducing bacteria and anaerobic methane oxidizing archaea use unknown mechanisms to convert seawater sulfate and seep methane into hydrogen sulfide and carbon dioxide (Aharon and Fu, 2000; Boetius et al., 2000; Joye et al., 2004). This biogenic sulfide supports free living thiotrophs as well as endosymbiotic thiotrophic bacteria in vestimentiferan tubeworms and some bathymodiolin mussels. Elevated CO_2 concentrations can lead to the precipitation of authigenic carbonates that form hard structures used as substratum for attachment by tubeworms. These carbonates can act as physical barriers slowing the release of hydrocarbons from sediments and allowing more time for microbial sulfide production.

GoM hydrocarbon seeps tend to be either brine seeps or oil seeps (Cordes et al., 2009). Brine seeps are formed by the dissolution of shallow salt diapirs (pillars) that have pressurized a hydrocarbon reservoir. Brine seeps have moderate seepage rates of high salinity brine (salinity up to 130) supersaturated with methane with little sulfide and lower abundances of complex hydrocarbons (Smith et al., 2000). Often the brine will pool and create seafloor lakes (e.g. NR1 in the Green Canyon; MacDonald et al., 1990) of high salinity that are ringed with methanotrophic bacteria and sometimes with *Bathymodiolus childressi* that house methanotrophic endosymbionts (Smith et al., 2000).

Some GoM seeps are associated with petroleum leakage with animals sometimes drenched in crude oil (Wade et al., 1989; McDonald, 1990; Sassen et al., 1994; Nix et al.,

1995). Wade et al. (1989) reported Green Canyon 272 sediment core PAH levels of up to 6800 ng/g. Mussels (presumably *Bathymodiolus childressi*) had total PAH concentrations up to 7530 ng/g (Wade et al., 1989), with lower molecular weight hydrocarbons being more prevalent in mussel tissue. *B. childressi* from petroleum seeps typically showed lower condition index and lower growth rate (Nix et al., 1995), both of which improved upon transplantation to brine seeps, sometimes out performing native brine seep mussels (Bergquist et al., 2004).

GoM seeps are commonly labeled by their location within Minerals Management Service lease blocks. Mussels from two GoM seep sites were used in this dissertation; Mississippi Canyon lease block 640 (MC-640) and Alaminos Canyon lease block 645 (AC-645). MC-640 is a brine seep that sits atop a 15m tall mound at a depth of 1420m (Brooks et al., 2008). Sediment cores taken from bacterial mats show elevated salinity of 75 – 88 and methane concentrations of 6 mM (Brooks et al., 2008). Several small brine pools and channels support bacterial mats and two species of mussel (*B. childressi* and *B. brooksi*).

Alaminos Canyon cuts across the continental slope from 1500m to 3000m and exposes alternating layers of evaporate and carbonate (Bryant et al., 1990; Cordes et al., 2007). AC-645 rests at 2200m near the eastern side of the canyon and supports two species of mussel (*B. brooksi* and *B. heckerae*) and abundant tubeworms (Cordes et al., 2007; Brooks et al., 2008) among fractured carbonates. Low methane concentrations (< 30

μM) were measured in sediment cores (Brooks et al., 2008). Stable carbon isotope data for authigenic carbonates suggest they were derived from the microbial degradation of crude oil (Roberts and Aharon, 1994). Mussels here were often covered in a white precipitate not seen at other GoM seeps (Brooks et al., 2008).

Gulf of Mexico Seep Mussels

The communities inhabiting GoM seeps visually resemble those at hydrothermal vents. Symbiont-bearing bathymodiolin mussels and vestimentiferan tubeworms are the biomass dominant and foundational megafauna upon which diverse epifauna rely (reviewed by Cordes et al., 2009).

The genus *Bathymodiolus* is represented in the Gulf of Mexico by three species (Cordes et al., 2009). *Bathymodiolus childressi* (also seen as *"Bathymodiolus" childressi*, Gustafson et al., 1998) is best studied and is found at seeps along the upper and lower Louisiana slope at depths less than 2200m. It contains only type 1 methanotrophic gamma-proteobacteria (Fisher et al., 1987) and gets most of its nutrition from these symbionts (Fisher and Childress, 1992) despite an ability to filter feed (Page et al., 1990; Pile & Young, 1992). Although it has a large depth range, *B. childressi* in the Gulf of Mexico represents a single panmictic population (Carney et al., 2006).

Bathymodiolus brooksi harbors both methanotrophic and thiotrophic endosymbionts, sometimes within the same vacuole (Fisher et al., 1993), and lives at

depths between 1080m and 3300m (Cordes et al., 2009). The proportion of each type of symbiont in *B. brooski* can differ with chemical conditions (Fisher et al., 1993; Duperron et al., 2007). *Bathymodiolus heckerae* (also seen as *B. heckeri*) is only found at the deepest sites, inhabiting seeps from 2200m to 3300m. It hosts four types of symbionts including a methanotroph, a methylotroph and two different thiotrophs (Duperron et al., 2007).

Recent phylogenetic studies of the Bathymodiolinae support morphological indications (Gustafson et al., 1998) that among the three seep species considered here, *B. childressi* belongs to separate group than the others (Jones et al., 2006a, b). *B. childressi* is ancestral to the others species that migrated to deeper water and diverged there (Jones et al. (2006a, b). *B. Brooksi* remained at deep seeps while the *Bathymodiolus thermophilus* clade colonized vents and radiated among vents from there. Their data suggest the *B. heckerae* group (which includes *B. azoricus* from Mid-Atlantic Ridge hydrothermal vents) diverged from the vent-inhabiting *B. brevior* (Chapter 2) group and recolonized deep seeps.

Agricultural Ditches

The geography of eastern NC includes the massive Albemarle and Pamlico Sounds and their associated rivers and drainages. The Pamlico Sound and its tributaries lie at the center of the North Carolina blue crab fishery and drains several counties with thousands of acres of cropland. Brackish water creeks that drain agricultural fields are

often less than 10 cm deep and 2 meters wide (personal observation) and are important habitats for juvenile and adult stage blue crabs (Posey et al., 2005), and their prey. Insecticides and herbicides applied to agricultural fields by tractor or plane can runoff into drainage ditches during large rain events shortly after application. They can also enter ditches by direct overspray or by wind drift. The genetic modification of crops for resistance to pesticides will likely lead to increased use of those particular pesticides and potentially elevated levels in estuarine systems.

Blue Crabs

The blue crab (*Callinectes sapidus*) is an ecologically and economically important estuarine and coastal species. It is a major predator and scavenger in coastal ecosystems along the east coast of the United States. Commercially, the blue crab fishery is North Carolina's largest, grossing over $21.4 million in 2007.

Blue crab larvae develop offshore in continental shelf waters before migrating inshore as settlement stage megalopae. Megalopae settle in submerged vegetation and metamorphose into juvenile crabs (Orth and Montfrans, 1987; Epifanio et al., 2003). Early stage juveniles then begin a secondary migration from the settling site upstream to the broader adult habitat (Reyns and Eggleston, 2004). Blue crabs can be found in water with salinities from 0 – 35.

Pesticides

In Chapter 4 the acute toxicity and sub-lethal effects of four insecticides and one herbicide in blue crabs are reported. Acephate, aldicarb, imidacloprid, lambda-cyhalothrin were assayed as both active ingredients alone and in commercial formulations. The commercial herbicide product Roundup® was only tested as a commercial product.

Acephate is a broad spectrum organophosphate foliar insecticide that is often applied to prevent thrips, aphids and infestations of other sucking insects. Organophosphate compounds are neurotoxins that inhibit the breakdown of acetylcholine, thus they keep neurons in a state of excitement (Eto, 1974). It is not acutely toxic to many crustaceans and other non-target organisms but is highly water soluble with a solubility of over 800,000 mg/L (PAN, 2008).

Aldicarb is an N-methyl carbamate that is used at planting to control thrips and nematodes (Bacheler et al., 2005). Like acephate, aldicarb is a cholinesterase inhibitor and is highly acutely toxic to a broad range of organisms especially crustaceans. It has a moderate water solubility of 5870 g/L making it a possible ground water contaminant (PAN, 2008).

Imidacloprid is a chloro-nicatinyl insecticide that is found in commercial products such as Trimax®, Provado®, and Goucho® (all Bayer Cropscience), which are either applied as a foliar spray or as a systemic seed treatment. It was developed to

mimic nicotine and as such is an acetylcholine receptor agonist causing neurotoxicity (Matsuda et al., 2001). It has a moderate water solubility of 514 μg/L and can runoff from cropland (Gupta et al., 2002).

Lambda-cyhalothrin (λ-cyhalothrin) is an extremely potent restricted use pyrethroid insecticide. Pyrethroids are synthetic variations on the natural pyrethrum insecticides produced by chrysanthemum flowers. They work by binding to and opening sodium channels on neuron axons, effectively paralyzing the subjects (He et al., 2008). Lambda-cyhalothrin binds tightly to soil and is not water soluble so is usually dissolved in petroleum or alcohol solvents for commercial applications.

Roundup® is a commercial herbicide with glyphosate as an active ingredient. Glyphosate is a water soluble phosphonoglycine that inhibits an enzyme required to produce the amino acids tryptophan, phenylalanine, and tyrosine (Amrhein et al., 1980). This contrasts greatly with the four insecticides tested here that all impact neuronal receptors and channels.

Chapter 2: Oxidative Stress Biomarkers in Lau and North Fiji Basin Hydrothermal Vent Invertebrates

Introduction

Deep-sea hydrothermal vents support ecosystems consisting primarily of endemic bacteria and invertebrates, often in endosymbiotic relationships. The physico-chemical conditions under which vent organisms thrive are generally considered to be among the most extreme environments on earth and are usually lethal to non-endemic organisms. Elevated concentrations of gases (H_2S, CH_4, H_2, CO_2) and metals (Fe, Mn, Zn, Pb, Cu, Co, Ag, Mg), low pH, sharp temperature gradients from 350°C to 2°C and often wide areas of temperatures elevated by ~10°C are common at vent sites around the globe. These conditions can be directly lethal and can indirectly induce the creation of reactive oxygen species. The mechanisms by which vent endemic macroinvertebrates maintain cellular homeostasis in such extreme conditions have only recently begun to be explored, most extensively in the northern Mid-Atlantic Ridge mussel, *Bathymodiolus azoricus*.

The sulfides and heavy metals of vent fluids can react with oxygen from ambient water to create reactive oxygen species (ROS) and lead to an imbalance of pro-oxidants and antioxidants in vent inhabitants. Such an imbalance is termed oxidative stress (Winston and Digiulio, 1991; Livingstone, 2001). Hydrogen sulfide reacts spontaneously with oxygen in ambient seawater to produce sulfate (Tapley et al., 1999), which is

energetically useless to thiotrophs. This reaction creates sulfur and oxygen radicals (Tapley et al., 1999) that can damage DNA (Pruski and Dixon, 2003), lipids, and other cellular molecules. Oxygen radicals form chain reactions that create more radicals and other reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$), and the highly reactive hydroxyl radical ($HO\cdot$) (reviewed by Fridovich, 1998).

Several metals such as Fe, Cu, Mn, and Zn are essential as components or cofactors of enzymes and respiratory pigments while others such as Hg, Co, and Pb have no known biological use (Stohs and Bagchi, 1995). All are toxic in excess. The toxicity of heavy metals partially lies in their ability to create ROS and in their ability to deplete cellular defenses (Stohs and Bagchi, 1995). Essential metals tend to be redox active and can directly create ROS through redox cycling. The Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + OH^-$, is one example of how iron can create hydroxyl radicals. Nonessential metals tend to be redox inactive and often impact cells by binding to and depleting metal sequestering compounds such as metallothioneins and glutathione, and other cellular components with available thiol groups (Stohs and Bagchi, 1995). Coastal mussels, *Mytilus edulis*, can regulate the bioaccumulation of some essential metals over wide concentrations, but cannot regulate nonessential metals (Langston et al., 1998). With depleted defenses, cells are at risk of oxidative damage from radicals produced during normal cellular respiration and from exogenous compounds.

Although much of the dissolved metal in hydrothermal fluid precipitates out of solution as metal-sulfides (forming the chimney and 'smoke' of black smokers)(Hsu-Kim et al., 2008), some of it remains bioavailable as evidenced by elevated metal concentrations in vent inhabitants. *Bathymodiolus azoricus* mussels from the Mid-Atlantic Ridge show highly elevated concentrations of Ag, Cd, Cu, Fe, Mn, and Zn as soluble form in gills and as insoluble forms in digestive glands (Cosson et al., 2008). The sequestration of metals as insoluble granules (Cosson et al., 2008) and the exportation into byssal thread (Kadar et al., 2006) could be methods of metal detoxification in vent mussels. Several other vent species have been shown to bioaccumulate metals. Most are either molluscs including the mussel *Bathymodiolus thermophilus* (Smith and Flegal, 1989) and clam *Calyptogena magnifica* (Roesijadi and Crecelius, 1984; Roesijadi et al., 1984), or crustaceans including shrimp *Rimicaris exoculata*, *Chorcaris chacei*, and *Mirocaris fortunata* (Colaco et al., 2006; Gonzalez-Rey et al., 2008) and crabs *Bythograea thermydron* (Cosson and Vivier, 1997) and *Segonzacia mesatlantica* (Colaco et al., 2006).

When ROS, especially HO·, contact cell membranes they initiate radical chain reactions among polyunsaturated lipids in cell membranes, a phenomenon known as lipid peroxidation (LPO). LPO can alter the structure and function of lipids and the membranes they compose, often resulting in cell death. Oxidized lipids form alkoxy radicals which can cleave off variously sized hydrocarbons and aldehydes including malondialdehyde (MDA) (Storey, 1996). MDA is used as a biomarker of oxidative

damage. Higher levels of MDA and other end products of lipid peroxidation that react with thiobarbituric acid (TBARS: thiobarbituric reactive substances) indicate that cellular defenses are not keeping pace with exogenous or endogenous ROS production. Since all aerobic organisms produce oxygen radicals naturally during cellular respiration, a suite of defense enzymes and molecules are produced to quench them.

Among the defense mechanisms are the superoxide dismutases (SOD) and catalase (CAT) enzymes, and the tripeptide glutathione. There are several different varieties of SODs, each with different metals in its active site, but all catalyze the reaction of $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. These are all very efficient enzymes that operate at diffusion-limited rates (Fridovich, 1998). SOD in the cytoplasm of eukaryotic cells have Cu and Zn in their active sites. Cu is believed to be the redox cycler while Zn is believed to be largely structural (Fridovich, 1998). Prokaryotes and eukaryotic mitochondria express a Mn-containing SOD that is unrelated to CuZnSOD (Fridovich, 1998). SOD dismutates one ROS but it makes another in hydrogen peroxide. H_2O_2 can be destroyed within cells by CAT, a heme group containing enzyme that changes $2H_2O_2$ into $O_2 + 2H_2O$. CAT is one of the most efficient enzymes known and can not be saturated by H_2O_2 (Lledías et al., 1998; Matés, 2000). The tripeptide glutathione (γ-glutamyl-cysteine-glycine) is one of the cell's major thiols, and serves to protect cells from endogenous and exogenous compounds (Meister and Anderson, 1983) including direct binding and detoxification of heavy metals (Singhal et al., 1987; Freedman et al., 1989; Viarengo and

Nott, 1993). It cycles back to its reactive reduced form (GSH) from its oxidized (GSSG) state with the help of enzyme glutathione reductase. Glutathione can also be bound to xenobiotics by glutathione S-transferase. Accumulation of metals and other xenobiotics can result in the depletion of reduced and total glutathione (Canesi et al., 1999).

Studies of antioxidant defenses in hydrothermal vent organisms are rare. Blum and Fridovich (1984) were first to report on antioxidant enzymes SOD, CAT, dianisidine peroxidase, and glutathione peroxidase in the vent tubeworm *Riftia pachyptila* and clam *Calyptogena magnifica* from the EPR. The MAR vent mussel *Bathymodiolus azoricus* has been the subject of several antioxidant defense studies. Bebianno et al. (2005) reported baseline *B. azoricus* CAT, SOD and LPO levels on the same order of magnitude as coastal mussels. Concentrations were higher in gill tissues than in mantle tissues, mirroring results seen in metal bioaccumulation. Metallothionein levels did not change amongst vent sites with different metal loads, supporting results from Rouse et al. (1998) and Geret et al. (1998) that metallothioneins do not play an elevated role in metal detoxification in vent mussels. *B. azoricus* has also been used in laboratory exposures of metals including Cd, Cu, and Hg for various lengths of time (Company et al., 2004, 2006, 2008). Exposure to Hg resulted in increased antioxidant enzyme activities in gill tissue while exposure to Cd and Cu resulted in decreased gill activities. *B. azoricus* from the Azores Triple Junction area of the MAR showed seasonal changes in antioxidant levels (Company et al., 2006). Enzyme activities in gills tissues showed direct relationships

with accumulated levels of Cu, Mn, soluble Ag, and insoluble Zn while enzymes in the mantle showed inverse relationships with accumulated levels of Cd, Zn, insoluble Zn, and soluble Mn (Company et al., 2006).

The only vent crustaceans to be assessed for antioxidant function are the two MAR vent shrimp *Rimicaris exoculata* and *Mirocaris fortunata* (Gonzales-Rey et al., 2007). They were found to have lower antioxidant enzyme activities than *B. azoricus* from the same vents, except for SOD which was of the same order of magnitude. Several ecological differences exist between vent mussels and crustaceans that could have implications for oxidative stress. Mussels are relatively immobile and contain chemoautotrophic endosymbionts that likely have their own antioxidant defenses. Vent crustaceans are primary consumers that can readily move from extreme conditions, do not have any endosymbionts, and, similar to non-vent crustaceans, should have the ability to mobilize blood pigment metals during molting cycles (Engel and Brouwer, 1991, 1993). It is unknown whether endosymbionts or metal mobilization have any influence on antioxidant defenses in hydrothermal vent invertebrates.

The South Pacific islands of Fiji are bounded to the Southeast by the Lau Basin (LB) and to the west by the North Fiji Basin (NFB), two back-arc basins (Fig. 2.1). The Eastern Lau Spreading Center and adjacent Valu Fa Ridge within the Lau Basin present a gradient in many physical characteristics including depth, spreading rate, bedrock type, and ultimately vent chemistry (Fouquet et al., 1991a,b; Martinez et al., 2006). From

north to south, ridge depth decreases from ~3000 to ~1700 m, spreading rate decreases from 97 to 39 mm/yr (Martinez et al., 2006), and bedrock changes from basalt to andesite (Hawkins, 1976). Due to these physical changes, vent chemistry changes including decreasing H_2S, Fe, and Zn concentrations from north to south (Hsu-Kim et al., 2008). Overall LB vent fluids are characterized as relatively high in dissolved Zn, Pb, As, Cu, and Cd, low pH (Fouquet et al., 1991a, b), and low in H_2S compared to mid-ocean ridge vent fluids.

The North Fiji Basin (NFB) is a mature back-arc basin (Kim et al., 2006) that began to form ~12 million years ago and is spreading at a rate of ~60 mm/yr (Auxende et al., 1995). Several high and low temperature hydrothermal vents have been described (Grimaud et al., 1991; Desbruyères et al., 1994; Ishibashi et al., 1994, Koschinsky et al., 2002). The Mussel Hill vent is a low temperature (<13 °C) vent site located at 2000 m depth on the Central Fiji Ridge near a triple junction (Koschinsky et al., 2002). Sampling of diffuse flow around Mussel Hill in 1998 (Koschinsky et al., 2002) indicated phase separation (subsurface boiling) was taking place with the vapor phase (enriched in gases and depleted in metals) being released, similar to nearby high temperature vents sampled in 1989 and 1991 (Grimaud et al., 1991; Ishibashi et al., 1994). NFB vent fluids have amongst the lowest concentrations of Fe and Mn in hydrothermal samples (Koschinsky et al., 2002). Low metal concentrations and high sulfide concentrations

make North Fiji Basin vents very suitable for chemoautotrophic ecosystems (Koschinsky et al., 2002).

Lau and North Fiji Basin vent ecosystems are dominated by the two provannid gastropods *Alviniconcha* sp. (Kojima et al., 2001) and *Ifremeria nautilei* (Bouchet and Waren, 1991) and by the mussel *Bathymodiolus brevior* (von Cosel et al., 1994; Desbruyères et al., 1994). All three molluscs contain endosymbiotic thiototrophic γ-proteobacteria within their hypertrophied gills (Endow and Ohta, 1989; Windoffer and Giere, 1997; Dubilier et al., 1998; Borowski et al., 2002; Suzuki et al., 2005, 2006a,b). Endosymbiosis necessitates that hosts live within diffuse flow vent effluent with sufficient H_2S to feed their symbionts. These three molluscs are often associated with each other and tend to live in zones around vents with *Alviniconcha* occupying the zone closest to the vent orifice followed by *I. nautilei* and *B. brevior* (Desbruyères et al., 1994; Henry et al., 2008; Waite et al., 2008; Podowski et al., 2009). Henry et al. (2008) explained this pattern as a consequence of the superior autotrophic potential and temperature tolerance of *Alviniconcha* compared to the lower sulfide tolerance of *I. nautilei* and the lower temperature tolerance of *B. brevior*. Consequently *Alviniconcha* lives in the zone with higher temperatures (9 – 20 °C) and higher concentrations of vent-derived chemicals than *I. nautilei* (6 – 14 °C) and *B. brevior* (3 – 6 °C) (temperatures from Waite et al., 2008).

The top predators in these vent ecosystems are the Brachyuran crab *Austinograea alayseaee* and the Anamuran spider crab *Paralomis hirtella*. Neither of these species contains endosymbionts and both are obligate predators/scavengers primarily of vent invertebrates. *A. alayseaee* crabs roam freely but are often found in and around gastropod and mussel beds well within physical and chemical influence of the vent fluid. They experience similar chemical conditions, at least most of the time, as *Alvinichoncha*, *I. nautilei*, and *B. brevior*, but retain the ability to quickly move from adverse physical and chemical conditions. Confamilial brachyuran crabs along the EPR, *Bythograea thermydron*, are known to travel away from the vent area to hatch their eggs and perhaps for other reasons (Perovich et al., 2003). It is not known whether *A. alayseaee* displays this behavior as well, but there is no reason to doubt that it can and does travel to non-vent area, at least periodically.

Tissue-specific activity of the enzymes CAT and SOD, the tripeptide GSH, and LPO were assessed in *B. brevior*, *I. nautilei*, *Alviniconcha* sp., and *A. alayseaee* collected from vent sites in the Lau and North Fiji Basins. This is the first report of oxidative stress biomarkers in *Alviniconcha* sp., *Ifremeria nautilei*, *Bathymodiolus brevior*, and *Austinograea alayseaee*, the first report of oxidative stress biomarkers in foot tissue of vent molluscs, and the first report of total glutathione levels in any vent species.

Methods

Collection

Specimens of the vent-endemic bathymodiolin mussel *Bathymodiolus brevior*, the provannid snails *Alviniconcha* sp. and *Ifremeria nautilei*, and the bythograeid crab *Austinograea alayseae* (Table 2.1) were collected from vent sites in the Lau and North Fiji Basins (Fig. 2.1) using the *ROV Jason II* operated from the *R/V Melville* during cruises TUIM-06-MV in 2005 and MGLN-07-MV in 2006. Not all species were collected from all of sites. Vent sites from which samples were collected include Tui Malila, ABE, Kilo Moana, and Hine Hina in the Lau Basin and from Mussel Hill in the North Fiji Basin. All samples were collected by scoop sampling or, in the case of bythograeid crab, by crab trap. Animals were placed in bioboxes for the remainder of the dive. Once aboard ship, samples were placed in 2–4°C surface seawater for transport into the shipboard laboratories. Mussels and snails were measured, removed from shells, and frozen intact in a -80°C freezer within 1 hour of arriving on board. Crabs were frozen whole.

Select tissues were dissected out of frozen samples, split into four subsamples for four different biomarker assays, weighed, and transferred to microcentrifuge tubes. Gill, foot, and mantle tissue samples were taken from all mussel and snail specimens. Gill, hepatopancreas, and cheli mussel tissue (specifically from the propodus) were taken from crabs. Hepatopancreas, or digestive gland, tissue was not taken from molluscs due to difficulty locating and accessing this tissue while keeping the specimen frozen. These

Table 2.1. Lau and North Fiji Basin vent sample data.

Species	Sampling Site	Depth (m)	Dive	N
Alviniconcha sp.	Kilo Moana, LB	2618	J2-140	3
	Tui Malila, LB	1895	J2-143	3
	Mussel Hill, NFB	1990	J2-150	3
Ifremeria nautilei	ABE, LB	2140	J2-231	8
	Mussel Hill, NFB	1990	J2-149	3
Bathymodiolus brevior	Tui Malila, LB	1895	J2-144	3
	Hine Hina North, LB	1817	J2-145	3
	Mussel Hill, NFB	1990	J2-149	3
Austinograea alayseae	ABE, LB	2140	J2-231	8

N, individuals; LB, Lau Basin; NFB, North Fiji Basin; J2-, *ROV Jason II* dive

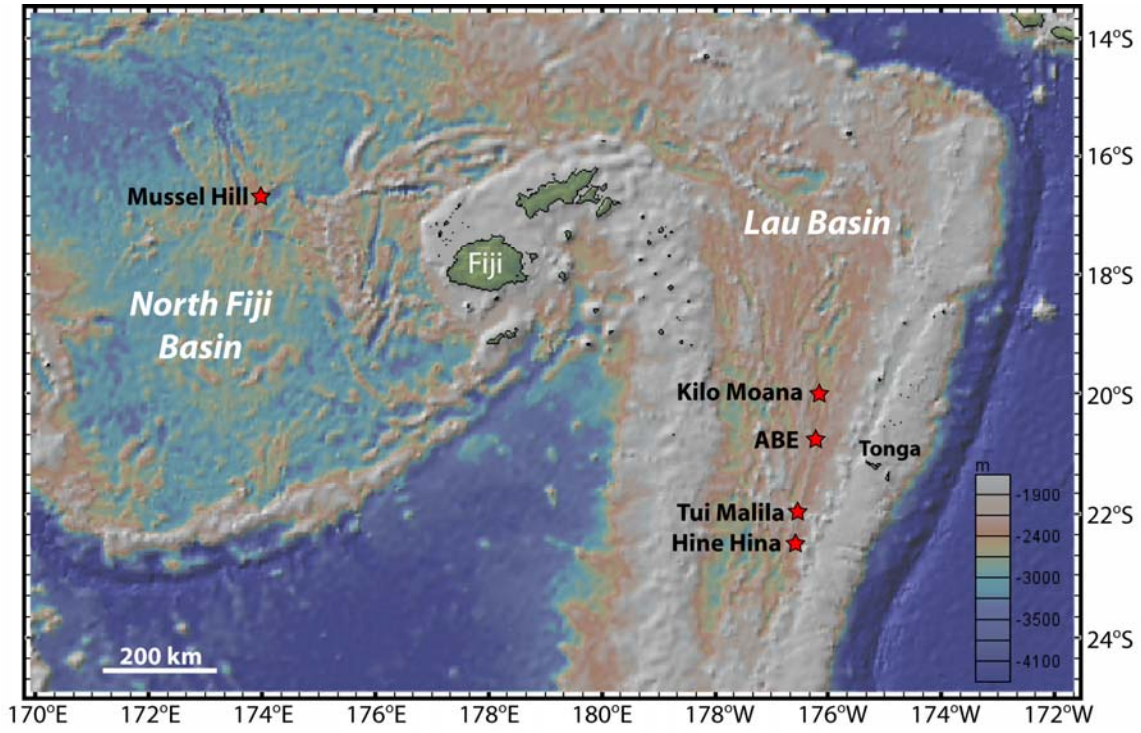

Figure 2.1. Map of Lau and North Fiji Basin vent sites. Drawn with GeoMapApp. Bathymetry from Ryan et al. (2009).

tissue choices parallel those tested in other similar studies (Company et al., 2004, 2006, 2007, 2008; Bebianno et al., 2005).

Biomarker Assays

Catalase, superoxide dismutase, total glutathione, and lipid peroxidation were assayed spectophotometrically for each of the tissue subsections for each specimen. Each of the samples used for the above assays, except for glutathione, was also assayed for supernatant protein using the Coomassie protein assay and all final oxidative stress biomarker concentrations are reported as per mg protein. The glutathione assay uses sulfosalysilic acid (SSA), which purges protein from supernatants during initial centrifugation; therefore glutathione is presented as per gram tissue wet weight. All assays were read on a Molecular Devices SpectraMax 190 or a SpectraMax M2 monochrometer-based microplate spectrophotometer using the Molecular Devices SoftMax Pro software.

Catalase

Catalase (CAT) activity was assayed using the Cayman Chemical Catalase Assay Kit (707002). This assay measures the rate at which catalase converts methanol and hydrogen peroxide into formaldehyde and water. The formaldehyde then reacts with the chromogen Purpald (4-amino-3-hydrazine-5-mercapto-1,2,4-triazole) and changes from colorless to purple. Tissue samples were homogenized on ice in 6 volumes of cold 50 mM potassium phosphate buffer containing 1 mM EDTA (pH = 7.0) and centrifuged

at 10,000 x g for 15 min at 4°C. The supernatant was removed and frozen at -80°C overnight for analysis of catalase and protein the following day. All samples were run in duplicate according to the manufacturer's instructions and catalase activity was read as an endpoint reading at 540 nm.

Total Glutathione

Total glutathione (reduced plus oxidized) was assayed using the DTNB-GSSG reductase recycling method following Anderson (1985) and Ringwood et al. (2003) as adapted for a 96-well microplate. Tissues were homogenized on ice in 6 volumes of 5% SSA and centrifuged at 6,000 x g for 12 min at 4°C. The supernatant was then diluted 1:2 with SSA and stored at 4°C until assayed within 24 hours. A cocktail of 0.238 mg/ml NADPH and 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was created. 195 µl of cocktail and 10 µl of sample were added to each well. The reaction was started with the quick addition of 6 µl of 50 unit glutathione reductase and the absorbance read at 405 nm for 2.5 min. The slope of the absorbance curve for each standard was plotted against concentration to make the standard curve. Total glutathione is reported as nmol of glutathione equivalents per gram wet weight.

Superoxide Dismutase

Cytosolic and mitochondrial superoxide dismutase (CSOD, MSOD) were assayed separately using the Cayman Chemical Superoxide Dismutase Assay Kit (706002). This endpoint assay measures the color change at 450 nm of a tetrazolium salt

radical detector as sample SOD dismutates superoxide produced by introduced xanthine oxidase after a 20 min incubation. Sample tissues were homogenized on ice in 5 volumes of cold 20 mM HEPES buffer (pH = 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. Homogenates were centrifuged at 1,500 x g for 5 min at 4°C. The supernatant was further centrifuged at 10,000 x g for 15 min at 4°C to separate the cytosolic (supernatant) from the mitochondrial (pellet) SOD. The pellet was resuspended in the same volume of homogenization buffer as the original sample. Both supernatant solutions were frozen at -80°C until assayed for SOD and protein within days. SOD activity standard curve was created using linearized rates, which were calculated by dividing the blank absorbance by each the absorbance of each standard. The linearized rate for each sample was similarly calculated (blank absorbance divided by sample absorbance) and back-calculated to SOD activity using the standard curve. One unit of SOD activity is defined as the amount of enzyme required for 50% dismutation of the superoxide radical. SOD is presented as units of SOD per mg of protein. Mitochondrial SOD was often near undetectable limits and was often back calculated as a negative number. Therefore only cytosolic SOD measurements will be discussed.

Lipid Peroxidation

Lipid peroxidation (LPO) was assayed following Ohkawa et al. (1979) and Ringwood et al. (2003) and as amended for use in a 96-well microplate. This assay is

often referred to as a Thiobarbituric Acid Reactive Substance (TBARS) assay as the vast majority of those substances are malondialdehyde molecules formed by lipid peroxidation. A standard curve was created using malondialdehyde tetraethylacetal heated for 60 min in a 50°C water bath. Tissue samples were homogenized on ice in 4 volumes of 50 mM potassium phosphate buffer (pH = 7.0) and centrifuged at 6,000 x g for 12 min at 4°C. Supernatants were decanted and stored on ice until assayed for LPO and protein within 2 hours. 100 µl of sample or standard were combined with 1400 µl of 0.375% thiobarbituric acid and 14 µl of 2% butylated hydroxytoluene, vortexed, and placed in a 100°C bath for 15 min. They were then centrifuged at 13,000 x g for 5 min at room temperature. 200 µl were plated in triplicate into a 96-well plate and absorbance was read at 532 nm.

The protein content of tissue homogenate supernatants used in all of the assays except glutathione was assayed using the Pierce Coomassie Protein Assay. 250 µl of Pierce Coomassie Blue reagent and 5 µl of sample homogenate supernatant were added to each well and absorbance read at 595 nm. A standard curve was made with bovine serum albumin and modeled as a 4-parameter curve per the manufacturer's instructions.

Analysis

Individual biomarker levels were compared within species and among tissues and vent sites using two-way analysis of variance (ANOVA) with Holm-Sidak post-hoc

tests. Separate two-way ANOVAs were run using SigmaPlot 11 (Systat Software) for each species-biomarker combination for the three molluscs. A One-way ANOVA (or ANOVA on Ranks when needed) was performed for each biomarker among tissues for the crab *Austinograea alayseae* because it was collected from one site.

A principal component analysis (PCA) was performed using biomarker levels from gill, foot, and mantle tissues from *Alviniconcha sp.*, *I. nautilei*, and *B. brevior*. Data were standardized prior to analysis since each biomarker is on a different scale. Biomarker measurements from *A. alayseae* were not used in this analysis because different tissues (hepatopancreas and skeletal muscle) were analyzed. PCA was performed using TIBCO Spotfire S+ 8.1 (TIBCO Software Co.).

Results

Alviniconcha sp.

Catalase

Catalase activity in *Alviniconcha* sp. (Fig. 2.2a) varied significantly across tissues ($p < 0.001$, $F_{2,16} = 22.940$) and across sites ($p = 0.057$, $F_{2,16} = 3.543$) and showed significant interaction between site and tissue ($p = 0.006$, $F_{4,16} = 5.480$). Foot tissue in *Alviniconcha* sp. always had the lowest catalase activity of the three tissues. Mantle and gill tissue showed significantly higher catalase activity than foot tissue at Kilo Moana ($p < 0.001$ for both) and Mussel Hill ($p < 0.001$ for both). At Tui Malila none of the tissues had

significantly different catalase activity from each other. Mantle was the only tissue that showed differences across sites. *Alviniconcha* mantle from Tui Malila had significantly lower catalase activity than mantle from Kilo Moana ($p < 0.001$, $t = 5.179$) and Mussel Hill ($p = 0.004$, $t = 3.332$).

Superoxide Dismutase

There was a significantly different activity of CSOD (Fig. 2.2b) among the tissues of *Alviniconcha* sp. ($p < 0.001$, $F_{2,16} = 22.542$). At all sites, mantle tissue had significantly higher CSOD activity than gill ($p < 0.001$, $t = 6.326$) and foot ($p < 0.001$, $t = 5.206$) tissue. The pattern of gill < foot < mantle held at all sites. No sites showed significant differences in CSOD activity ($p = 0.413$, $F_{2,16} = 0.935$).

Total Glutathione

There were significant differences in the total glutathione (Fig. 2.2c) concentration among *Alviniconcha* sp. tissues ($p < 0.001$, $F_{2,15} = 98.598$). The same pattern of tissue levels was present at each sampling site: gill >> mantle > foot. There was no significant difference in glutathione concentrations among the sites ($p = 0.668$; $F_{2,15} = 0.415$).

Lipid Peroxidation

Mean lipid peroxidation in *Alviniconcha* (Fig. 2.2d) ranged from 0.69 nmol MDA/mg protein in foot tissue from Kilo Moana to 10.22 nmol MDA/mg protein in gill tissue from Mussel Hill. The Mussel Hill gill measurements of 10.22 nmol MDA/mg

protein was more than five fold higher than any other site or tissue mean. This value alone accounts for significant differences among sites ($p < 0.001$, $F_{2,14} = 58.813$) and among tissues ($p < 0.001$, $F_{2,14} = 67.621$).

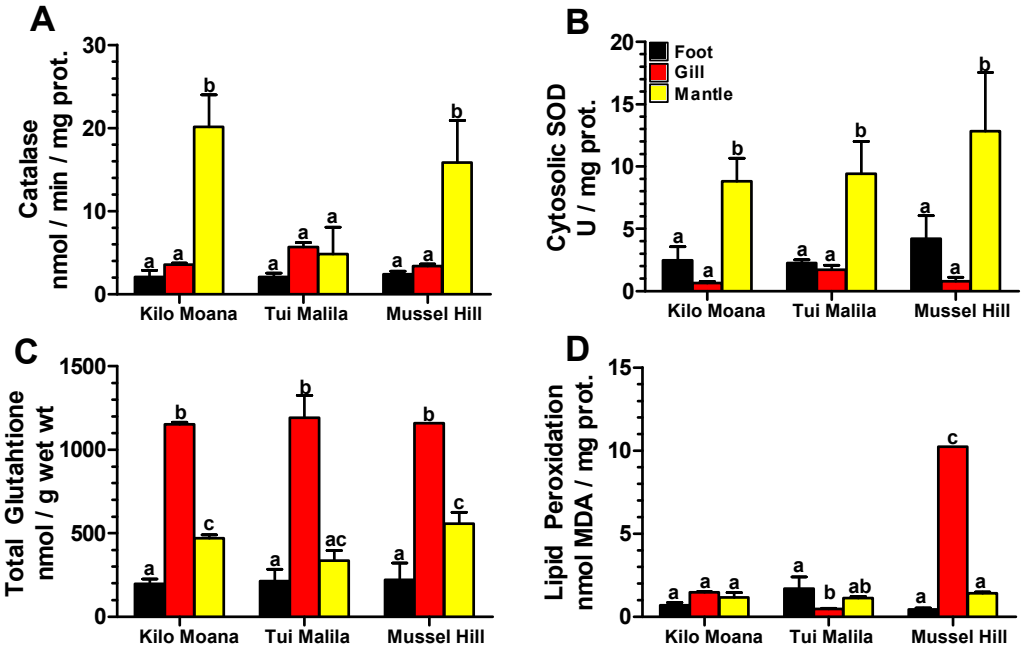

Figure 2.2. Mean (±SEM) biomarker levels measured in *Alviniconcha* sp. Catalase (A), CSOD (B), total glutathione (C), and lipid peroxidation (D). Different letters represent significant differences within biomarkers ($p<0.05$).

Ifremeria nautilei

Catalase

Ifremeria nautilei was collected from ABE and from Mussel Hill. Similar to *Alviniconcha*, *I. nautilei* foot tissue had the lowest mean catalase activity and mantle

tissue had the highest (Fig. 2.3a). There were no significant differences among tissues ($p = 0.082$, $F_{2,26} = 2.758$) or sites ($p = 0.502$, $F_{1,26} = 0.464$).

Superoxide Dismutase

Mean cytosolic SOD activity in mantle tissue was the highest CSOD activity of any species measuring 16.05 U/mg protein at ABE and 17.17 U/mg protein at Mussel Hill (Fig. 2.3b). Mantle tissue activity was significantly higher than gill activity ($p = 0.011$, $F_{2,26} = 5.451$). CSOD activity was not significantly different between the two collection sites ($p = 0.951$, $F_{1,16} = 0.00382$).

Glutathione

Similar to *Alviniconcha*, *Ifremeria* gill tissue showed the highest total glutathione concentrations of the three tissues (Fig. 2.3c), although not significantly higher ($p = 0.063$, $F_{2,25} = 3.087$). Total glutathione concentrations were significantly higher at the ABE site than at Mussel Hill ($p < 0.001$, $F_{1,25} = 23.676$). All of the total glutathione measurements were higher at ABE than any from Mussel Hill.

Lipid Peroxidation

Ifremeria nautilei showed generally elevated levels of lipid peroxidation in comparison to the other species. Mean TBARS concentrations ranged from 1.56 nmol MDA/mg protein to 3.33 nmol MDA/mg protein (Fig. 2.3d). *Ifremeria* from Mussel Hill had significantly higher concentrations of TBARS than those from ABE ($p = 0.037$, $F_{1,25} =$

4.832). *Ifremeria* tissues were not significantly different from each other ($p = 0.612$, $F_{2,25} = 0.500$).

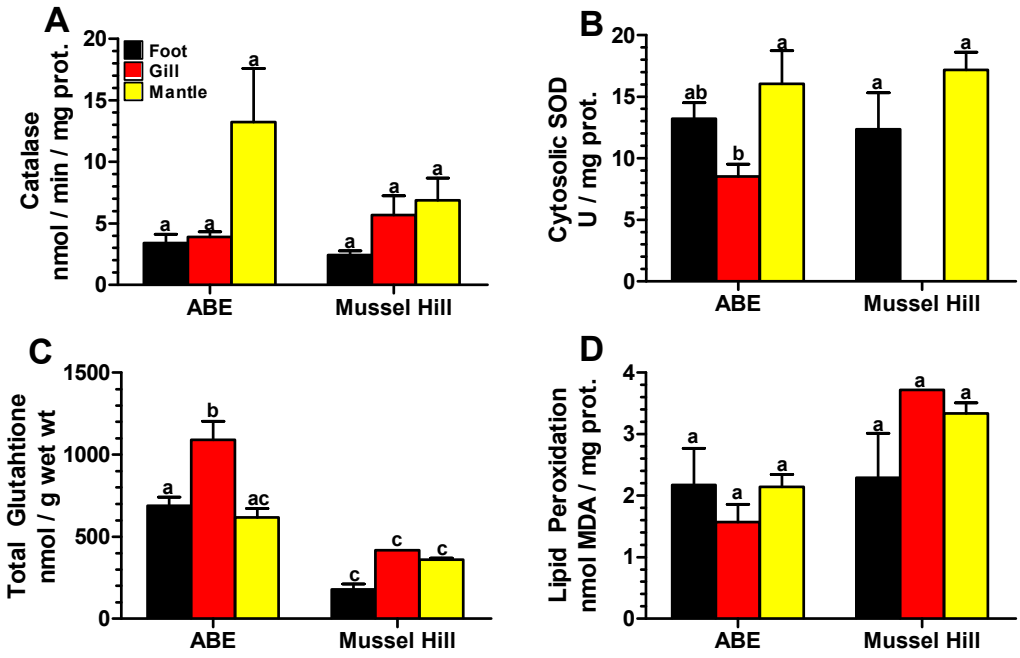

Figure 2.3 Mean (±SEM) biomarker levels measured in *Ifremeria nautilei*. Catalase (A), CSOD (B), total glutathione (C), and lipid peroxidation (D). Different letters represent significant differences within biomarkers ($p<0.05$).

Bathymodiolus brevior

Catalase

Catalase activity in *B. brevior* (Fig. 2.4a) varied significantly among the three tissues measured ($p = 0.02$, $F_{2,17} = 4.989$). Mean activities in the foot (8.36 – 10.24 U/mg

protein) and gill (2.57 – 12.84 U/mg protein) were significantly higher ($p = 0.024$, $t = 2.755$; $p = 0.016$, $t = 2.678$, respectively) than in mantle tissues (1.78 – 2.72 U/mg protein). Mantle tissue always produced the lowest mean catalase activity at all three sampling sites Tui Malila, Hine Hina, and Mussel Hill. There was no significant difference in catalase activity among the collection sites ($p = 0.269$, $F_{2,17} = 1.419$).

Superoxide Dismutase

Mitochondrial SOD values were often below the level of detection and so this section will deal exclusively with cytosolic SOD. CSOD activity (Fig. 2.4b) differed significantly across tissue ($p < 0.001$, $F_{2,18} = 21.351$) and across sites ($p = 0.022$, $F_{2,18} = 4.742$) and there was significant interaction between these two variables ($p = 0.014$, $F_{4,18} = 4.235$). In contrast to catalase activity, CSOD activity in the mantle was significantly higher than in the gills at all three collection sites as well as higher than foot tissue at two sites. CSOD activity in foot tissue is different at Mussel Hill. Here foot activity is three fold higher ($p < 0.001$) than at the other sites, while activity in gill and mantle tissues remains similar to levels from the other sites.

Glutathione

Concentrations of total glutathione (Fig. 2.4c) varied significantly across tissue ($p = 0.018$, $F_{2,18} = 5.026$) and site ($p = 0.03$, $F_{2,18} = 4.299$) but there was no significant interaction ($p = 0.171$, $F_{4,18} = 1.808$). Glutathione levels were always lowest in mantle tissue and higher in gill and foot tissues. Hine Hina mussels had the lowest mean mantle

concentration (481.64 nmol/g ww) as well as the highest mean gill (1880.36 nmol/g ww) and foot (1509.42 nmol/g ww) concentrations of all of the mussels measured.

Lipid Peroxidation

Lipid peroxidation levels (Fig. 2.4d) did not significantly differ among sites ($p = 0.939$, $F_{2,13} = 0.0635$) or among tissues ($p = 0.377$, $F_{2,13} = 1.053$). Mean concentrations of TBARS ranged from 0.088 nmol MDA/mg protein in foot tissue from Mussel Hill to 1.702 nmol MDA/mg protein in mantle tissue from Tui Malila.

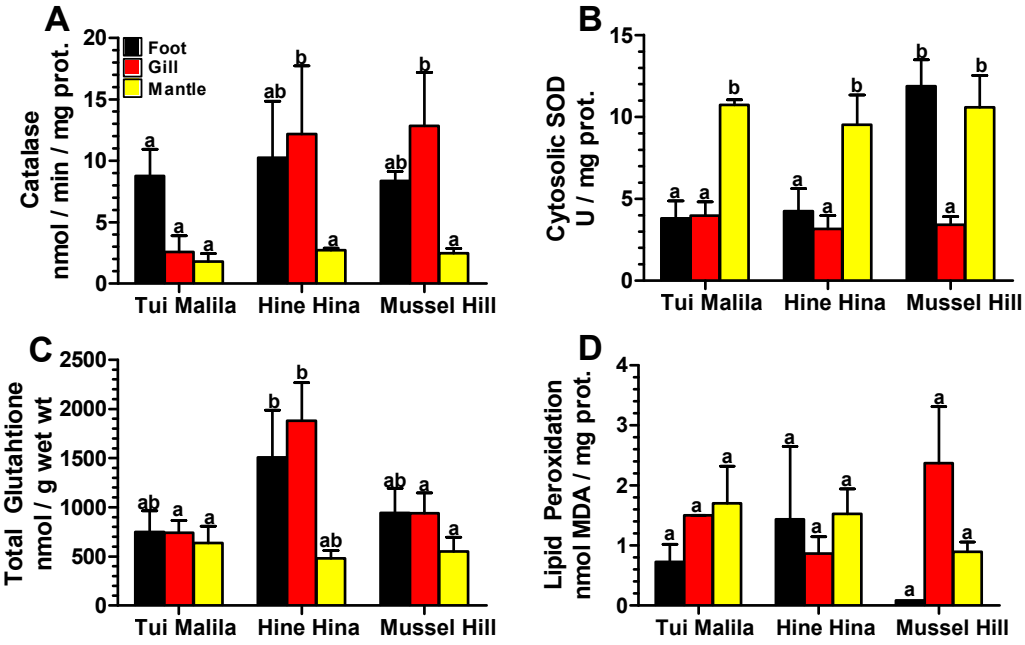

Figure 2.4. Mean (±SEM) biomarker levels measured in *Bathymodiolus brevior*. Catalase (A), CSOD (B), total glutathione (C), and lipid peroxidation (D). Different letters represent significant differences within biomarkers ($p<0.05$).

Austinograea alayseae

Catalase

Austinograea alayseae was only collected from the ABE site and so only comparisons between gill, muscle, and hepatopancreas tissues were made. There was a significant difference in catalase activity among the three tissues ($p < 0.001$, $F_{2,21} = 10.508$; Fig. 2.5a). Muscle had significantly higher mean catalase activity (1.65 U/mg protein) than gill (0.68 U/mg protein; $p < 0.001$, $q = 6.101$) and hepatopancreas (0.87 U/mg protein; $p = 0.006$, $q = 4.950$).

Superoxide Dismutase

CSOD from *A. alayseae* (Fig. 2.5b) ranged from 7.42 U/mg protein in muscle tissue to 12.07 U/mg protein in gill tissue. Cytosolic SOD did not differ significantly among tissues in *A. alayseae* from ABE ($p = 0.094$, $F_{2,20} = 2.670$).

Glutathione

Total glutathione concentrations in crab tissues were compared with a non-parametric ANOVA on ranks. Tissues did not show any significant differences in GSH levels ($p = 0.119$, $H_2 = 4.265$; Fig. 2.5c).

Lipid Peroxidation

Lipid peroxidation was also analyzed with a non-parametric ANOVA on ranks. Tissues did show a significant difference in TBARS ($p < 0.001$, $H_2 = 16.415$; Fig. 2.5d).

Hepatopancreas concentrations (3.57 nmol/mg protein) were significantly higher than muscle (0.49 nmol/mg protein; $p < 0.05$, $q = 5.6$) and gill (0.95 nmol/mg protein; $p < 0.05$, $q = 3.85$).

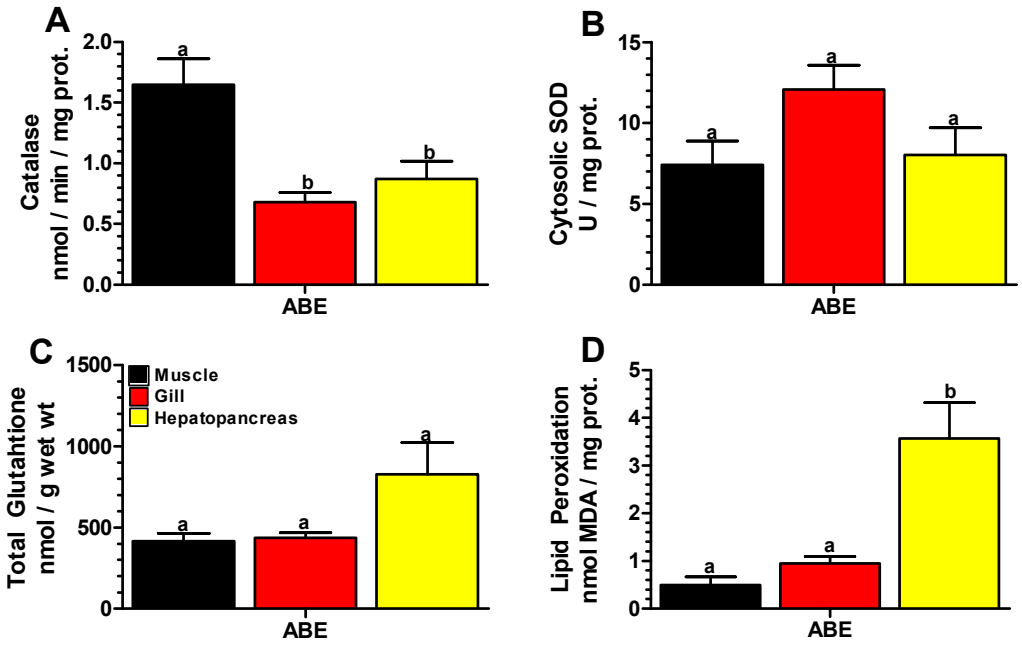

Figure 2.5. Mean (±SEM) biomarker levels measured in *Austinograea alayseae*. Catalase (A), CSOD (B), total glutathione (C), and lipid peroxidation (D). Note different tissues were measured in this species. Different letters represent significant differences within biomarkers ($p<0.05$).

Mussel Hill

Catalase

Mussel Hill is the one site from which all three molluscs were collected, which allows for comparison among them. There was significant interaction between tissue

and species in the catalase results ($p = 0.002$, $F_{4,15} = 7.213$; Fig. 2.6a). Overall across species there was no significant difference in catalase activity among the tissues ($p = 0.093$, $F_{2,15} = 2.790$). The greatest differences among species are seen in mantle tissue. *Alviniconcha* had significantly higher mantle catalase activity than *Ifremeria* ($p = 0.016$, $t = 2.713$) or *Bathymodiolus* ($p = 0.001$, $t = 4.047$). Within the gill tissue, *B. brevior* had the highest activity, significantly higher than *Alviniconcha* ($p = 0.012$, $t = 2.871$). All three tissues from *I. nautilei* had intermediate catalase activity compared to *Alviniconcha* and *B. brevior*.

Cytosolic SOD

Across the three species CSOD activity in foot tissue and mantle tissue were significantly higher than gill tissue ($p = 0.018$, $p < 0.001$, respectively; Fig. 2.6b). There was little difference among the three molluscs in terms of CSOD activity within respective tissues. Within the respective tissues there were few difference among the species. The only difference was that *Bathymodiolus brevior* had significantly higher gill CSOD activity than *Alviniconcha* ($p = 0.031$, $t = 4.893$). No *I. nautilei* gill tissue was assayed for CSOD activity due to small specimens.

Total Glutathione

Overall there was no significant difference in total glutathione concentration among the tissues ($p = 0.062$, $F_{2,13} = 3.472$; Fig. 2.6c). Significant differences were seen within the foot tissue. *Bathymodiolus brevior* had significantly higher total glutathione

concentrations than *Alviniconcha* (p = 0.003, t = 3.701) and *I. nautilei* (p = 0.004, t = 3.506). No other differences within the tissues were seen among species.

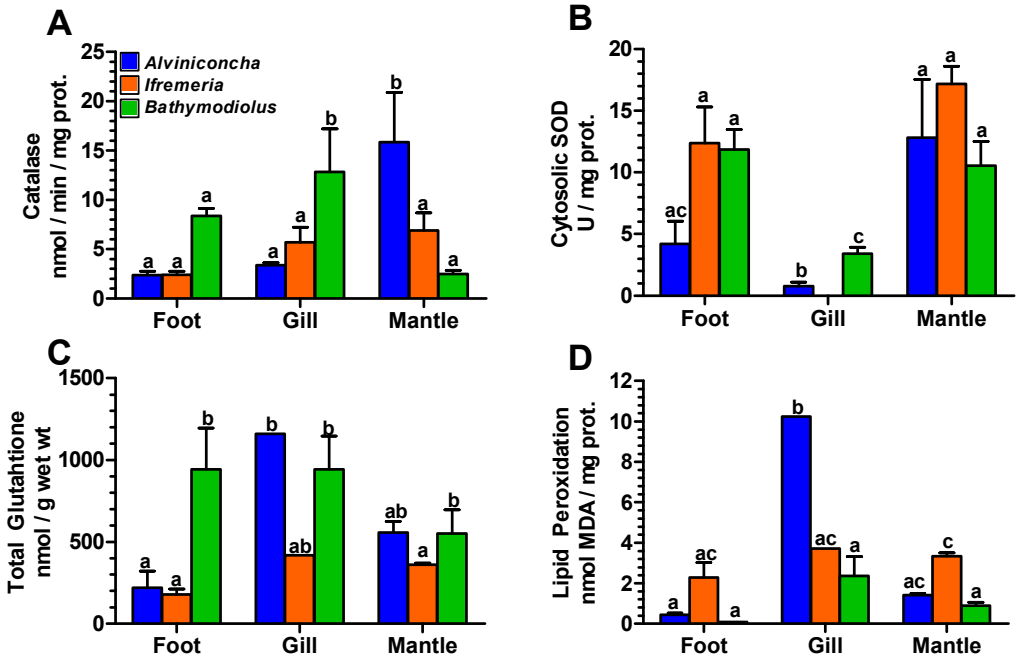

Figure 2.6. Mean (±SEM) biomarker levels measured in *Alviniconcha* sp., *I. nautilei*, and *B. brevior* from Mussel Hill. Catalase (A), CSOD (B), total glutathione (C), and lipid peroxidation (D). Different letters represent significant differences within biomarkers (p<0.05).

Lipid Peroxidation

Statistical differences in lipid peroxidation (Fig. 2.6d) at Mussel Hill were seen among species (p = 0.002, $F_{2,10}$ = 12.721) and tissues (p < 0.001, $F_{2,10}$ = 27.354). Overall gill tissue had significantly more TBARS than foot or mantle. Among gill tissue samples *Alviniconcha* gill showed significantly higher levels of lipid peroxidation than *Ifremeria* (p

< 0.001, t = 4.907) and *Bathymodiolus* ($p < 0.001$, $t = 7.253$). The mean *Alviniconcha* gill reading of 10.22 nmol MDA/mg protein was the highest mean level of lipid peroxidation in the entire study. *Ifremeria* mantle tissue had significantly higher TBARS concentrations than *Bathymodiolus* ($p = 0.010$, $t = 3.187$) and *Alviniconcha* ($p = 0.049$, $t = 2.246$).

Principal Component Analysis

A PCA was performed on the standardized data and produced three significant components that cumulatively explained 78.5% of the variation. Loadings from the first two components, which cumulatively explained 53.6% of the variation, are plotted in Fig 2.7a. Component 1 was strongly positively influenced by cytosolic SOD values in all tissues, and catalase, glutathione and lipid peroxidation in the mantle. Component 2 was strongly positively influenced by glutathione and catalase in foot tissue as well as cytosolic SOD in gill and foot tissue.

A plot of the PCA scores shows data clustering by species (Fig. 2.7b) with moderate overlap among collection sites. *Alviniconcha* shows the tightest cluster. *Ifremeria* and *Bathymodiolus* form clusters of similar shape that trend in opposite directions. *Bathymodiolus* from Hine Hina show the largest spread and a single sample from Tui Malila overlaps with the *Ifremeria* group.

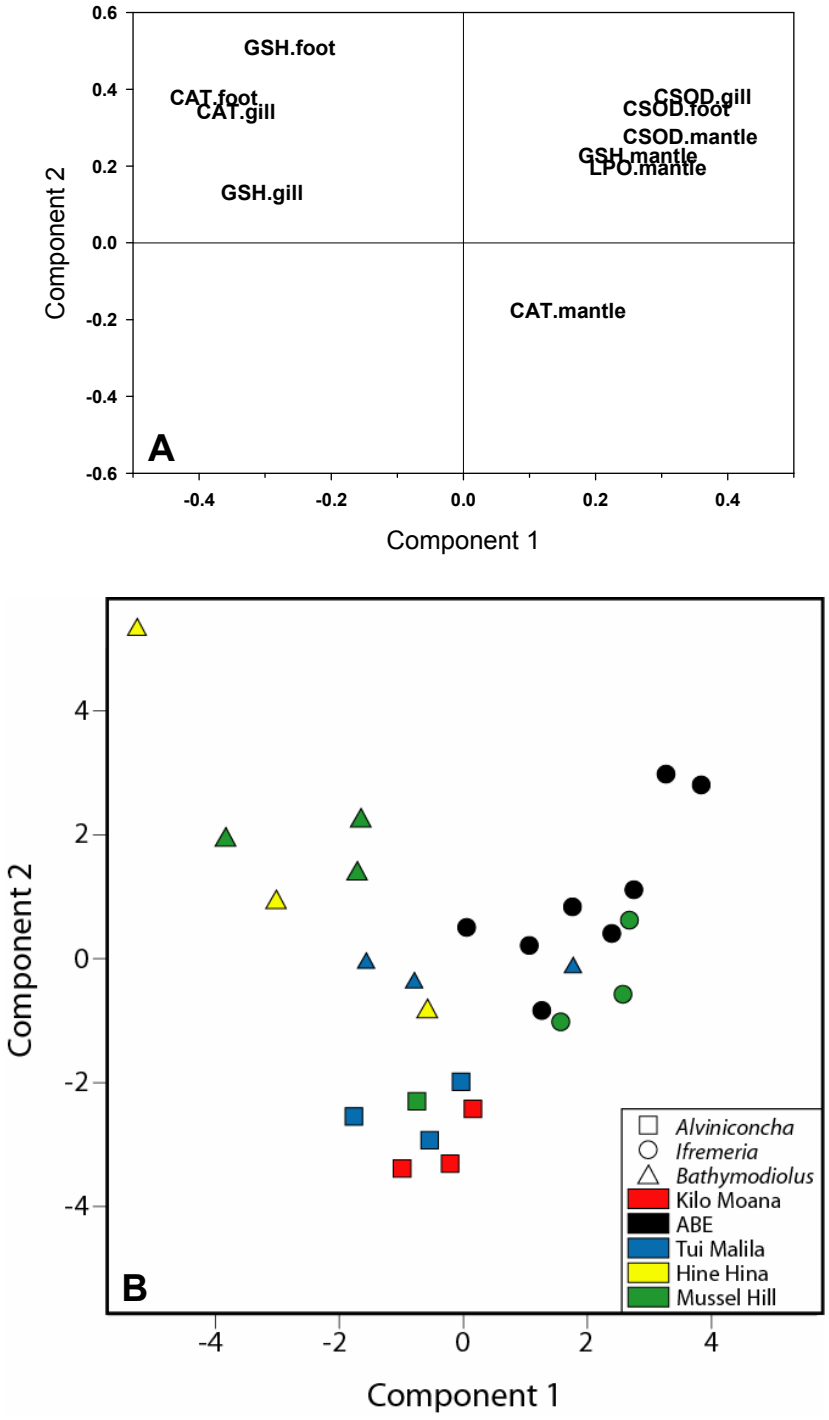

Figure 2.7. Loadings (A) and scores (B) of a PCA performed with vent mollusc gill, foot, and mantle tissue.

Discussion

Among the three vent molluscs assayed in this study, values for each of the four biomarkers were generally of the same respective order of magnitude. There were some differences in which tissues showed high or low levels of certain biomarkers among the three molluscs. The gastropods, *Alviniconcha* sp. (Fig. 2.2a) and *Ifremeria* (Fig. 2.3a), showed high catalase activity in mantle tissue compared to gill and foot tissue while the opposite was seen in *B. brevior* (Fig. 2.4a). *Ifremeria* (Fig. 2.3b) and *B. brevior* (Fig. 2.4b) had very similar patterns of high mantle cytosolic SOD activity compared to gill and foot tissue while in *Alviniconcha* sp. (Fig. 2.2b) there was much less difference among tissues. *Alviniconcha* sp. also had a different pattern of total glutathione levels among tissues with significant differences among the three tissues at all three sampling sites (Fig. 2.2c). *Alviniconcha* sp. gill tissue had about twice as much total glutathione than mantle tissue and four times as much as foot tissue. *Ifremeria* and *B. brevior* showed only one case in which tissues within a site had significantly different total glutathione. At all other sites there were no significant glutathione differences among tissues. All three molluscs show similar lipid peroxidation (Fig. 2.2d, 2.3d, 2.4d) patterns among tissues with little difference among tissues within a site and even among sites. The glaring exception is extremely high level of lipid peroxidation seen in *Alviniconcha* sp. gill tissue from Mussel Hill (Fig. 2.2d).

The biomarker activities and concentrations reported here are generally of the same order of magnitude as those reported for other vent endemics with the exception of lipid peroxidation. Bebianno et al. (2005) reported activities of SOD (~12 – 19 U/mg prot.) and catalase (~13 – 29 nmol/min/mg prot.) in *B. azoricus* gill tissue that is higher than molluscan gill values from Lau and North Fiji Basin (~1 – 8 U/mg prot. and ~3–13 nmol/min/mg prot.). Catalase and CSOD activities in mantle tissue, however, are very similar among Lau and North Fiji basin molluscs and *B. azoricus*. Compared to only *B. brevior*, *B. azoricus* does show higher mantle catalase activity (~6 – 8 nmol/min/mg prot. vs. ~1 – 3 nmol/min/mg prot.). Lipid peroxidation in Lau and North Fiji Basin molluscs was an order of magnitude higher than *B. azoricus* as well as molluscs from polluted sites. Bebianno et al. (2005) report lipid peroxidation values in the range of 0.072 – 0.186 nmol/mg protein for gill and 0.042 – 0.117 nmol/mg protein in mantle tissue. This pales in comparison to the present study values of 0.47 – 10.22 nmol/mg protein in Lau Basin molluscan gill tissue and 0.89 – 3.33 nmol/mg protein in mantle tissue. So despite these two groups (MAR mussels and LB/NFB molluscs) facing generally similar environmental challenges and having similar catalase and SOD activities, the antioxidant defenses of Lau and North Fiji Basin vent molluscs do not prevent oxidative damage.

Total glutathione levels have not been measured before in any vent endemic species. Glutathione concentrations in Lau and North Fiji Basin molluscs ranged from

197.34 nmol/g ww in *Alviniconcha* foot to 1509.42 nmol/g ww in *B. brevior* foot with most measurements well above the 400 nmol/g ww that Ringwood et al. (1999) use as a cut off between stressed (lower values) and unstressed estuarine oysters.

Austinograea alayseae is the first vent brachyuran crab (true crab) to be assayed for antioxidant biomarkers and is the only highly mobile species in this study as well as the only one without gill endosymbionts. *A. alayseae* was only collected from the ABE site and muscle, gill, and hepatopancreas tissue was assayed. *A. alayseae* showed very low catalase activity across tissues but pronounced CSOD activity in all three tissues, especially gill tissue (Fig. 2.5a,b). Gills had low levels of total glutathione compared to the Lau Basin molluscs but hepatopancreas, not surprisingly, had the highest levels of glutathione and lipid peroxidation. Catalase and CSOD activity in all tissues of *A. alayseae* is on the same order of magnitude as, although lower than, whole homogenates of the vent shrimp *Mirocaris fortunata* and *Rimicaris exoculata* from Rainbow on the Mid-Atlantic Ridge (Gonzalez-Rey et al., 2007).

All of the vent-dominant molluscs, including bathymodiolid mussels and provannid gastropods, contain intracellular endosymbiotic bacteria within their gills. Most of them are thiotrophic sulfur oxidizing or methanotrophic proteobacteria that oxidize reduced compounds in order to fix carbon organically. Sulfide oxidation produces ROS (Tapley et al., 1999) so the symbionts must have some antioxidant defenses to protect against them. Antioxidant biomarker levels measured in mollusc gill

tissue surely include a contribution from symbiont cells. The role gill symbionts play in metal detoxification for the hosts remains unclear (Cosson-Mannevy et al., 1988; Company et al., 2004; Bebianno et al., 2005). Laboratory chemical exposures of *B. azoricus* that have purged (or at least reduced) their symbionts under non-chemosynthetic conditions could help answer this question.

Symbiont-bearing *Ifremeria nautilei* and symbiont-free *Austinograea alayseae* were collected from ABE. *Ifremeria* gill catalase activity was ~8 fold higher than *Austinograea*. *Ifremeria* gill total glutathione levels were about twice those for *Austinograea*. CSOD and lipid peroxidation levels, however, were very similar between the two.

The unbalanced sampling of opportunity used in this study precluded a parametric multivariate ANOVA approach to statistical testing due to missing data. A principal component analysis (PCA) was performed to get a broad picture view of all of the biomarker levels from all of the tissues from each of the sites. The PCA scores show that overall antioxidant levels are segregated by species and not by site (Fig. 2.7b). Thus the species, and the evolutionary history that created it, play a larger role in determining the antioxidant response of a vent mollusc than the vent site from which it comes. The distinct physiological tolerances and preferences of these three dominant vent molluscs (Henry et al., 2008) suggests that each species has its own niche that it occupies at each vent. The microhabitats from which the specimens were collected could have been very

similar across vents, no matter the end-member fluid composition. Unfortunately, concomitant chemical characterizations were not possible during collection.

Mussel Hill

Mussel Hill in the North Fiji Basin was the only site from which all three molluscs were collected. Nonetheless, they almost certainly were exposed to different chemical microenvironments with *Alviniconcha* sp. exposed to the highest concentration of vent effluent and *B. brevior* the lowest (Waite et al., 2008; Podowski et al., 2009). If all three species have similar defense mechanisms and capabilities, then the expected order of biomarker levels is *Alviniconcha* > *Ifremeria* > *B. brevior*. This pattern was generally not seen (Fig. 2.6). The only instances in which it was clearly seen was in mantle catalase activity (Fig. 2.6a), and lipid peroxidation (Fig. 2.6d) in gills. In both cases *Alviniconcha* sp. levels were significantly higher than *Ifremeria* and *B. brevior*, which did not differ from each other. Similarity of antioxidant and oxidative stress biomarkers among the species suggests that either *Alviniconcha* sp. is using oxidative defense mechanisms other than the ones assayed in this study, or that each species' niche is of equal oxidative potential. If it is assumed that Mussel Hill vent effluent has not changed dramatically since last analyzed in 1998 (Koschinsky et al., 2002) then this site should be considerably less stressful than Lau Basin sites due to lower metal concentrations. The factors making

Mussel Hill oxidatively stressful to vent molluscs could be biological rather than chemical.

Van Dover et al. (2007) reported an epizootic fungal infection of *B. brevior* mussels at Mussel Hill. They describe a fungal infection of black yeast resulting in highly necrotic gill tissue in advanced cases (23% of their sample) and small brown spots in early cases (55% of their sample). It is possible that specimens used in the present study were afflicted with that fungal infection, since these samples and the Van Dover et al. (2007) samples were collected during the same ROV dives and possibly from the same patches within Mussel Hill. No visible signs of infection were seen in any Mussel Hill samples used in the present study but no histological analysis was performed. Van Dover et al. (2007) never found infection in any other macrofauna (including *Alviniconcha* sp. and *I. nautilei*) from Mussel Hill or in *B. brevior* from Lau Basin vent sites Kilo Moana, ABE, and Tui Malila.

Fungi are thought to be opportunistic pathogens, infecting stressed or weakened individuals (Guarro et al., 1999; Van Dover et al., 2007). If environmental factors at Mussel Hill induce oxidative stress in endemic molluscs, it might predispose them to pathogenic infection by agents that are normally kept in check by unstressed individuals.

At Mussel Hill, molluscan gill tissue showed the highest levels of lipid peroxidation among the assayed tissues (Fig. 2.6d), which is in contrast to the tissue

patterns of lipid peroxidation seen at vent sites in the Lau Basin (Fig. 2.2d, 2.3d, 2.4d). It is impossible to determine at this time whether any increased lipid peroxidation at Mussel Hill is caused by environmental conditions or by unseen fungal infection. Further research into the Van Dover et al. (2007) fungus should be performed with a look at whether its pathogenicity is aided by environmentally-induced oxidative stress and/or causes oxidative stress in its hosts.

Deep-sea hydrothermal vents create highly variable environments in which temperature, metal concentration, and sulfide concentration can fluctuate rapidly. The organisms that have evolved to thrive under these conditions must be able to cope with this variability. The three molluscs studied here have different physiological tolerances for temperature and sulfide (Henry et al., 2008; Waite et al., 2008; Podowski et al., 2009). While the overall similarity of antioxidant biomarkers within species and across sites suggests that their respective physiological tolerances keep each species within a narrow chemical environment, their antioxidant defenses appear to not always be able to prevent lipid peroxidation.

Conclusions

The antioxidant and oxidative stress biomarkers catalase, CSOD, total glutathione, and lipid peroxidation were measured in tissues of *Alviniconcha* sp., *Ifremeria nautilei*, *Bathymodiolus brevior* and *Austinograea alayseae* collected from vents sites

in the Lau and North Fiji Basins. Species- and tissue-specific biomarker levels were generally similar across chemically distinct vent sites with some exceptions. *Alviniconcha* sp. showed high activities of catalase and CSOD in mantle tissue as well as high total glutathione levels in gill tissue. *Ifremeria* showed high mantle catalase and CSOD like *Alviniconcha* but also showed high CSOD in foot tissue. The catalase tissue pattern of *Bathymodiolus brevior* was the opposite of the two gastropods with low mantle activity and high foot and gill activity tissue. Its CSOD tissue pattern resembled *Alviniconcha* while its total glutathione patterns match *Ifremeria*. Lipid peroxidation in all three was similar in most tissues. *Austinograea alayseae* catalase activity in all tissues was about 8 fold lower than mollusc activity. All other biomarkers were similar to molluscs.

Catalase and CSOD activities in mollusc gills were about half those in *B. azoricus* from the Mid-Atlantic Ridge (Bebianno et al., 2005), but activities in mantle tissue were similar. Lipid peroxidation, a biomarker of oxidative stress, was at least an order of magnitude higher in Lau and NFB molluscs than *B. azoricus* (Bebianno et al., 2005).

Principal component analysis of all mollusc biomarker measurements grouped individuals by species rather than by site. PCA showed very little overlap among species. The microhabitats from which the specimens within a species were collected could have been very similar across vents, no matter the fluid composition.

Chapter 3: Oxidative Stress Biomarkers in Gulf of Mexico Seep Mussels with Comparison to Vent Mussels

Introduction

The partial reduction of molecular oxygen to reactive intermediates is a constant process in all cells of aerobic organisms (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1999). Reactive oxygen species (ROS) include the free radicals superoxide ($\cdot O_2^-$) and hydroxyl ion ($\cdot OH$), and the non-radical hydrogen peroxide (H_2O_2). A vast network of ubiquitous antioxidants and enzymes has evolved to protect cells from the harmful effects of ROS and maintain cellular homeostasis. Exposure to xenobiotics that either increase the production of ROS or hinder their removal can lead to an imbalance in pro-oxidants and antioxidants and result in oxidative damage to essential cellular components like DNA, proteins, and lipids (Halliwell and Gutteridge, 1999). Antioxidants like glutathione, enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, together with oxidative damage have been increasingly used as biomarkers of pollution in marine teleost fish and molluscs (reviewed by Winston and Di Giulio, 1991; Livingstone, 2001). Changes in oxidative stress biomarkers in coastal mussels have resulted from environmental and laboratory exposures to several different chemical classes including redox active metals (e.g. Viarengo et al., 1998; Canesi et al., 1999; Vlahogianni and Valavanidis, 2007; Giarratano et al., 2010) and polycyclic aromatic

hydrocarbons (PAHs) (e.g. Livingstone et al., 1990; Solé et al., 2007; Bebianno and Barreira, 2009).

The mode of action of many toxic substances is the production of reactive oxygen species (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1999). The oxidation of sulfide to sulfate, as done by thiotrophic endosymbionts, produces ROS (Tapley et al., 1999). PAHs are known to be harmful to estuarine organisms. In mussels PAHs are usually metabolized by mixed function oxygenases into quinones (Stegeman, 1985; Livingstone et al., 1988) which can redox cycle and create ROS that could lead to oxidative stress.

Deep sea cold seeps and hydrothermal vents present distinct extreme chemical conditions that are theoretically conducive to the production of ROS and consequently oxidative stress in their endemic inhabitants. The broad dominance of bathymodiolin mussels at vents and seeps around the world allows for comparison among congeneric species that have evolved under extreme conditions. In this study antioxidant and oxidative stress biomarkers were measured in three bathymodiolin mussel species collected from two Gulf of Mexico seeps (Alaminos Canyon and Mississippi Canyon). Results are compared to biomarker levels measured in *Bathymodiolus brevior* from two Lau Basin (Tui Malila and Hine Hina) and one North Fiji Basin (Mussel Hill) vent site.

The seeps of the Gulf of Mexico and the hydrothermal vents of the Lau and North Fiji Basin share elevated concentrations of sulfide and methane which are used as

fuel by thiotrophic and methanotrophic free-living and endosymbiotic bacteria. Both environments are highly spatially variable with mussels from the same beds sometimes being exposed to different chemical milieu (Bergquist et al., 2005).

Gulf of Mexico seeps tend to be either brine seeps or oil seeps that are sedimented and have low diffuse flux over long time spans. Brine seeps have moderate seepage rates of high salinity brine (salinity up to 130) supersaturated with methane with little sulfide and lower abundances of hydrocarbons (Smith et al., 2000). Often the brine will pool and create seafloor lakes (e.g. NR1 in the Green Canyon; MacDonald et al. (1990)) of high salinity that are ringed with methanotrophic bacteria and sometimes with *Bathymodiolus childressi* that house methanotrophic endosymbionts (Smith et al., 2000). Reduced compounds used for chemosynthesis are associated with the pore water in the sediment with very little present in the local bottom water.

Gulf of Mexico seeps are sometimes associated with petroleum leakage with animals sometimes drenched in crude oil (Wade et al., 1989; McDonald, 1990; Sassen et al., 1994; Nix et al., 1995). Wade et al. (1989) reported Green Canyon 272 sediment core PAH levels of up to 6800 ng/g. Mussels (presumably *Bathymodiolus childressi*) had total PAH concentrations up to 7530 ng/g (Wade et al., 1989), with lower molecular weight hydrocarbons being more prevalent in mussel tissue. *B. childressi* from petroleum seeps typically showed lower condition index and lower growth rate than *B. childressi* from saline seeps (Nix et al., 1995). Both condition index and growth rate improved upon

transplantation to brine seeps, sometimes out-performing native brine seep mussels (Nix et al., 1995; Bergquist et al., 2004).

Hydrothermal vents are usually situated on exposed crust with high fluxes of focused or diffuse venting. The longevity of vents is usually on the order of decades. Lau Basin hydrothermal vent fluids are characterized by elevated heavy metal concentrations (Fe, Mn, Zn, Pb, Cu, Co, Ag, Mg), higher concentrations of sulfide than methane, and low pH (Fouquet et al., 1991).

Limited study of the antioxidant defenses of seep mussels have been published to date. Willett et al. (1999) reported higher activity of the enzymes aryl hydrocarbon hydroxylase and glutathione S-transferase in *B. childressi* gill and hepatopancreas collected from oil seeps than from brine seeps. Similar results for aryl hydrocarbon hydroxylase were reported by McDonald (1990). Several studies of hydrothermal vent inhabitants have been published including many with the vent mussel *Bathymodiolus azoricus* (Company et al., 2004, 2006a,b, 2007, 2008; Bebianno et al., 2005). Baseline activities of catalase, superoxide dismutase, and amounts of lipid peroxidation in *B. azoricus* collected from several sites in the northern Mid-Atlantic Ridge are on the same order of magnitude as coastal mussels (Bebianno et al., 2005; Company et al., 2006). Gill tissue tended to have higher activities of antioxidant enzymes and higher concentrations of accumulated metals than mantle tissue. Exposure of *B. azoricus* to elevated concentrations of Hg resulted in increased antioxidant activities in gill tissue while

exposure to Cu and Cd resulted in decreased activities (Company et al., 2006). Metallothioneins appear not to play a large role in detoxification of metals (Geret et al., 1998; Rousse et al., 1998; Bebianno et al., 2005).

The genus *Bathymodiolus* is represented in the Gulf of Mexico by three species (reviewed by Cordes et al., 2009). *Bathymodiolus childressi* (also seen as *"Bathymodiolus" childressi*, Gustafson et al., 1998) is best studied and is found at seeps along the upper Louisiana slope at depths less than 2200m. It contains only type 1 methanotrophic gamma-proteobacteria (Fisher, 1987) and gets most of its nutrition from these symbionts (Fisher and Childress, 1992) despite an ability to filter feed (Page et al., 1990; Pile, 1992). Despite its large depth range, *B. childressi* in the Gulf of Mexico represents a single panmictic population (Carney et al., 2006).

Bathymodiolus brooksi harbors both methanotrophic and thiotrophic endosymbionts, sometimes within the same vacuole (Fisher et al., 1993), and lives at depths between 1080m and 3300m (Cordes et al., 2009). The proportion of each type of symbiont in *B. brooski* can differ with chemical conditions (Fisher et al., 1993; Duperron et al., 2007). *Bathymodiolus heckerae* (also seen as *B. heckeri*) is only found at the deepest sites, inhabiting seeps from 2200m to 3300m. It hosts four types of symbionts including a methanotroph, a methylotroph and two different thiotrophs (Duperron et al., 2007).

Lau and North Fiji Basin hydrothermal vents are home to *Bathymodiolus brevior*, which hosts thiotrophic gamma-proteobacterial endosymbionts (von Cosel et al., 1994;

Desbruyères et al., 1994; Dubilier et al., 1998). *B. brevior* is often found at temperatures between 3 and 6°C (Waite et al., 2008; Podowski et al., 2009) in weak diffuse flow. *B. brevior* has a low temperature threshold of approximately 18°C and might utilize thiosulfate rather than hydrogen sulfide (Henry et al., 2008) as has been proposed for *Bathymodiolus thermophilus* from vents on the East Pacific Rise (Belkin et al., 1986).

The co-occurrence of *B. childressi* with *B. brooksi* and *B. heckerae* at certain seeps allows for some insight into how evolutionary background affects antioxidant activities. Recent phylogenetic studies of the Bathymodiolinae support morphological indications (Gustafson et al., 1998) that among the four species considered here, *B. childressi* is the most distantly related (Jones et al., 2006a, b). Jones et al. (2006a, b) report that *B. childressi* is ancestral to the others, which migrated to deeper water and diverged there. *B. brooksi* diverged and remained at deep seeps while the *Bathymodiolus thermophilus* group colonized vents and radiated from there. Their data suggest the *B. heckerae* group (which includes *B. azoricus* from the Mid-Atlantic Ridge) diverged from the vent inhabiting *B. brevior* group and recolonized deep seeps.

Here I present basal levels of the four oxidative stress biomarkers catalase, superoxide dismutase, total glutathione and lipid peroxidation measured in three bathymodiolin mussel species from two Gulf of Mexico seep sites. These will be directly compared to the same biomarkers measured in *B. brevior* from Western Pacific vents.

Methods

Collection

Three mussel species were collected from two brine seeps in the Gulf of Mexico (Table 3.1, Fig. 3.1) using the submersible *Alvin* in 2006 (cruise AT-15-03). Specimens of *Bathymodiolus childressi* were collected from Mississippi Canyon lease block 640 (MC-640). *Bathymodiolus brooksi* were collected from MC-640 and from Alaminos Canyon lease block 645 (AC-645), and *Bathymodiolus heckerae* were collected from AC-645. All mussels were collected by grab samples and brought to the surface in closed insulated boxes. Once aboard ship, mussels were shucked and frozen whole at -80°C until processed for analysis on shore. GoM seeps are commonly labeled by their location within Minerals Management Service lease blocks.

MC-640 is a brine seep that sits atop a 15m tall mound at a depth of 1420m (Brooks et al., 2008). Sediment cores taken from bacterial mats show elevated salinity of 75 – 88 and methane concentrations of 6 mM (Brooks et al., 2008). Several small brine pools and channels support bacterial mats and two species of mussel (*B. childressi* and *B. brooksi*).

Alaminos Canyon cuts across the continental slope from 1500m to 3000m and exposes alternating layers of evaporate and carbonate (Bryant et al., 1990; Cordes et al., 2007). AC-645 rests at 2200m near the eastern side of the canyon and supports two species of mussel (*B. brooksi* and *B. heckerae*) and abundant tubeworms (Cordes et al.,

2007; Brooks et al., 2008) among fractured carbonates. Low methane concentrations (< 30 μM) were measured in sediment cores (Brooks et al., 2008). Stable carbon isotope data for authigenic carbonates suggest they were derived from the microbial degradation of crude oil (Roberts and Aharon, 1994). Mussels here were often covered in a white precipitate not seen at other GoM seeps (Brooks et al., 2008).

Biomarker Assays

Four gill, foot, and mantle tissues sub-samples were dissected out of each mussel, measured for wet weight, and homogenized in a glass-glass homogenizer in the respective buffers of each of four spectrophotometric biomarker assays; catalase, superoxide dismutase, total glutathione, and lipid peroxidation. Protein was measured in each homogenized supernatant (except total glutathione) and final biomarker assay values are reported as per mg protein. The homogenization buffer used in the total glutathione assay eliminates protein from the supernatant so total glutathione values are reported as per gram wet weight of homogenized tissue. Samples were run in duplicate or triplicate. All absorbances were measured on Molecular Devices SpectraMax M2 or SpectraMax 190 microplate readers and analyzed with its SoftMax Pro software.

Table 3.1. Seep and vent mussel collection data.

Species	Habitat	Sampling Site	Depth (m)	Dive	N
Bathymodiolus childressi	Seep	Mississippi Canyon 640	1414	A-4182	6
Bathymodiolus brooksi	Seep	Mississippi Canyon 640	1414	A-4182	4
	Seep	Alaminos Canyon 645	2208	A-4197	8
Bathymodiolus heckerae	Seep	Alaminos Canyon 645	2208	A-4197	3
Bathymodiolus brevior	Vent	Tui Malila, LB	1895	J2-144	3
	Vent	Hine Hina North, LB	1817	J2-145	3
	Vent	Mussel Hill, NFB	1990	J2-149	3

N, individual; LB, Lau Basin; NFB, North Fiji Basin; A-, Alvin dive; J2-, *ROV Jason II* dive

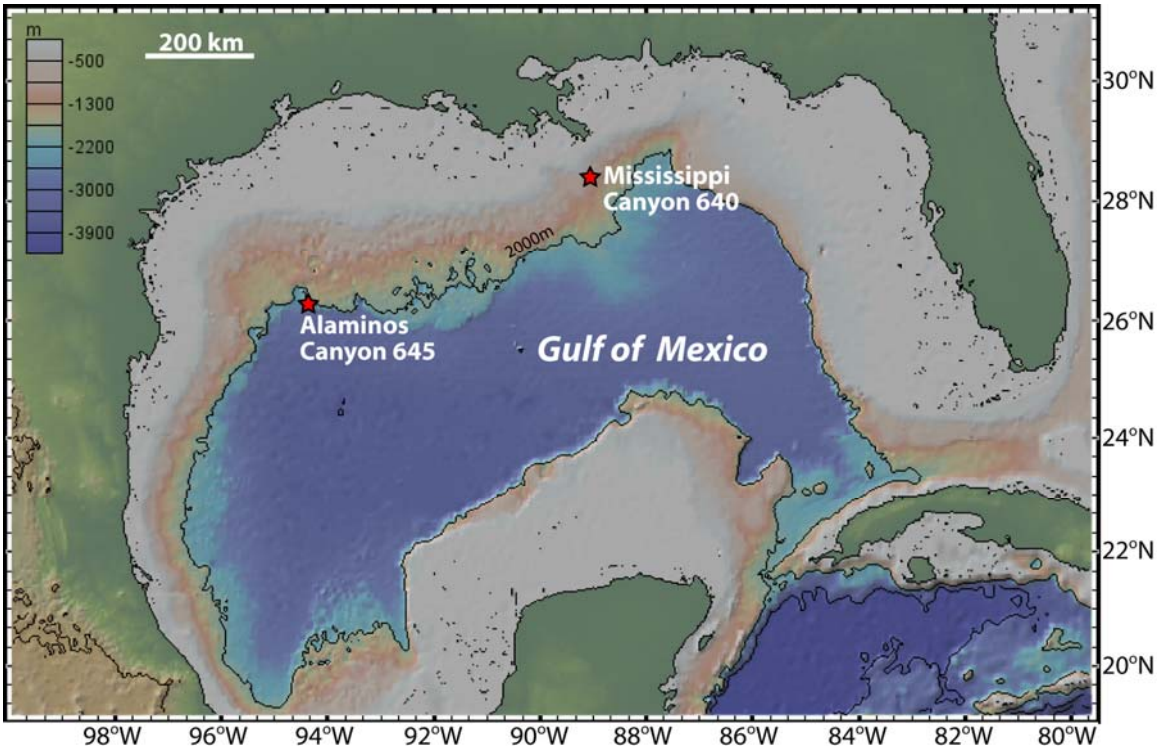

Figure 3.1. Location of seep sampling sites in the Gulf of Mexico. For location of vent sampling sites see Fig 2.1.Drawn with GeoMapApp. Bathymetry from Ryan et al., 2009.

Catalase

Catalase activity was assayed using the Cayman Chemical Catalase Assay Kit (707002). Tissue sub-samples were homogenized in 6 volumes of cold 50 mM potassium phosphate buffer with 1 mM EDTA (pH = 7.0) and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was removed and to it was added methanol and hydrogen peroxide. Tissue catalase converted these to formaldehyde. The chromogen Purpald (4-amino-3-hydrazine-5-mecapto-1,2,4-triazole) was added to react with the formaldehyde to produce a purple color an the absorbance was read at 540 nm.

Superoxide Dismutase

Cytosolic and mitochondrial SOD activities were measured using the Cayman Chemical Superoxide Dismutase Assay Kit (706002). Tissues were homogenized in 5 volumes of cold 20 mM HEPES buffer (pH = 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose and then centrifuged at 1,500 x g for 5 min at 4°C. The supernatant was isolated and further centrifuged at 10,000 x g fro 15 min at 4°C to separate the cytosolic SOD (new supernatant) from the mitochondrial SOD (new pellet). The pellet was resuspended in homogenization buffer and both samples frozen at -80°C until assayed within days. To produce superoxide radicals and detect them the enzyme xanthine oxidase and a tetrazolium salt radical detector were added, respectively. Samples were incubated at room temperature for 20 min. The color change of the tetrazolium salt was measured as an endpoint at 450 nm. Linearized rates were

calculated by dividing the absorbance of the blank by the sample absorbance. One unit of SOD activity is defined as the amount of enzyme required for a 50% dismutation of the superoxide radical.

Total Glutathione

Total glutathione was assayed following Ringwood et al. (2003) as adapted for a 96-well plate. Tissues were homogenized in 6 volumes of 5% sulfosalicylic acid and centrifuged at 6,000 x g for 12 min at 4°C and the supernatant diluted 1:2 with sulfosalicylic acid. A cocktail of 0.238 mg/ml NADPH and 10 mM 5,5'-dithiobis(2-nitobenzoic acid) was made and 195 µl of it mixed with 10 µl of sample. The reaction was started with the quick addition of 6 µl of 50 unit glutathione reductase. Absorbance at 405 nm was read over 2.5 min and the slope was used to calculate total glutathione concentration from a standard curve.

Lipid Peroxidation

The thiobarbituric acid reactive substances assay for lipid peroxidation was modified from Ringwood et al. (2003) for use in a microplate reader. Tissue sampled were homogenized in 4 volumes of 50 mM potassium phosphate buffer (pH = 7.0) and centrifuged at 6,000 x g for 12 min at 4°C. A mixture of 100 µl of supernatant, 1400 µl of 0.375% thiobarbituric acid, and 14 µl of 2% butylated hydroxytoluene was incubated in a 100C water bath for 15 min. Samples were centrifuged at 13,000 x g for 5 min ad room temperature and the absorbance of the supernatant was measured at 532 nm.

Protein

The protein content of each biomarker assay homogenate supernatant (except for total glutathione) was measured using the Pierce Coomassie Protein Assay. 225 µl of Pierce Coomassie Blue reagent and 5 µl of sample supernatant were added to each well and absorbance read at 595 nm. A standard curve was made with a bovine serum albumin dilution series and modeled as a 4-parameter curve per the company instructions.

Analysis

Individual biomarkers were compared within tissues and among all specie-sites using one-way ANOVAs with Tukey multiple pairwise comparison post-hoc tests. These analyses were run on SigmaPlot 11 (Systat Software). Two principal component analyses were performed on standardized gill and mantle biomarker levels (Gotelli and Ellison, 2004; Sinha et al., 2009). The first PCA was performed using only biomarker from seep species while the second included biomarker data from *B. brevior* collected from Lau and North Fiji Basin vents. PCA does not include individuals for which any variable is missing. Therefore foot tissue was not included in this PCA because the many missing foot biomarker values would have reduced the overall number of data points. PCAs were performed using TIBCO Spotfire S+ 8.1 (TIBCO Software Co.).

Results

Biomarker levels for *B. brevior* have already been presented in Chapter 2 and so will not be presented in this section. For ease of comparison *B. brevior* data are presented again in Figures 3.2 and 3.3 together with new biomarker data from seeps. No foot tissue from *B. heckerae* was tested because this tissue was buried deep within the visceral mass of the specimens and it would have compromised the rest of the specimens against future analyses. Both PCAs are reported in this results section since neither has been presented elsewhere.

Catalase

The activity of catalase in seep mussel was tissue specific and site specific (Fig. 3.2a). Gill tissues generally had the highest catalase activities while mantle tissues in all species had the lowest. *B. childressi* and *B. brooksi* from MC-640 each had gill catalase activity significantly higher (9 fold) than their respective mantle activity ($p = 0.025$ and $p = 0.024$, respectively). Alternatively, neither of the two species (*B. brooksi* and *B. heckerae*) from AC-645 showed any significant difference among tissues despite a 5- and 2-fold difference between gill and mantle.

Mantle showed very little variability across the two seep sites and across the three seep species and ranged from 1.74 ± 0.43 nmol/min/mg protein for *B. childressi* from MC-640 to 2.14 ±0.41 nmol/min/mg protein in *B. brooksi* from MC-640. Catalase

activity in foot tissue did not differ significantly among species but ranged from 5.10 ± 1.31 nmol/min/mg protein in *B. brooksi* at MC-640 to 15.07 ± 6.18 nmol/min/mg protein in *B. brooksi* at AC-645.

Cytosolic SOD

Similar to catalase, CSOD was consistently lowest in mantle tissue (3.23 – 11.66 U/mg protein) and higher in foot (58.79 – 67.85 U/mg protein) and gill (10 – 74 U/mg protein) tissue (Fig. 3.2b). Gill tissue showed significant difference between seep sites with CSOD activity in MC-640 species (between 3- and 7-fold) higher than in AC-645 species. For both species from MC-640 (*B. childressi* and *B. brooksi*) gill and foot CSOD activity was significantly higher than mantle tissue. Alternatively, *B. brooksi* from AC-645 Gill CSOD activity was significantly lower than foot activity and similar to mantle activity.

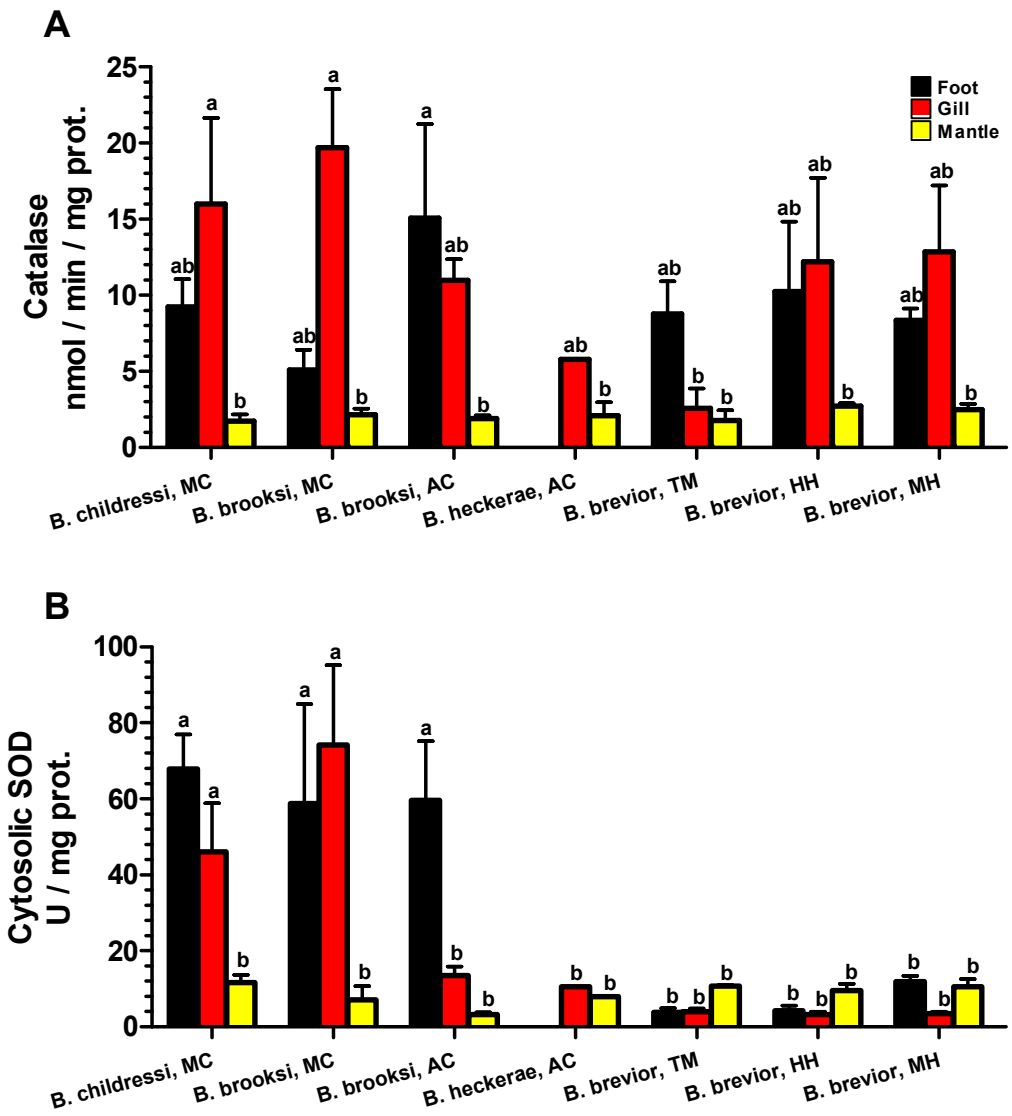

Figure 3.2. Catalase activity (A) and CSOD activity (B) in seep and vent mussels. Different letters represent significant differences ($p<0.05$).

Total Glutathione

No significant difference in total glutathione levels were seen among tissues, species or seep site (Fig. 3.3a). Total glutathione levels ranged from 491.69 – 899.33 nmol/g wet wt. in foot tissue, from 401.69 – 1147.76 nmol/g wet wt. in gill tissue and from 734.73 – 891.81 nmol/g wet wt. in mantle tissue. Despite a lack of significant differences a difference in pattern is seen between sites. At MC-640 mean gill levels are lower than mean mantle levels while at AC-645 this pattern is reversed.

Lipid Peroxidation

Lipid peroxidation levels were similar across all tissues, species and sites, but for one significant difference (Fig. 3.3b). *B. childressi* gills from MC-640 showed significantly higher lipid peroxidation levels (6.92 ± 1.01 nmol/mg protein) than all other gill samples (2 – 3-fold; all $p < 0.001$) as well as all other tissue samples (all $p < 0.044$). Despite a lack of significant differences among the other tissue, mantle tissue (0.81 – 1.36 nmol/mg protein) always had lower mean lipid peroxidation levels than gill (1.97 – 3.03 nmol/mg protein, excluding *B. childressi* mentioned above) and foot tissue (1.85 – 2.91 nmol/mg protein).

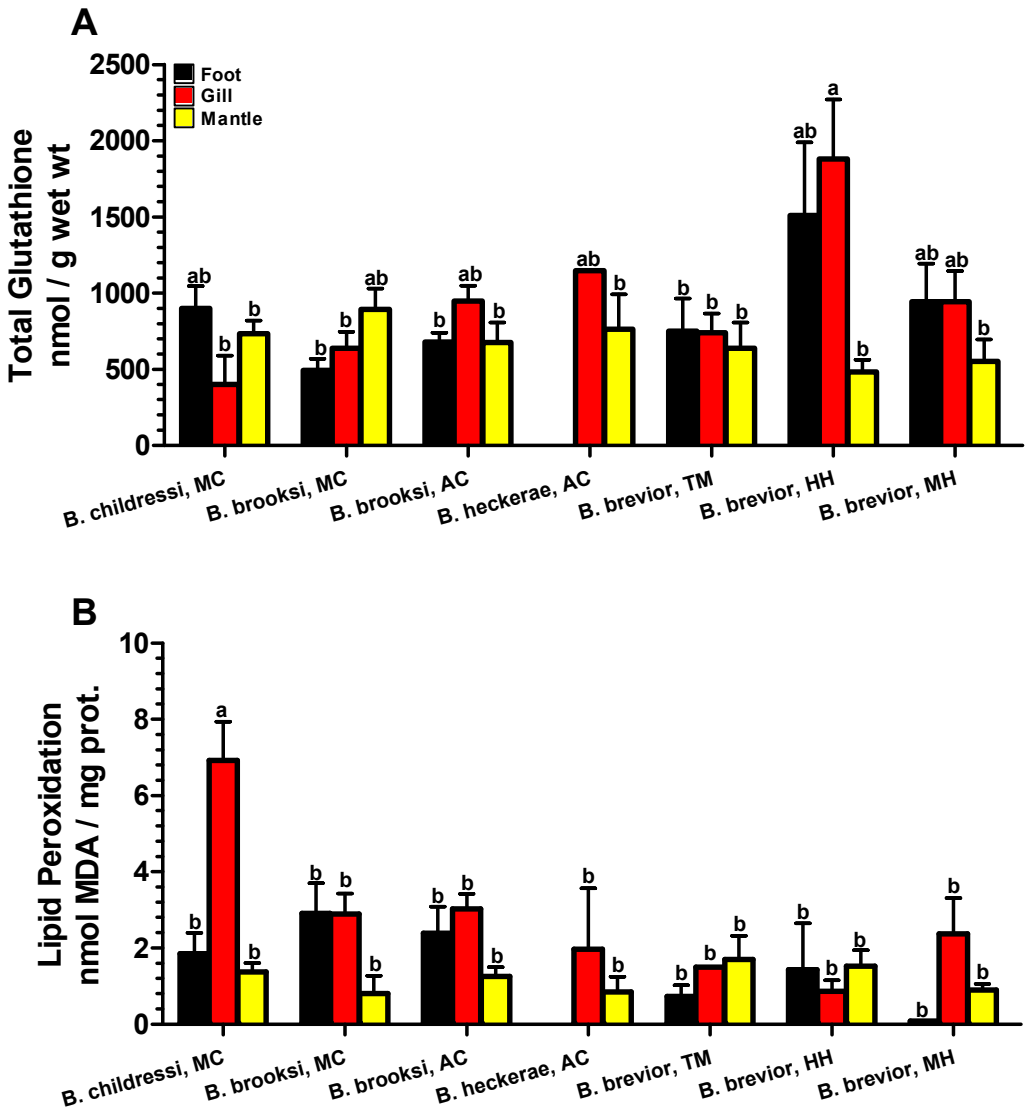

Figure 3.3. Total glutathione (A) and lipid peroxidation (B) in seep and vent mussels. Different letters represent significant differences ($p<0.05$).

Principal Component Analysis

Seep Mussels

PCA of seep mussel gill and mantle tissue biomarker levels produced three significant components that together explained 75% of the variation. The first principal component (Fig 3.4a) was heavily positively influenced by CSOD levels in both the gill and mantle and heavily negatively influenced by glutathione in the gill (not graphed due to absence from second component). The second component was heavily positively influenced by lipid peroxidation in both tissues and heavily negatively influenced by gill catalase and CSOD.

The plot of PCA scores seen in Fig 3.4b shows tighter clustering of mussels from AC-645 than those from MC-640. Component 1 differentiates samples by site with MC-640 mussels generally having higher scores than AC-645 mussels. An alternative interpretation of component 1 is that is generally differentiates *B. childressi* from *B. brooksi* and *B. heckerae* with one *B. brooksi* and one *B. childressi* outlier. *B. childressi* from MC-640 generally shows little variation on component 1 (with one outlier) while shows great spread on component 2. Component 2 does not differentiate any natural grouping.

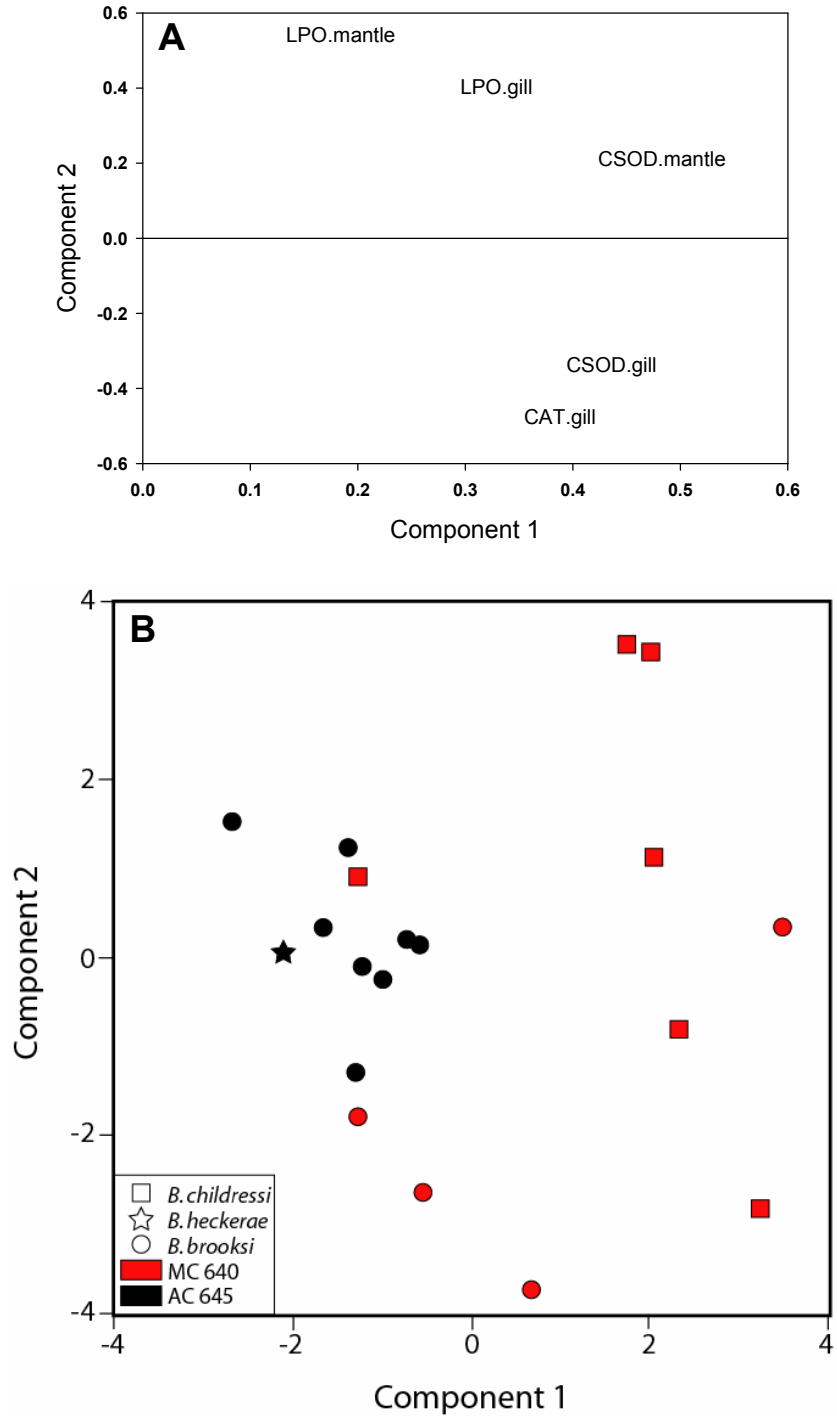

Figure 3.4. Loadings (A) and score (B) of a PCA performed with seep mussel gill and mantle tissue.

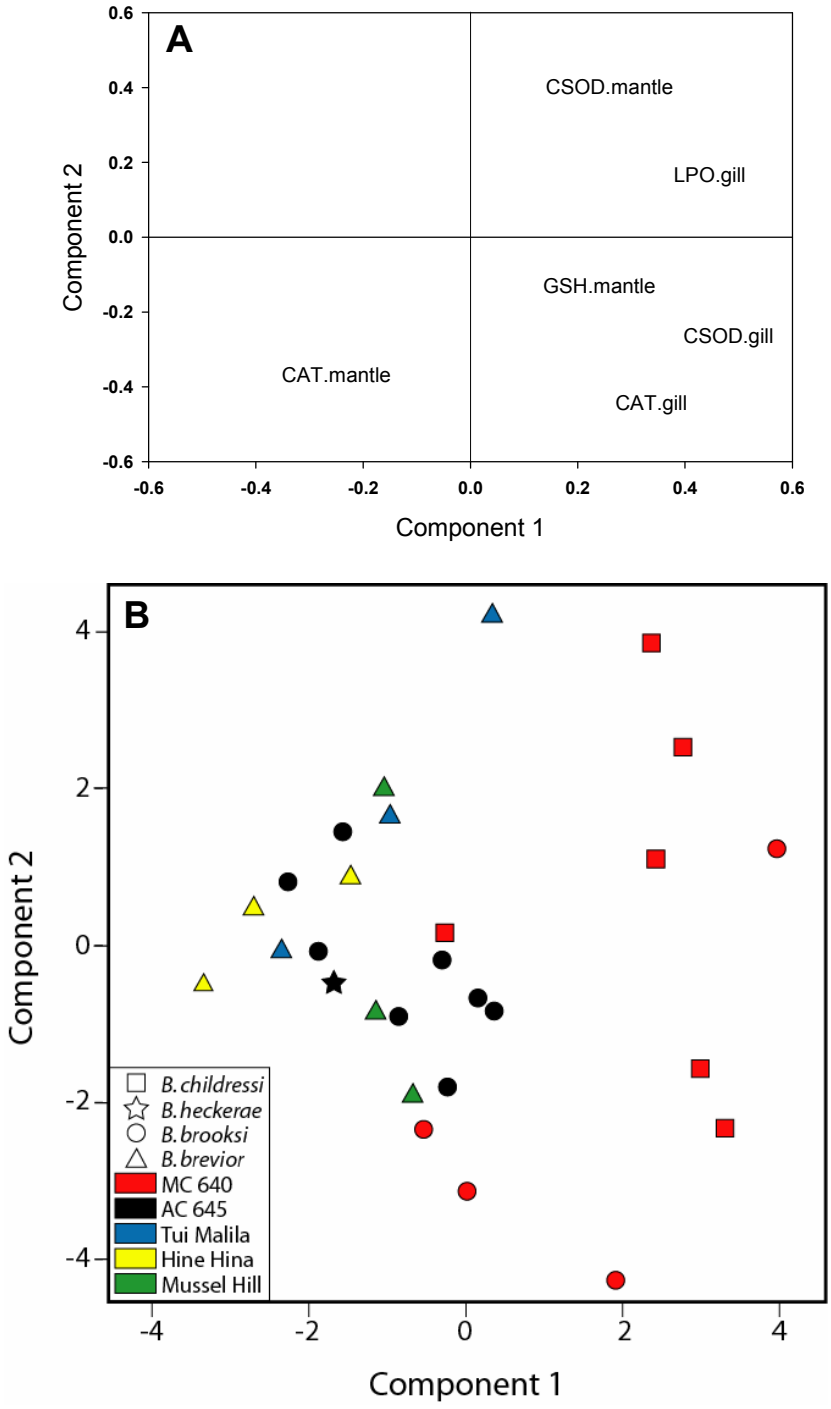

Figure 3.5. Loadings (A) and score (B) of a PCA performed with seep and vent mussel gill and mantle biomarker levels.

Seep and Vent Mussels

When oxidative stress biomarkers from *B. brevior* collected from the Lau and North Fiji Basins are included with seep mussel biomarkers a similar picture is produced (Fig 3.5a,b). Three significant principal components explained 69.9% of the original variation. The first two are presented in Figure 2.5a. Component 1 was heavily influenced by gill levels of CSOD, lipid peroxidation, and catalase, while negatively influenced by catalase in mantle tissue. Component 2 was positively influenced by mantle CSOD and lipid peroxidation in gill tissue and negatively influenced by catalase in gill and mantle.

The plot of PCA scores (Fig 3.5b) shows similar placement of seep samples to the first PCA (Fig 3.4b). The added vent biomarker data overlaps closely with *B. brooksi* and *B. heckerae* from AC-645. *B. childressi* remains differentiated from the rest by component 1. Component 2 again does not clearly differentiate any natural grouping of mussels.

Discussion

The Bathymodiolinae are found at deep-sea chemosynthetic sites all around the world (Van Dover, 2000) and are one of the most well studied groups in the deep sea. Their inclusion within the well studied and cosmopolitan Mytilidae makes this group a particularly good choice for exploration of antioxidant defenses and oxidative stress.

Shallow water mussels have been used as sentinels for marine and freshwater pollution for years (Goldberg et al., 1978) and they continue to be the primary group of marine invertebrates used in exotoxicological studies (reviewed by Livingstone, 2001). An extended line of research has been performed on basal levels of antioxidant biomarkers in the Mid-Atlantic Ridge vent mussel *Bathymodiolus azoricus* (Cosson, 1997; Bebianno et al., 2005; Company et al., 2007) as well as their responses to laboratory exposures to various metal solutions (Company et al., 2004, 2006, 2008). Data presented here represent the first report of basal antioxidant biomarker levels from three Bathymodiolinae species from Gulf of Mexico seeps.

The three mussel species used in this study came from two seep sites, Mississippi Canyon lease Block 640 (MC-640) and Alaminos Canyon lease Block 645 (AC-645) (Table 3.1, Fig. 3.1). MC-640 is a brine seep with sediment salinity reaching 88 and sediment methane concentrations of at least 6 mM (Brooks et al., 2008). AC-645 is a deep seep site that supports large tubeworm communities and hosts abundant authigenic carbonate that Roberts and Aharon (1994) attribute to microbial degradation of crude oil, although no visible signs of macro seepage of oil can be seen.

Catalase activity showed tissue-specific and site-specific differences with significantly higher gill and foot activity than mantle (Fig. 3.2a). This pattern matches what is seen in *B. brevior* from Lau and North Fiji Basin vents and *B. azoricus* from the Mid-Atlantic Ridge (Bebianno et al., 2005; Company et al., 2006, 2008). Catalase activities

in seep mussels reached a maximum of 19.69 ± 3.86 nmol/min/mg protein in *B. brooksi* gill tissue from MC-640, which is at the lower end of the range reported in *B. azoricus* (13.7 – 56.1 nmol/min/mg protein)(Bebianno et al., 2005).

Seep mussels showed a site specific pattern in gill activity of cytosolic SOD. Mussels from MC-640 showed 3 – 7 fold higher gill activity than mussels from AC-645 (Fig. 3.2b). Foot tissue showed high activity at both sites while mantle CSOD activity was consistently low. The foot and gill CSOD activities from MC-640 were 11 – 23 fold higher than foot and gill activity in *B. brevior* and 2 – 10 fold higher than gill activity in *B. azoricus* (Bebianno et al., 2005; Company et al., 2006b). No difference in mantle activity of CSOD was seen across seep sites, or across Lau Basin and Mid-Atlantic Ridge sites (Bebianno et al., 2005; Company et al., 2006b).

Total glutathione remained consistent across species and sites, with no significant difference at seeps. This agrees well with levels seen in *B. brevior* from Tui Malila and Mussel Hill. Glutathione levels in foot and gill of *B. brevior* from Hine Hina, an odd site with no appreciable focused venting, were almost 2 fold higher than the highest mean levels from seeps (Fig. 3.3a).

Seep mussels showed moderately high levels of lipid peroxidation with *B. childressi* gill tissue particularly so (Fig. 3.3b). As with other biomarkers foot and gill tissue tended to have slightly higher lipid peroxidation levels than mantle, although never significantly higher. No differences among tissues were seen in *B. brevior*,

although mantle levels were never the lowest. Bebianno et al. (2005) and Company et al. (2006b) reported *B. azoricus* lipid peroxidation in gills almost always higher than in mantle. What is most striking though is that seep and Lau Basin vent mussels had levels between 0.89 – 6.92 nmol MDA/mg protein (with most values between 1 – 3 nmol MDA/mg protein) while Mid-Atlantic Ridge mussels showed between 0.042 – 0.186 nmol MDA/mg protein (Bebianno et al., 2005), up to a 164-fold difference. The control values of lipid peroxidation during laboratory exposures were of similar values to Bebianno et al. (2005) (Company et al., 2006a, 2008).

The PCA performed on standardized biomarker data of only the three seep species shows tight clustering by *B. brooksi* from AC-645 while *B. brooksi* from MC-640 shows considerably higher degree of variation. Five out of the six *B. childressi* data points align nicely on component 1 and show a large spread in component 2. The tight clustering of *B. brooksi* at AC-645 and not MC-640, together with large spread in *B. childressi* from MC-640 suggests that Mississippi Canyon seep site presents a wider range of microhabitats than does Alaminos Canyon.

Inclusion of biomarker data from *B. brevior* into a new PCA (Fig. 3.5b) produced similar groupings to the first PCA (Fig. 3.4b) for the three seep species. *B. brevior* individuals cluster around *B. brooksi* from AC-645 with minimal spread on component 1 and moderate spread on component 2. *B. brevior*, *B. heckerae*, and to some degree *B.*

brooksi show overall antioxidant defense differences from *B. childressi*. This fits with the evolutionary history of the Bathymodiolinae as reported by Jones et al. (2006).

Another difference is that *B. childressi* is the only species assayed here with only methanotrophic endosymbionts (Fisher et al., 1987). *B. brooksi* and *B. heckerae* possess both methanotrophic and thiotrophic symbionts and *B. brevior* contains only thiotrophic symbionts (Fisher et al., 1993; Dubilier et al., 1998; Duperron et al., 2007). Thus *B. childressi* might be more prone to oxidative damage by sulfide than those mussels with thiotrophic endosymbionts.

GoM seeps and deep-sea hydrothermal vents support related organisms in analogous ecosystems, yet they differ in their physical, chemical, and temporal characteristics. Seeps tend to have diffuse flux of hydrocarbon-rich fluid over centuries while vents tend to have highly variable flux of hot metal- and sulfide-rich fluid that can generally last from weeks to decades (Van Dover, 2000). Despite these differences, seep and vent mussels show similar antioxidant biomarker levels, except for CSOD. High levels of lipid peroxidation in seep and LB and NFB mussels suggest their antioxidant defenses may not always be able to prevent oxidative damage.

Conclusions

Biomarker levels in the seep mussels *Bathymodiolus childressi, B. brooksi*, and *B. heckerae* were similar across species except for elevated foot and gill cytosolic SOD in

mussels from MC-640 compared to those from AC-645. Gill and foot CSOD activity in MC-640 mussels was 3 – 7 fold higher than seep mussels from AC-645. All seep and vent mussels consistently showed very low levels of catalase, CSOD, and lipid peroxidation in their mantle tissue. There were no differences in total glutathione levels across all seep and Lau and NFB vent mussels.

The PCA performed on standardized biomarker data of only the three seep species differentiated by species with *B. childressi* isolated from *B. brooksi* and *B. heckerae*. *B. childressi* shows considerable variation on component 2 score and is somewhat isolated from the other mussel species. Whether this reflects *B. childressi*'s more distant relationship to the other mussel species or is perhaps due to its lack of a thiotrophic endosymbiont can not be determined.

Chapter 4. Toxicity and Sub-lethal Effects of Common Insecticides and an Herbicide to Blue Crab Megalopae and Juveniles

Introduction

Swan Quarter shedder Dell Newman has experienced precipitous die off of peeler crabs associated with spring rains and permanent soft crabs in mid summer. These times coincided with approximate times of aerial insecticide and/or herbicide applications to cotton. Much is known about the toxicity of pesticides to non-target model organisms. When the non-target organism is of great ecological and economic importance, like the blue crab in North Carolina, it deserves its own assessment of toxicity.

Marine fisheries and agriculture are immensely important to the state of North Carolina (NC), contributing significantly to its economy and culture. In 2007 the NC blue crab fishery grossed over $21.4 million (including hard, peelers, and soft blue crabs) making it the most lucrative marine fishery in the state (NCDMF, 2008). Tobacco is still the most profitable crop in the state with 2007 sales of $586 million followed by corn ($305 million), soybeans ($274 million), and cotton ($252 million) (NCDA&CS, 2008a).

The Pamlico Sound and its tributaries lie at the center of the North Carolina blue crab fishery but also drain several counties with thousands of acres of cropland. Saltwater creeks that drain agricultural fields are important habitats for juvenile and

adult stage blue crabs (Posey et al., 2005), and their prey. Blue crabs inhabit the creeks, rivers, sounds, and coastal oceans of NC, in waters with salinities between 0 and 35. After mating in spring and summer, gravid females migrate downstream and out of the estuary to release eggs. Dispersal stage zoeae larvae then travel offshore to feed before returning onshore as settlement stage megalopae in late summer and fall. Megalopae use flood tide transport to migrate upstream (Welch et al., 1997). They settle and metamorphose to the first juvenile stage in submerged vegetation. Secondary dispersal away from initial settlement sites to their broader estuarine habitat occurs in early juvenile stages (between J1 and J4; Reyns and Eggleston, 2004). The shallow brackish tidal ditches and creeks so common in eastern NC are home to abundant juvenile and adult blue crabs and their prey. They are adjacent to residential and agricultural areas and are often less than 10 cm deep and 2 meters wide (personal observation).

Hyde, Beaufort, and Pamlico counties directly border the Pamlico and Neuse Rivers and Pamlico Sound and contain countless saltwater creeks and drainage ditches. In these three counties a total of 32,450 acres were planted with cotton in 2007 and 100,800 acres were planted with soybean (NCDA&CS, 2008b). All crops together in these three counties contributed over $150 million to the 2007 economy. With two of North Carolina's most important economic engines in close proximity it behooves the scientific community to learn as much as possible about any negative effects of one on the other so that minimally disruptive solutions can be developed.

As tobacco production in North Carolina ebbs, cotton and soybean production is surging in coastal counties. Cotton is usually planted in April and May and is often treated at planting with the carbamate aldicarb (Temik 15G®) or the chloro-nicatinyl imidacloprid (Gaucho®, Trimax®) to prevent thrip infestations (Bacheler personal communication; Bacheler et al., 2005; NCDA&CS, 2005). In the summer, cotton leaves are often sprayed with a pyrethroid insecticide such as lambda-cyhalothrin (Karate® or Warrior® brands) or an organophosphate such as acephate (Orthene®) to prevent thrips, aphids, loopers, and lygus (Bacheler et al., 2005; NCDA, 2005). These insecticides can be extremely toxic to aquatic organisms (PAN, 2008), especially crustaceans, which are in the same phylum as insects.

Pesticide runoff or spray drift from airplane application has the potential to negatively affect blue crabs by directly killing individuals by acute or chronic toxicity, by ingestion of contaminated sediment, or by killing their prey. Pesticides could have population level effects by altering the success and timing of metamorphosis. During metamorphosis from megalopae to juvenile, crustaceans go through elaborate morphological and physiological changes. As in all hard-shelled animals, blue crab metamorphosis (as well as all other moltings) require the shedding of protective exoskeletons and brief periods as soft crabs. The elevated energetic and physiological demands during metamorphosis and molting leave crabs vulnerable. We assay the

effects of pesticide exposure on the duration of the megalops stage, and the success of metamorphosis.

Some pesticides are known to induce oxidative stress, which is the cellular inbalance of pro-oxidants and anti-oxidants. Oxygen is required by all aerobic organisms in order to produce energy by cellular respiration. Oxygen radicals (with an unpaired electron in its outer shell) and other reactive oxygen species are natural byproducts of cellular respiration that can damage and oxidize proteins, DNA, and lipids in cell membranes. Under normal physiological conditions, these reactive oxygen species are quenched by a comprehensive system of cellular defenses comprised of several enzyme and non-protein antioxidants. One of those defenses is the antioxidant tripeptide glutathione, one of the most abundant cellular non-protein thiols. Reduced glutathione can donate one reducing equivalent (H^+ + 1 electron) from its thiol group to reduce and stabilize reactive oxygen species; can be bound directly to xenobiotics by glutathione S-transferase for excretion; and can play roles in immune responses.

We chose to test the toxicity of the commercial formulations as well as their active ingredients alone to megalopae post larvae, juveniles, and peeler blue crabs. Commercial formulations of pesticides and herbicides often include solvents and surfactants that are toxic to non-target organisms or act synergistically with the active ingredients (Giesy et al., 2000; Howe et al., 2004; Wang et al., 2005; Brausch et al., 2007; Brausch and Smith, 2007). Much of the cotton, soybeans, and corn have been genetically

modified to be resistant to the herbicide glyphosate, the active ingredient in the product Roundup®. Such crops are termed "Roundup Ready®." With more Roundup Ready crops, more use of Roundup and glyphosate is expected. The toxicity of Roundup was assessed because it is not recommended for aquatic application (manufacturer's instructions) and is known to impact aquatic animals (Giesy et al., 2000). We assayed the toxicity to blue crabs of the active ingredient insecticides acephate, aldicarb, imidacloprid, and λ-cyhalothrin and the herbicide glyphosate (Fig 4.1).

Acephate (Fig. 4.1A) is a broad spectrum organophosphate foliar insecticide that is often applied to prevent thrips, aphids and infestation of other biting insects. Organophosphate compounds are known neurotoxins that inhibit the breakdown of acetylcholine, thus they keep neurons in a state of excitement (Eto, 1974). It is not acutely toxic to many crustaceans and other non-target organisms but is highly water soluble with a solubility of over 800,000 mg/L (PAN, 2008). Acephate has a residual systemic activity of 10 – 15 days and half lives in aerobic and anaerobic soils of < 3 and 6 days respectively. In 2007 39% of NC cotton acreage was treated at a rate of 0.481 lbs/A for a total of 122,000 pounds of acephate, up from 85,000 pounds in 2005 (NCDA&CS, 2005; 2008c). We used granular Ortho Orthene® Fire Ant Killer which is 50% acephate.

Aldicarb (Fig. 4.1B) is an N-methyl carbamate that is used at planting to control thrips and nematodes (Bacheler et al., 2005). Like acephate, aldicarb is a cholinesterase inhibitor and is highly acutely toxic to a broad range of organisms especially

crustaceans. As such is it classified as a controlled use pesticide that requires a special permit to purchase and use. A 96 hour LC_{50} value of 200 µg/L (95% CI: 200 – 250 µg/L) was reported for the amphipod *Echinogammarus tabaldii* (Pantani et al., 1997). It has a moderate water solubility of 5870 g/L making it a possible ground water contaminant (PAN, 2008). Aldicarb, often in its most popular commercial formulation Temik 15G® (Bayer Cropscience), was used on 35% of the NC cotton acreage in 2007 at a rate of 0.756 lbs/A for a total of 132,000 lbs (NCDA&CS, 2008c). Since Temik is applied at planting in May, its presence in the environment coincides with the blue crab peeler season in the spring. We acquired Temik but were unable to get the granular formulation to solubilize in a reasonable amount of time in the laboratory so we were unable to do a full analysis of its toxicity to blue crab megalopae and juveniles. We did however assay the active ingredient aldicarb.

Imidacloprid (Fig. 4.1C) is a chloro-nicatinyl insecticide that is found in commercial products such as Trimax®, Provado®, and Goucho® (all Bayer Cropscience), which are either applied as a foliar spray or as a systemic seed treatment. It was developed to mimic nicotine and as such is an acetylcholine receptor agonist causing neurotoxicity (Matsuda et al., 2001). It has a moderate water solubility of 514 µg/L and can runoff from cropland (Gupta et al., 2002). It has been found in concentrations as high as 0.4 µg/L in Black Brook, New Brunswick, Canada (Hewitt et al., 2005) and 0.13 mg/L in Croton River, NY (Phillips and Bode, 2004). Imidacloprid toxicity in crustaceans

ranges broadly with LC_{50} values as low as 38.0 µg/L for *Americamysis bahia* (OPP, 2000) to 17,360 µg/L for *Daphnia magna* to 361,000 µg/L for *Artemia* brine shrimp (Song et al., 1997). Trimax Pro® was assayed here and is a foliar imidacloprid formulation targeted specifically for cotton that contains 40.7% active ingredient.

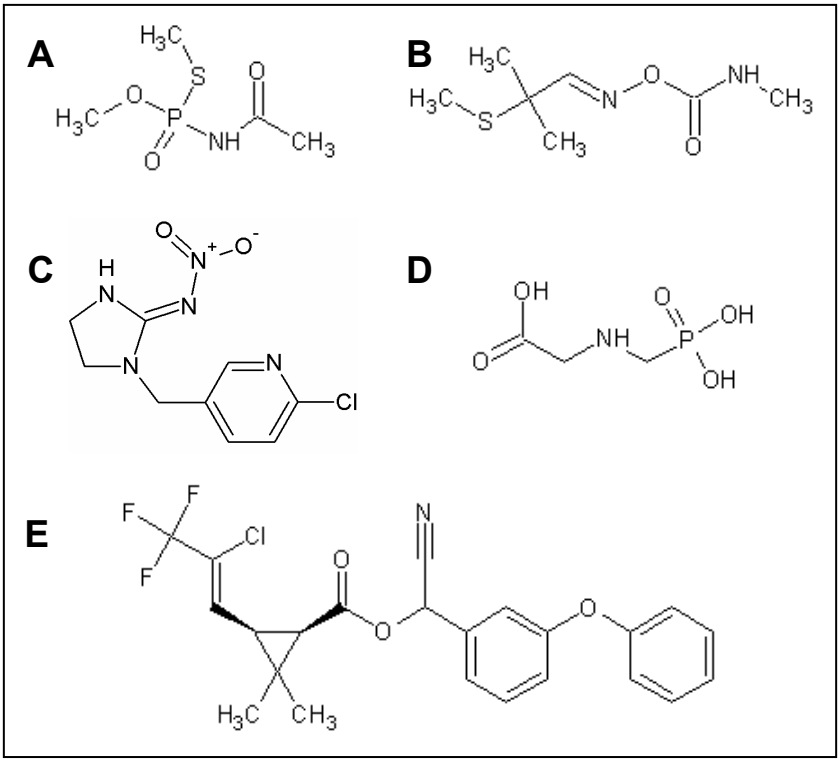

Figure 4.1. Chemical structures of active ingredient pesticides acephate (A), aldicarb (B), imidacloprid (C), glyphosate (D), and λ-cyhalothrin (E).

Lambda-cyhalothrin (λ-cyhalothrin; Fig. 4.1E) is an extremely potent restricted use pyrethroid insecticide. Pyrethroids are synthetic variations on the natural pyrethrum insecticides produced by chrysanthemum flowers. They work by binding to and

opening sodium channels on neuron axons, effectively paralyzing the subjects (He et al., 2008). Lambda-cyhalothrin binds tightly to soil and is not water soluble so is usually dissolved in petroleum or alcohol solvents for commercial applications. Crustacean LC_{50} values for λ-cyhalothrin range from 0.0041 µg/L for opossum shrimp (*Americamysis bahia*; OPP, 2000) to 0.36 µg/L for *Daphnia magna* (Mokry and Hoagland, 1990). Commercial formulations of λ-cyhalothrin are marketed under the brand names Karate® or Warrior® and are foliar sprays. A total of 4,000 lbs was applied to 17% of the NC cotton acreage in 2007 at a rate averaging 0.028 lbs/A (NCDA&CS, 2008c). An additional 3,000 lbs was applied to soybean fields in 2007. The Karate with Zeon Technology® (Syngenta) foliar spray used in this study contained 22% a.i. dissolved in petroleum-based solvents. The Zeon technology is micro-encapsulation of the insecticide inside 2.5 µm diameter polyurea capsules.

The genetic modification of several crops including cotton, corn, and soybeans with glyphosate resistant genes has greatly changed which herbicides are used on crops with a trend towards glyphosate-based products. Roundup Ready cotton has been available since 1997 and over 90% NC cotton is now of this variety. A total of 951,000 lbs of glyphosate-based products (including glyphosate-isopropylamine salt, the form used in Roundup) were applied to at least 86% of NC cotton fields in 2007 at a rate averaging 0.733 lbs/A per application (NCDA&CS, 2008c). In 2006 86% of NC soybean fields were treated with a total of 1,791,000 lbs of glyphosate isopropylamine salt. Glyphosate (Fig.

4.1D) is a very water soluble (900,000 mg/L) phosphonoglycine herbicide that adsorbs strongly to soil and organic particles in water (PAN, 2008). Toxicity is low in the crayfish *Orconectes nais* with 96 hr LC_{50} values of 7,000 μg/L (Mayer and Ellersieck, 1986). Glyphosate's mode of action is the inhibition of an enzyme required to produce the amino acids tryptophan, phenylalanine, and tyrosine (Amrhein et al., 1980). This contrasts greatly with the four insecticides tested here that all impact neuronal receptors and channels.

The Pamlico-Albemarle system is the state's most important blue crab fishery and we must understand anthropogenic influences on that system. If these pesticides prove acutely toxic, developmentally toxic, or oxidatively stressful to blue crabs, the next step will be to find alternatives that complement the economic importance of both blue crabs and agriculture to North Carolina. Here we present data from acute toxicity experiments performed with both commercial formulation pesticides and their active ingredients on megalops, juvenile, and peeler stage blue crabs as well as data on the effects of active ingredient compounds on the metamorphosis of megalopae and levels of oxidative stress biomarkers.

Methods

Collection

Blue crab (*Callinectes sapidus* Rathbun) megalopae were collected from the water column as they migrated inshore during summer 2008 nighttime rising tides. Two 0.75 m diameter plankton nets were deployed for one hour straddling the maximum flood tide current from the NOAA sampling platform located beneath the Pivers Island Bridge, Beaufort, NC. Plankton samples were sieved to remove ctenophores and visually identified blue crab megalopae were sorted out. Megalopae were maintained in filtered aged estuarine seawater (ASW; salinity 35, 25 °C) on an ambient light: dark cycle and reared in large glass finger bowls. Each finger bowl contained 100 – 200 individuals in ~800 mL of ASW. Filtered and aged estuarine seawater was used to mimic natural conditions as closely as possible while minimizing any potential effects of chemical cues. Immediately after daily water change megalopae were fed ~5 mL (~1500 individuals) of newly hatched brine shrimp (*Artemia*) nauplii. Newly molted juvenile (J1) blue crabs and dead megalopae were removed from the finger bowls daily. Megalopae were used in toxicity testing within three days of collection or allowed to molt to juveniles. This method of collecting megalopae from near-oceanic water ensured that the crabs had not been exposed to any significant amount of pesticide for several weeks.

Juvenile blue crabs were reared in similar conditions to megalopae but were housed in lower densities (50 individuals max) and were fed crushed shrimp pellets (dry

fish food) daily. A few strips of nylon window screening were added to each bowl to minimize interactions among juveniles, including cannibalism. First stage (J1) through fourth stage (J4) juveniles were reared and used in acute toxicity assays.

Peelers were purchased from Newman Sea Food in Swan Quarter, NC and transported to Beaufort by car in coolers with wet burlap similar to the way peelers are commercially transported. The smallest and earliest stage peelers (white line peelers) were chosen to minimize the chance of molting during the 24 hr toxicity assays. Peelers were kept overnight in large water tanks with aerated sand-filtered estuarine seawater with salinity lowered to 20 to match the conditions at Newman Seafood. All toxicity testing commenced the day after transportation to allow for acclimation to the local conditions and decrease stress induced by transport. Peeler crabs proved very fragile subjects with many deaths and molts during the overnight acclimation. Only the commercial product Orthene® and the active ingredients λ-cyhalothrin and aldicarb were tested before the peeler season ended. Since no clear results were seen from any of the peeler assays and their monetary cost, amount of pesticide waste generation, and time for transportation were all high, it was decided not to perform any further assays with peeler crabs.

Experimental Solutions

Since the commercial pesticides being applied to agricultural and residential fields usually contain other ingredients and surfactants in addition to the active pesticide compound we decided to test both the commercial formulation and the active ingredient alone. Karate and Temik 15G are restricted use pesticides and were obtained and used under the supervision of Twyla Michelle Blickley, NC Pesticide Board Pesticide Applicator License number 026-26323.

Active ingredient insecticides (Fig. 4.1) used for megalopae and juvenile acute toxicity and time to metamorphosis assays were acquired through Fisher Scientific and were manufactured by Chem Service (West Chester, PA). The four insecticides were dry powder acephate (O,S-Dimethyl acetylphosphoramidothioate; 99.3% purity), aldicarb (2-methyl-2-(methylthio)propionaldehyde O-methylcarbamoyloxime; 99.0% purity), imidacloprid (*N*-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide; 99.5% purity) and λ-cyhalothrin ((RS)-alpha-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2,-dimethylcyclopropanecarboxylate; 99% mix of isomers). Powder Ortho Orthene® Fire Ant Killer (Ortho Group, Marysville, OH) with 50% acephate was purchased from Lowe's Home Improvement. Experimental samples of granular Temik 15G® (15% aldicarb) and liquid Trimax Pro® (40.80% imidacloprid) were acquired from Bayer CropScience (Kansas City, MO). We were unable to acquire scientific samples of Karate from its manufacturer (Syngenta Crop Protection, Greenville, NC) so a 50 mL

sample of Karate with Zeon Technology® was graciously donated by Open Grounds Farm (Carteret County, NC). Roundup Pro® Concentrate (Monsanto Company, St. Louis, MO; 50.2% glyphosate isopropylamine salt) was purchased from Lowe's Home Improvement.

All commercial products were kept in their original bottles or as with Karate transferred to a 50 ml conical tube and kept in the dark. All active ingredients were kept in the dark to prevent photolysis. Working stocks were made immediately before use in glass vials and kept in a light-tight metal container for at most one week before being discarded and new working stocks made as needed. Toxicant dilutions were then made from these working stocks immediately before use and diluted with ASW. If a solvent other than ASW was used in the working stock, dilutions were spiked with additional solvent so that the concentration of solvent in all dilutions was identical. Concentrations of commercial products in this study are presented as the concentration of active ingredient.

24 hr Acute Toxicity Testing

Between two and six rounds of static non-renewal 24 hr acute toxicity assays were conducted for each experimental toxicant. A broad range of concentrations was tested in the initial round followed by progressively narrower ranges in later rounds focusing towards concentrations killing 50% of the crabs. Only one round of toxicity

testing was used with peeler crabs due to their cost, fragility, amount of toxicant needed, and waste disposal. Each round included several concentrations of pesticide as well as aged seawater (ASW) control and a solvent control if a solvent was used in addition to ASW. Test animals in a given round were allocated to their respective containers with a small amount of ASW while pesticide dilutions were made. Crabs were haphazardly taken from all of the available rearing finger bowls to minimize any effects of rearing condition on the toxicity results. The ASW was removed and replaced with toxicant. Test containers were covered to prevent evaporation and the number of dead crabs counted after 24 hrs. No feeding, aeration, or mixing took place during the 24 hr assay. Death was judged by lack of movement of the appendages and antennae in response to shaking the container. Dead juveniles were usually upside down with legs curled.

Individual megalopae were transferred to glass test tubes in groups of 5 so that each pesticide concentration per round had 3 replicates of 5 megalopae. Each test tube contained 10 ml of test solution. Juveniles were tested in cell-culture-treated polystyrene 24-well microplates with one individual per well in 1.5 ml of test solution. One complete 24-well microplate was used per tested concentration. Peelers were housed individually in 0.5 gallon glass pickle jars containing 500 ml of test solution. Three replicate groups of 5 individual peelers were tested at each concentration.

Acute toxicity was modeled as four-parameter log dose-response nonlinear regressions using GraphPad Prism 5 (GraphPad Software, San Diego, CA). To avoid the

logarithm of zero, ASW and solvent controls were entered into models as concentration at least 3 log units below the lowest experimental concentration. This was 0.0001 µg/L for λ-cyhalothrin and Karate, 0.001 µg/L for imidacloprid, Trimax, and aldicarb, and 1 µg/L for Orthene, acephate, and Roundup. Since 100% mortality was reached with all test solutions, model maxima were constrained to a value of 100%. If control mortality was zero for a toxicant then its model was constrained to a minimum of 0%. No lower constraints were placed on models of toxicants with control mortalities greater than zero. LC_{50} values with 95% confidence intervals were calculated from each model. Statistical differences between LC_{50} concentrations of megalopae and juveniles in the same toxicant and between the same ontogenetic stage in the commercial pesticide and its active ingredient were tested using one-way analysis of variance.

Time to Metamorphosis

After completing and modeling the 24 hr acute toxicity assays the concentration modeled to kill 20% of the crabs (LC_{20}) was used as the single concentration to test the effects of pesticides on the time to metamorphosis from the megalopae stage to the J1 stage. Megalopae were collected as above but were used in this assay within 24 hr of collection. Only visually identified intermolt megalopae of a single cohort (Forward et al., 1996) were used in this assay. Thirty intermolt megalopae were used per pesticide treatment and each was housed individually in glass test tubes filled with 2 ml of test

solution. By housing megalopae individually in glass it eliminated any potential effects of plasticizers, cannibalism, or changes in density with removal of juveniles. Test solutions were made using estuarine seawater (ESW) so that any natural chemical cues would be present. All tubes were dosed only once at the beginning of the experiment and kept on an ambient light:dark cycle.

Water was not changed and megalopae were not fed during the experiment. Based on previous research by Forward and colleagues (1996, 2001; R.B. Forward pers. comm.) we hypothesized that pesticides would delay metamorphosis so we therefore used estuarine water to speed up control metamorphosis.

Two separate rounds of time to metamorphosis (TTM) assays were completed. The first round tested ESW control (control 1), 32000 µg/L acephate, 240 µg/L aldicarb, and 4.8 µg/L imidacloprid. The second round tested ESW control (control 2), 0.15 µg/L λ-cyhalothrin, and 5500 µg/L Roundup Pro. Megalopae were checked approximately every 6 hours until all megalopae had either died as megalopae or molted to juveniles. The time period in which any individual died or molted (or both) was recorded. Statistical analyses were performed using GraphPad Prism 5 and done between experimental results and those of their respective control; i.e only within round 1 and within round 2.

Time to metamorphosis data were analyzed using three different methods: ANOVA on the mean TTM (Forward et al., 1994, 1996, 1997); Log-rank Mantal-Cox metamorphosis analysis (*sensu* "survival analysis", similar to Tankersley and Wieber,

2000); and ANOVA of regression-derived ET_{50}. For the first method, all of the times (hr) of metamorphosis for each treatment were averaged together (Forward et al., 1994, 1996, 1997; Wolcott and De Vries, 1994; Brumbaugh and McConaugha, 1995; O'Connor and Judge, 1997; Fitzgerald et al., 1998; Gebauer et al., 1998; O'Connor and Gregg, 1998; Rodriquez and Epifanio, 2000). Only those megalopae that successfully molted to the juvenile stage, whether alive or dead when checked, were included in the calculation of the mean time to metamorphosis. Two one-way ANOVAs (one for each round) with Dunnets post-hoc tests were employed to compare the mean TTM.

We employed a "survival analysis" on metamorphosis as our second method of TTM analysis similar to Tankersley and Wieber (2000). We replaced "death" in the traditional survival analysis with "metamorphosis." This method has the benefit of taking into account megalopae that die during the experiment when calculating the percentage of megalopae or juveniles at each point. Those megalopae that die are censored but included in the analysis. A log-rank Mantel-Cox test (Peto and Peto, 1972) was employed for each round of testing.

The third method of TTM analysis mirrored our acute toxicity analysis. TTM was modeled using a 4-parameter curve similar to the dose-response curve. The percentage of megalopae metamorphosed at each time point, as calculated during the survival analysis (above), was used as the input data. The times modeled to produce 50% molting

for each treatment (ET_{50}: Effective Time 50) were compared using two separate one way ANOVAs with Bonferonni-corrected pairwise post-hoc comparisons.

Fisher's exact tests were used to compare the frequencies of megalopae that molted to J1 for each treatment. Likewise tested were the frequencies of molted juveniles found dead when first checked after molting. These tests were performed using SigmaPlot 11.

Oxidative Stress Biomarkers

The oxidative stress biomarkers glutathione (GSH) and lipid peroxidation (LPO) were measured in intermolt megalopae exposed to LC_{20} levels of each of the active ingredient insecticides and commercial Roundup as in the TTM experiments. GSH was used as a biomarker of antioxidant defense and LPO was used as a biomarker of oxidative damage. A single cohort of intermolt blue crab megalopae was collected by plankton net from the Pivers Island Bridge, NC on 30 September 2009. Megalopae were sorted, visually identified as intermolt, fed *Artemia* nauplii, and kept overnight at room temperature in filtered aged estuarine seawater (ASW; salinity 35) in groups of approximately 300. The following day all of the megalopae were combined and live swimming megalopae were arbitrarily assigned to pesticide treatments in groups of approximately 20 megalopae. Groups of megalopae were transferred to their respective 25 x 150 mm borosilicate culture tubes, associated water removed and 15 ml of

appropriately spiked ASW was added. Culture tubes were covered with parafilm and housed under ambient conditions in a fume hood for 24 hrs.

Following the exposure, each group was examined and the number of megalopae alive and dead was noted. Dead megalopae were removed and only live megalopae were used in further analysis. The remaining live megalopae were split evenly into two groups (one for each GSH and LPO analysis), transferred to 1.5 ml microcentrifuge tubes minus associated transfer water, and frozen at -80°C until processed for analysis. GSH and LPO were analyzed spectrophotometrically following protocols adapted from Ringwood et al. (2003) used elsewhere in this dissertation. Kruskal-Wallis ANOVAs on Ranks with Dunn's pairwise comparisons were performed using SigmaPlot 11 to test for differences in GSH and in LPO among the pesticide treatments.

GSH and LPO were also measured in juvenile and newly adult blue crabs collected from clean water and a ditch draining agricultural land. Biomarkers were measured in the gill, cheli muscle, and hepatopancreas of each crab. The first group of crabs were collected from the Great Ditch which drains Lake Mattamuskeet in Hyde County, NC and connects it the Pamlico Sound. Both Lake Mattamuskeet and the Great Ditch are almost entirely surrounded by and drain agricultural lands growing cotton, corn and soybean (D. Rittschof personal observation). Ten juvenile blue crabs (five male, five female, mean CW = 106.3 mm) were collected on 6 October 2009 using the chicken

necking method. Crabs were placed in individual Tupperware containers and placed on ice during transport back to DUML where they were frozen intact until processing for analysis.

The second and third groups of crabs were collected from the Rachel Carson Estuarine Research Reserve (RCERR), Beaufort, NC. This is a clean site that experiences semi-diurnal tides and fluctuates between oceanic water from Beaufort Inlet and Newport River effluent. The Newport River estuary has a relatively low level of agriculture compared to Lake Mattamuskeet and Hyde County.

Ten juvenile blue crabs (10 female, mean CW = 50.7 mm) were collected from RCERR and reared in the laboratory in ASW for several months. These crabs were fed daily with pieces of fish and housed individually at room temperature. The salinity was lowered from 35 to 2 over one week and kept at salinity 2 for three days before being sacrificed. This was done to closely match the salinity of the Great Ditch and remove salinity as a confounding factor. The third group of crabs were all female (mean CW = 134.5 mm) and were collected from RCERR as peelers. They were reared individually in running ESW (salinity ~30 – 35 PSS) for several days and allowed to molt and mate before being sacrificed and frozen one week thereafter.

No data transformations would make our data normally distributed so non-parametric Kruskal-Wallis ANOVAs on Ranks with Dunn's pairwise comparisons were performed using SigmaPlot 11 within tissues for juvenile crabs. GSH and LPO levels in

megalopae were tested with Kruskal-Wallis ANOVAs on Ranks with Dunn's method of comparing pesticides to ASW control.

Results

Acute Toxicity

Results of 24 hr acute toxicity tests are presented for megalopae and juveniles by commercial insecticide and its active ingredient. Acute toxicity results of peeler crabs are presented separately. All LC_{50} values are reported as the modeled value followed by the 95% confidence interval in parentheses. All concentrations are expressed in µg/L, equivalent to parts per billion (ppb).

Table 4.1. Pesticide properties and acute toxicities to blue crabs.

				Water	24 hr LC_{50} (µg/L = ppb)	
Compound	**Form.**	**Class**	**Type**	**Soluble**	**Megalopae**	**Juveniles**
Karate®	Comm.	Pyrethroid	I	Yes/No	0.526	3.565
λ-Cyhalothrin	A.I.	Pyrethroid	I	No	0.2233	2.701
Trimax Pro®	Comm.	Chloro-nicatinyl	I	Yes	312.7	816.7
Imidacloprid	A.I.	Chloro-nicatinyl	I	Yes	10.04	1112
Aldicarb*	A.I.	Carbamate	I	Yes	311.6	291.1
Orthene®	Comm.	Organophosphate	I	Yes	61210	191300
Acephate	A.I.	Organophosphate	I	Yes	50380	137300
Roundup Pro®*	Comm.	Phosphonoglycine	H	Yes	6279	316000

Form., Formulation; Comm, Commercial; A.I., Active Ingredient; I, Insecticide; H, Herbicide; ppb, parts per billion

*Aldicarb was only tested as AI and Roundup Pro was only tested as a commercial product.

Karate and λ-cyhalothrin

Karate with Zeon technology and its active ingredient λ-cyhalothrin were the most potent of the insecticides tested. LC_{50} values for megalopae exposed to Karate was 0.526 µg/L (95% CI, 0.351 – 0.789 µg/L) and 3.565 µg/L (1.721 – 7.385 µg/L) for juveniles (Fig. 4.2). Treatment with λ-cyhalothrin produced similar results with a megalopa LC_{50} of 0.2233 µg/L (0.1833 – 0.2720 µg/L) and a juvenile LC_{50} of 2.701 µg/L (2.215 – 3.294 µg/L). LC_{50} values for juveniles exposed to λ-cyhalothrin was significantly greater than that for megalopae ($p < 0.0001$, $F_{1,29} = 68.7$). There was no significant difference between Karate and λ-cyhalothrin LC_{50} values for either megalopae ($p = 0.075$, $F_{1,23} = 3.478$) or juveniles ($p = 0.4413$, $F_{1,28} = 0.6101$).

Trimax and Imidacloprid

Trimax and its active ingredient imidacloprid were the next most potent insecticide after Karate and λ-cyhalothrin. LC_{50} values for megalopae exposed to Trimax was 312.7 µg/L (95% CI, 222.4 – 439.9 µg/L) and 816.7 µg/L (692.9 – 962.6 µg/L) for juveniles (Fig. 4.2). Imidacloprid was significantly more toxic than Trimax ($p < 0.0001$, $F_{1,51} = 42.68$) to megalopae with an LC_{50} value 10.04 µg/L (6.381 – 15.79 µg/L). Exposure of juveniles to imidacloprid produced an LC_{50} of 1112 µg/L (841.9 – 1468 µg/L), which was not significantly different than that for Trimax® ($p = 0.0969$, $F_{1,17} = 3.087$). Both Trimax (p

= 0.002, $F_{1,44}$ = 17.07) and imidacloprid ($p < 0.0001$, $F_{1,24}$ = 42.87) were significantly more toxic to megalopae than to juveniles.

Aldicarb

We had difficulty getting pelletized Temik 15G into solution so we only present results for its active ingredient aldicarb. Aldicarb had toxicity to blue crabs similar to the toxicity of Trimax and imidacloprid. LC_{50} concentrations of megalopae and juveniles treated with aldicarb were 311.6 µg/L (281.6 – 344.8 µg/L) and 291.1 µg/L (227.7 – 372.3 µg/L), respectively (Fig.4.2). Aldicarb was the only tested insecticide that showed no significant difference in toxicity between megalopae and juveniles ($p = 0.8554$, $F_{1,55}$ = 0.0335).

Orthene and Acephate

Orthene and its active ingredient acephate were by far the least potent of the insecticides tested. The LC_{50} value for Orthene-exposed megalopae was 61,210 µg/L (48,500 – 77,260 µg/L) between 2 and 4 orders of magnitude less potent than the other insecticides (Fig. 4.2). The LC_{50} of Orthene for blue crab juveniles (191,300 µg/L; 95% CI 141,100 – 259,000 µg/L) was significantly higher than for megalopae ($p = 0.0069$, $F_{1,42}$ = 8.077). The toxicity of acephate to megalopae (LC_{50} 50,380 µg/L; 95% CI 44,300 – 57,300 µg/L) did not differ significantly from that of Orthene ($p = 0.1151$, $F_{1,90}$ = 2.531). Acephate was significantly more toxic to megalopae than to juveniles ($p = 0.004$, $F_{1,64}$ = 8.892) with

a juvenile LC_{50} of 137,300 µg/L (132,800 – 141,900 µg/L). Acephate was significantly more toxic to juveniles than Orthene ($p = 0.0149$, $F_{1,18} = 7.247$).

Roundup

The toxicity of the herbicide Roundup Pro was significantly higher to megalopae than to juveniles ($p < 0.0001$, $F_{1,41} = 48.63$). The LC_{50} value for megalopae exposed to Roundup was 6,279 µg/L (5,937 – 6,640 µg/L) and that for juveniles was 316,000 µg/L (167,000 – 595,200 µg/L) (Fig. 4.2).

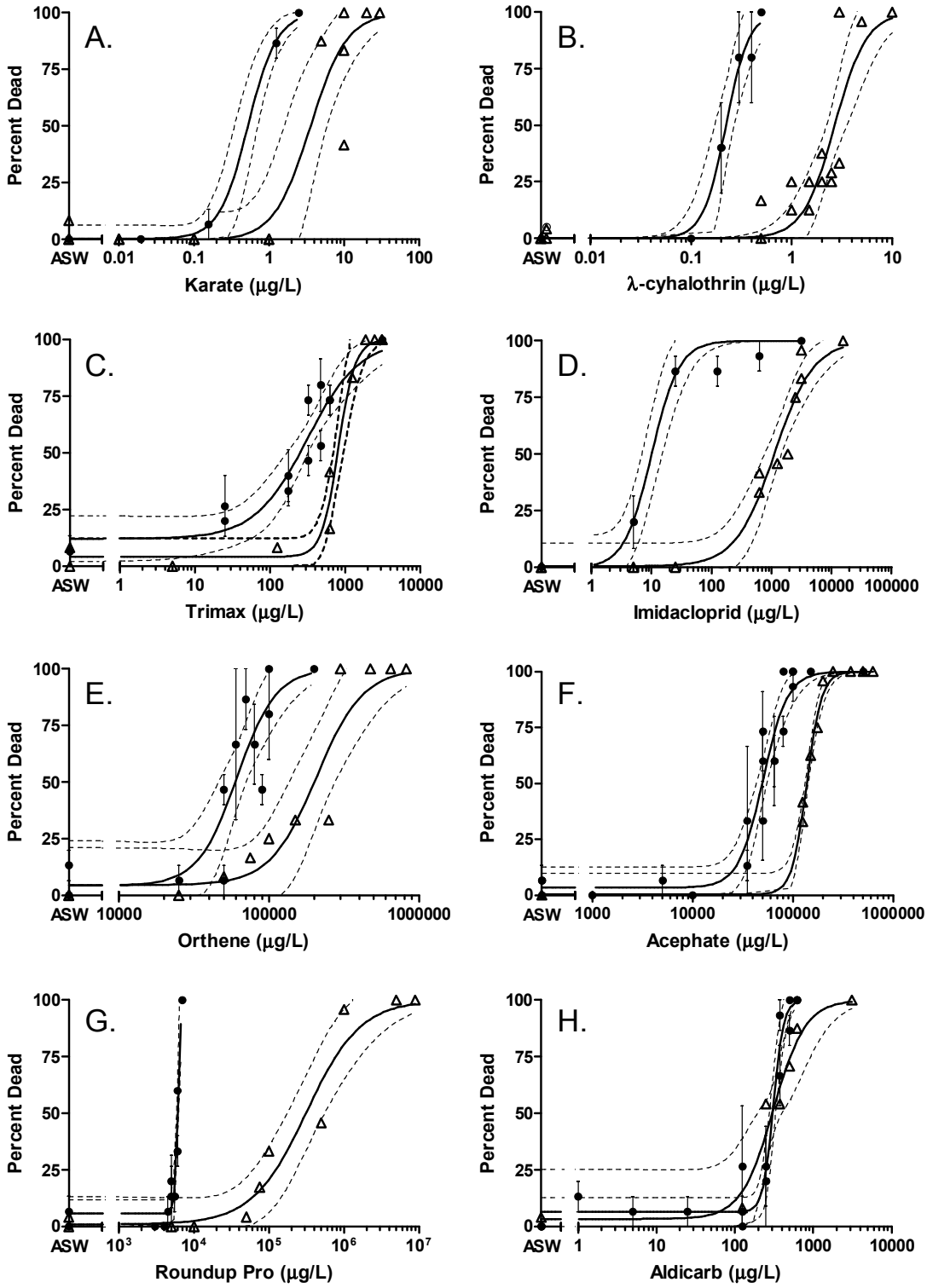

Figure 4.2. Mortality of megalopae (●) and juveniles (Δ) exposed to pesticides. Commercial formulations are presented in the left column and active ingredients in the right. Best fit model (solid line), 95% confidence interval (dotted line).

Peelers

Mortality was surprisingly high among control (both seawater and solvent) treatment crabs with levels of mortality similar to those seen at the highest tested concentrations (Fig. 4.3). No clear trend in the data could be determined and no LC_{50} values could be calculated due to the extremely high control mortality levels, which tended to be similar to mortality levels seen in experimental treatments for that round of testing. No tested concentrations of Orthene killed more than 27% of the crabs even at 1 g/L which made the water milky in appearance. Of 38 total crabs that we noted molted during the experiments, only 2 survived (both at low Orthene levels).

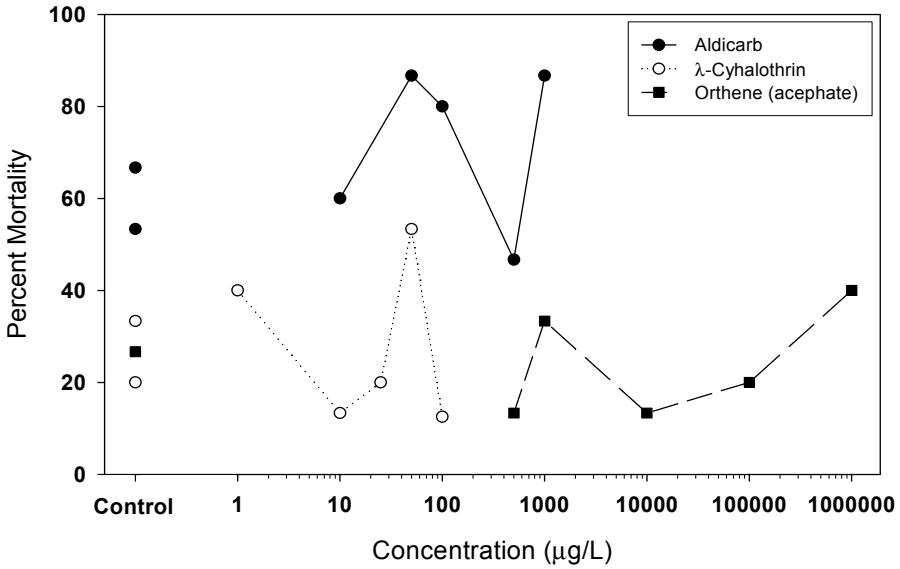

Figure 4.3. Mortality of peeler blue crabs to aldicarb (●), lambda-cyhalothrin (○), and Orthene (■).

Time to Metamorphosis

Roundup was the only treatment that significantly reduced mean TTM ($p < 0.05$, $q = 2.646$) and ET_{50} ($p = 0.002$; Fig. 4.4) but its metamorphosis curve was not significantly different than Controls (Fig. 4.5). Round 1 tested acephate, aldicarb, and imidacloprid. There was no significant difference in mean TTM ($p = 0.978$, $F_{3,85} = 0.0653$; Fig. 4.4), metamorphosis curves ($p = 0.6990$, $\chi^2 = 1.428$, 3 df; Fig. 4.5), or ET_{50}s ($p = 0.091$, $F_{3,35} = 2.265$; Fig. 4.4). Mean TTM ranged from 64.14 hr for aldicarb to 67.13 hr for acephate. ET_{50}s ranged from 55.81 hr for aldicarb to 66.90 hr for acephate.

Round 2 tested λ-cyhalothrin and Roundup. There were significant differences in mean TTM ($p = 0.0165$, $F_{2,68} = 4.362$; Fig.4.4) and ET_{50} ($p = 0.0070$, $F_{2,19} = 6.515$; Fig. 4.4) but no differences among survival curves ($p = 0.3309$, $\chi^2 = 2.212$, 2 df; Fig. 4.5). Mean TTM for round 2 ranged from 63.38 hr for Roundup to 85.03 hr for λ-cyhalothrin. ET_{50} for Roundup and λ-cyhalothrin were 52.06 hr and 81.25 hr, respectively. For both rounds all ET_{50} values were lower (quicker) than their corresponding mean TTM.

Of the 30 intermolt megalopae per treatment 86.2% and 96.7% of control 1 and 2, respectively, molted to juveniles. Only treatment with imidacloprid (56.7%; $p = 0.0204$) and λ-cyhalothrin (58.6%; $p = 0.0004$) resulted in significantly fewer megalopae molting than their respective controls (Fig 4.6a). None of the newly molted juveniles in either control treatment were dead when first checked after molting. Treatment with the three insecticides acephate, aldicarb, and imidacloprid significantly increased the frequency of

juveniles that died after molting compared to control 1 (Fig. 4.6b). Aldicarb proved most deadly to the molting crabs with 91.3% ($p < 0.0001$) of J1s dead when first checked after molting. Treatment with acephate and imidacloprid resulted in 37.5% ($p = 0.0005$) and 41.2% ($p = 0.0007$) of the juveniles dying after metamorphosis, respectively. The commercial herbicide product Roundup Pro ($p = 0.04$) significantly increased mortality after molting compared to control 2.

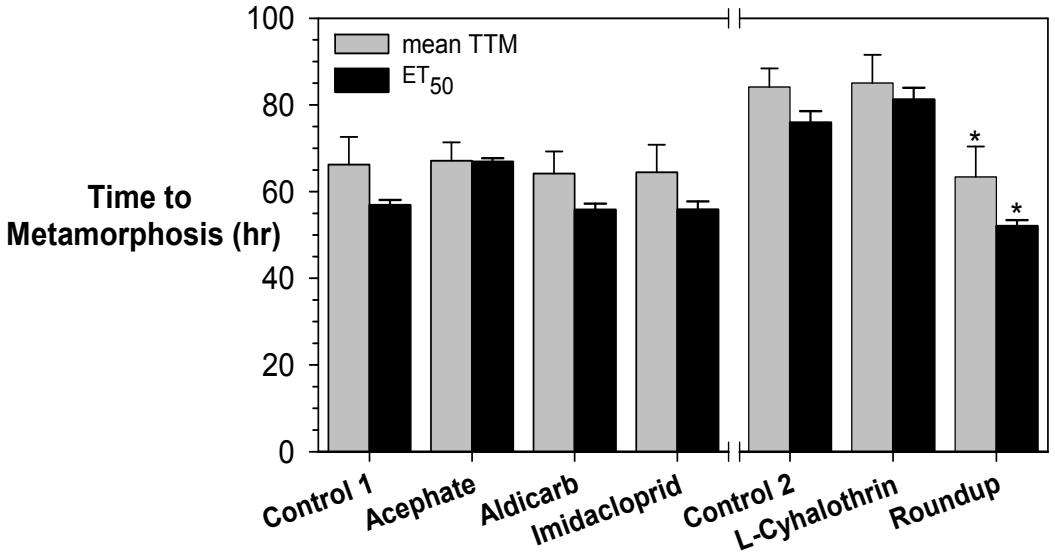

Figure 4.4. Mean time to metamorphosis (± SEM) and modeled ET_{50}. An axis break separates group 1 from group 2 and statistical analyses were only performed within groups. Significance: *p<0.05.

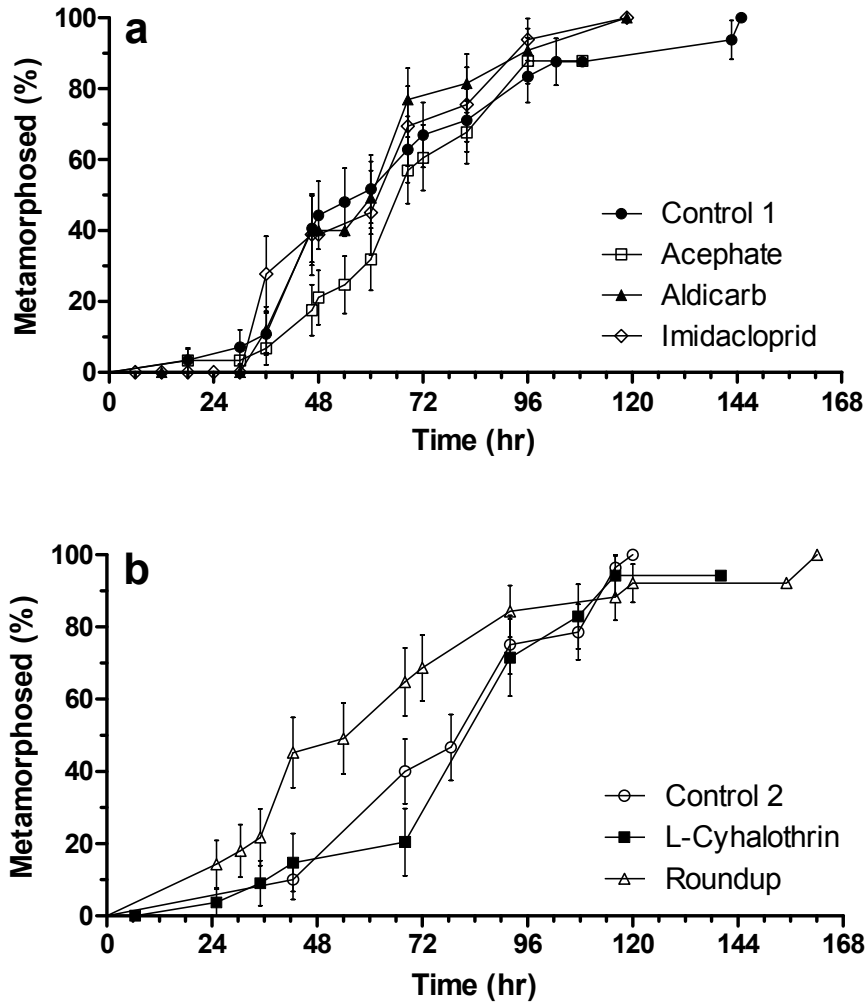

Figure 4.5. Metamorphosis curves (with SEM) for round 1 (a) and round 2 (b). These data have been censored and take into account megalopae that died during the experiment.

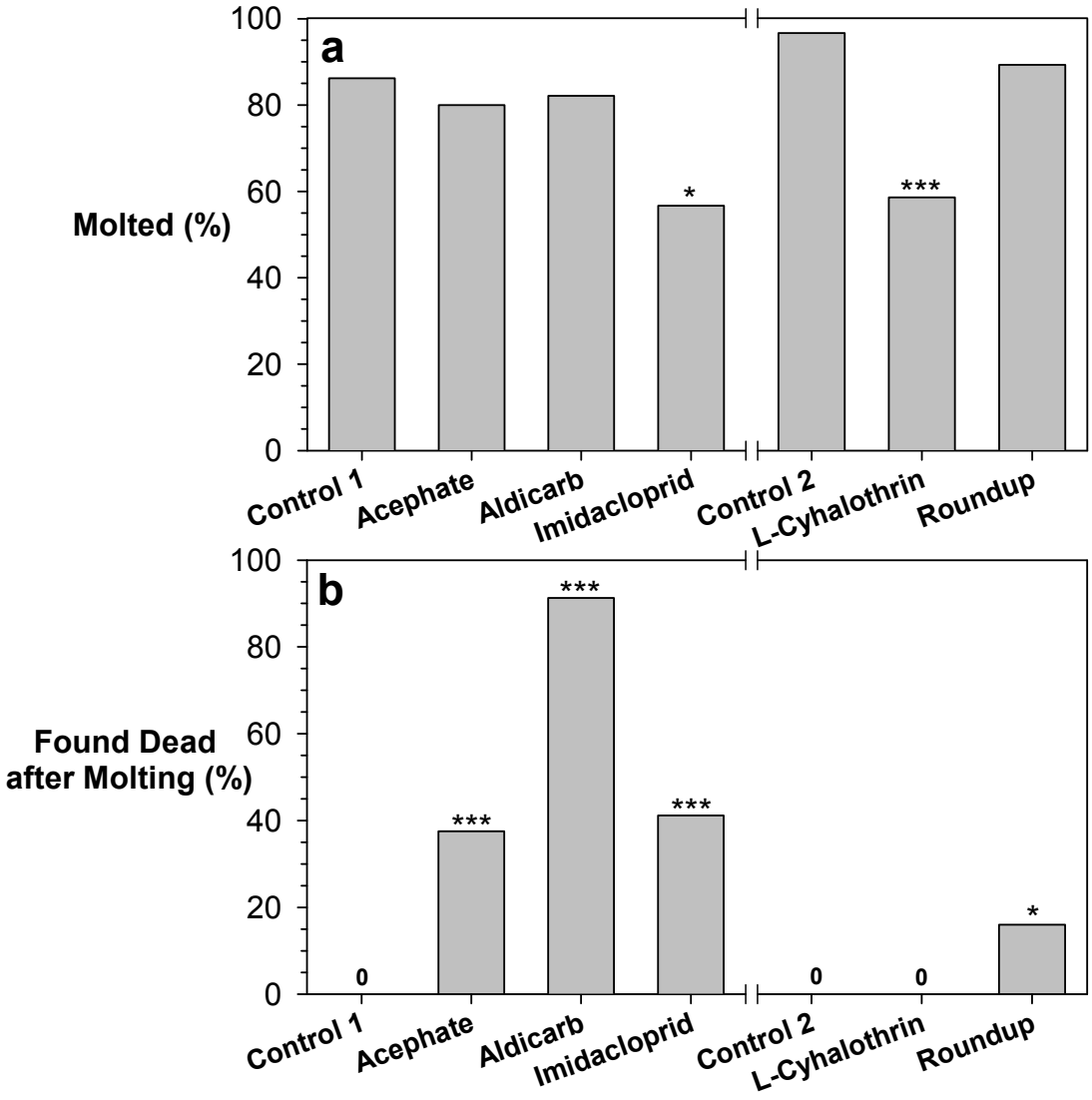

Figure 4.6. Moltings (a), and molting mortality (b) from TTM experiments. Axis breaks separate group 1 from group 2 and statistical analyses were only performed within groups. Significance: *p<0.05, ***p<0.0005.

Oxidative Stress Biomarkers

There were significant differences in total glutathione levels among 24 hr pesticide exposures of megalopae ($p= 0.001$, $H_6= 22.174$) but no differences were significant in post-hoc tests (Fig. 4.7). Exposure to aldicarb produced the highest mean GSH levels (332.674 nmol/g ww) while exposure to acephate produced the lowest (200.115 nmol/g ww) (Fig. 4.7a). Methanol was used a solvent for λ-cyhalothrin. Interestingly treatment with methanol alone produced lower GSH levels than either ASW controls or λ-cyhalothrin (with methanol), although not significantly so ($p> 0.05$).

No significant differences in lipid peroxidation levels were seen $p= 0.568$, $H_6= 4.810$) among megalopae exposed for 24 hr at LC_{20} levels (Fig. 4.7). Exposure to Roundup produced the highest mean level of lipid peroxidation in megalopae (4.186 nmol MDA/mg protein). Methanol exposure produced the lowest levels of LPO with a mean of 1.820 nmol MDA/mg protein, just slightly lower the ASW control levels (2.011 nmol MDA/mg protein). All tested pesticides produced higher mean LPO levels than ASW in megalopae, although none were significantly higher. There was generally an inverse relationship between toxicity to megalopae and LPO levels. The more toxic (lower LC_{50}) a pesticide is to megalopae, the lower the mean LPO level is.

GSH levels in juvenile and newly adult blue crabs were tissue- and site-dependent. Within each site hepatopancreas levels (mean 261.69 to 989.33 nmol/g ww) were always higher than muscle levels (mean 302.38 to 582.17 nmol/g ww) which were

higher than gill levels (mean 55.58 to 176.27 nmol/g ww), although these differences were not statistically tested (Fig. 4.8a). Among the groups, lab-reared crabs had higher tissue-specific GSH levels than Great Ditch crabs and higher than Beaufort-reared crabs (Fig. 3.8a).

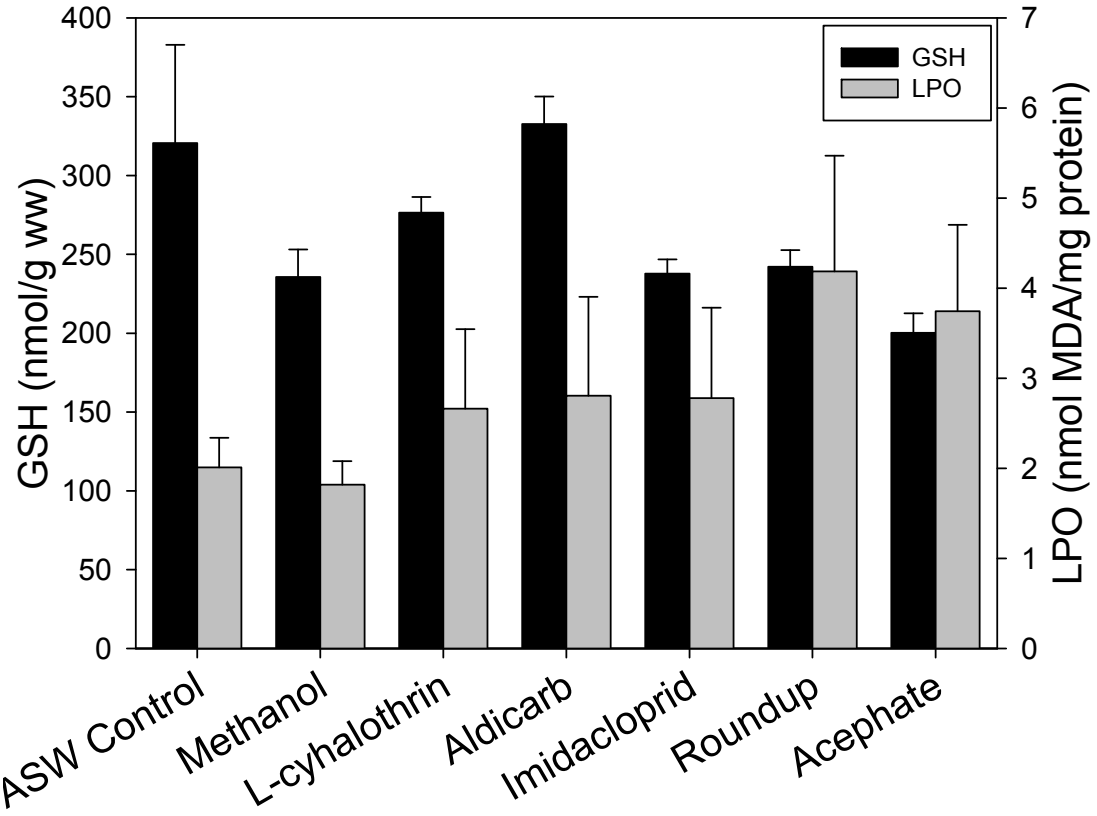

Figure 4.7. Mean (±SEM) total glutathione and lipid peroxidation in whole grouped megalopae exposed for 24 hr.

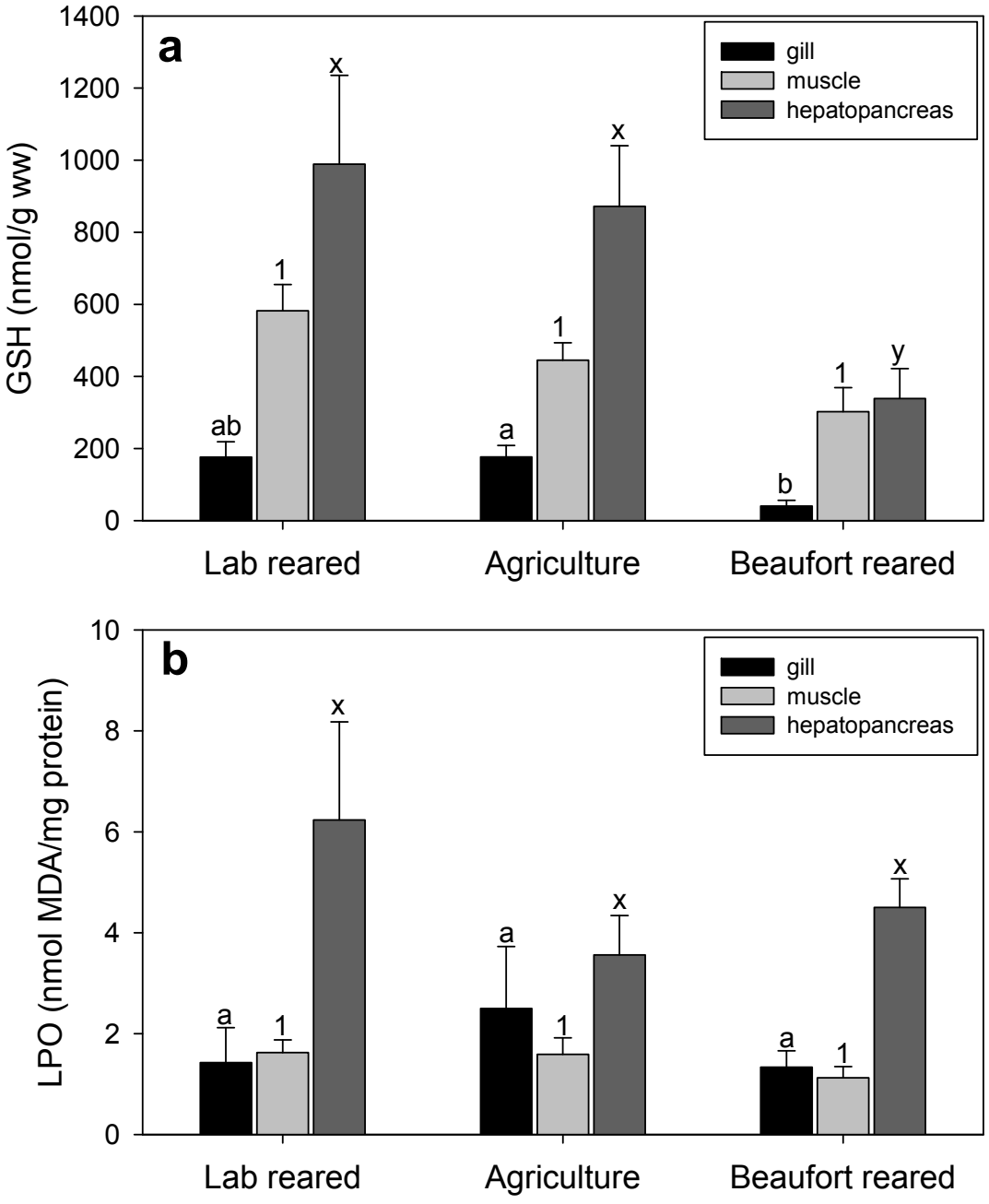

Figure 4.8. Mean (±SEM) Total glutathione (a) and lipid peroxidation (b) of gill, muscle, and hepatopancreas tissue from blue crabs reared in the lab or collected from agricultural canal. Different letter or numbers within each tissue and biomarker represent $p<0.05$.

Hepatopancreas GSH levels from lab reared and Great Ditch crabs were significantly higher than Beaufort reared crabs (Q= 2.769 and Q= 2.642, respectively). There were no significant difference among muscle GSH levels (p= 0.052, H_2= 5.917). Gill tissue from Great Ditch crabs were significantly higher than Beaufort gill (Q= 2.858).

Lipid peroxidation followed a tissue-specific pattern similar to GSH with hepatopancreas levels higher than muscle and gill levels (Fig. 4.8b). There were no significant differences in LPO levels among the sites for gill (p= 0.867, H_2= 0.286), muscle (p= 0.514, H_2= 1.331) or hepatopancreas (p= 0.101, H_2= 4.586).

Discussion

Similar patterns of relative acute toxicity are seen in four active ingredient insecticides to blue crab megalopae and juveniles with the pattern: λ-cyhalothrin > imidacloprid ≈ aldicarb > acephate (Fig. 4.9). All tested pesticides, except aldicarb, were significantly more toxic to megalopae than to juveniles. There was no statistical difference between the aldicarb LC_{50} values for megalopae and juveniles. Foran et al. (1985) reported significantly greater toxicity of aldicarb to juvenile *Daphnia laevis* than adults. Imidacloprid was 100-fold more toxic to blue crab megalopae than to juveniles. The Roundup LC_{50} value for juveniles was ~50-fold higher than for megalopae. Roundup was the least toxic compound to juveniles, 1.65-fold less toxic than the least toxic insecticide Orthene.

There was agreement among the toxicities of the tested commercial formulations and their respective active ingredients. Exceptions to this trend were imidacloprid, which was significantly more toxic to megalopae than Trimax; and acephate, which was significantly more toxic to juveniles than Orthene (Fig. 4.9).

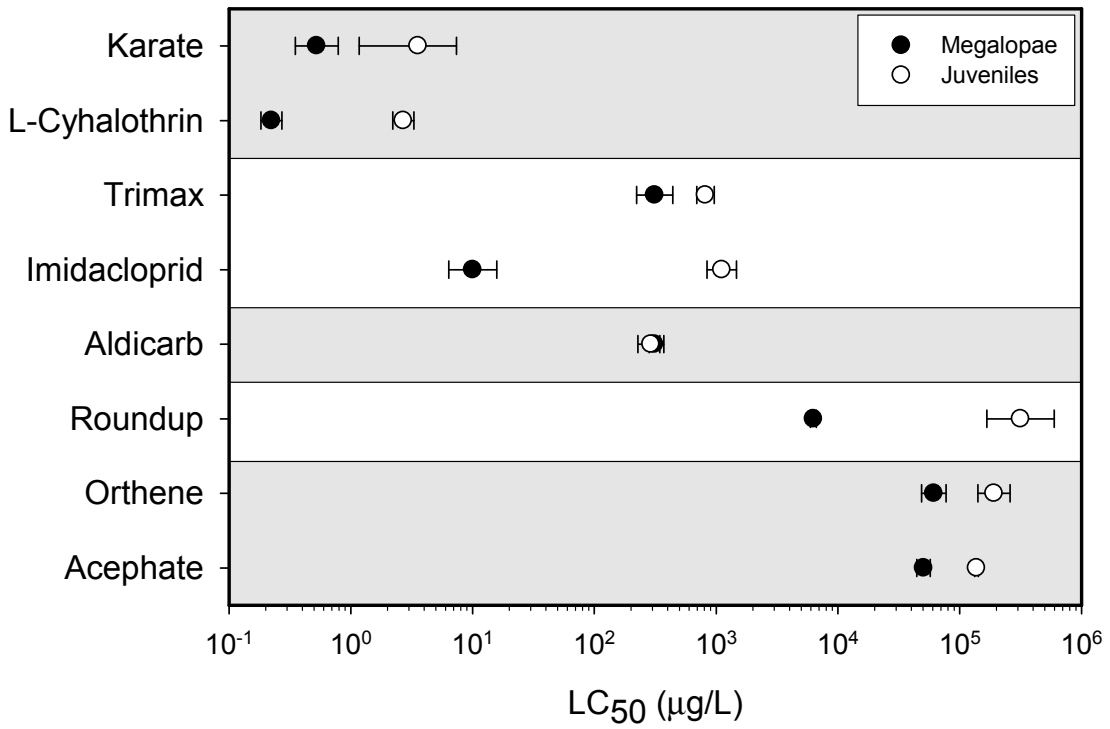

Figure 4.9. LC_{50} (± 95%CI) values for megalopae (●) and juveniles (○). Background shading groups commercial products with their active ingredient.

Blue crab megalopae and juveniles were an order of magnitude less sensitive to acephate and Orthene than the brown shrimp *Penaeus aztecus* (Office of Pesticide Programs [OPP], 2000) or *Gammarus pseudolimnaeus* (Woodward and Mauck, 1980). Acute toxicities of aldicarb to megalopae and juveniles were similar to *Echinogammarus*

tibaldii (96 hr LC_{50}: 220 µg/L; Pantani et al., 1997) and *Daphnia laevis* (Foran et al., 1985) but three orders of magnitude greater than *Artemia salina* brine shrimp (Barahona and Sanchez-Fortun, 1999). Blue crabs showed similar sensitivity to imidacloprid to *Americamysis bahia* mysid shrimp but three orders of magnitude greater than *Daphnia magna* (OPP, 2000). Lambda-cyhalothrin was by far the most acutely toxic compound tested. Mokry and Hoagland (1990) reported 48 hr Karate LC_{50} values for *Daphnia magna* and *Cerrodaphnia dubia* first instars as 1.04 and 0.30 µg/L, respectively. These values match up well with the blue crab megalopae LC_{50} values presented here. Blue crab megalopae were 50 times more sensitive to Roundup than juveniles as well as most other crustaceans including *Uca pugilator* (OPP, 2000), *Hyalella azteca* (Henry et al., 1994), and *Gammarus pseudolimnaeus* (Folmer et al., 1979).

Blue crab megalopae can delay metamorphosis until chemical cues indicating suitable juvenile habitat are sensed (review by Forward et al., 2001). Gebauer et al. (1999) showed that delays of metamorphosis resulted in smaller and later molting crabs thus reducing their fitness. Chemicals such as ammonium and predator odor delay metamorphosis in estuarine crabs, as do extremes in salinity and oxygen concentration. We anticipated that all pesticides would delay metamorphosis in blue crab megalopae on the basis that pesticides would be a toxic stressor in a settlement area and as such tested them in combination with and against estuarine water controls. Estuarine water

contains odors and chemical cues that accelerate metamorphosis in blue crab megalopae (Forward et al., 1994, 1996; Wolcott and De Vries, 1994).

Time to metamorphosis experiments were dosed at the LC_{20} level only at the beginning of the experiment. Since most metamorphosis happened about 60 – 80 hours after dosing, it is likely that the most labile pesticides had broken down sometime before the end of the experiment. Since we used the LC_{20} concentrations, we expected on average 6 out of the 30 initial megalopae in each treatment to die in the first day. Our procedure mimicked a pulsed pesticide application, or pesticide runoff by a short rain event. Traditional methods of TTM analysis using ANOVAs of mean TTM might not be adequate to deal with expected mortality and loss from the experimental system during the experiment. Metamorphosis curve analysis and the ET_{50} modeling take into the account the megalopae that die by including them into the calculation of percentage molted until they die. This perhaps makes these two methods of analysis more suitable for TTM assays with toxic compounds than traditional mean TTM. The inclusion of dead megalopae in the ET_{50} calculation is responsible for making those values lower than their respective mean TTM. Most megalopae mortality occurred at the beginning of experiments and thus any remaining successful molts accounted for an increased percentage change in the percent molted value. This leads to a steeper curve than would be modeled if only the final number of molted juveniles was used in the calculation of percentages. Steeper lines produced lower ET_{50} values.

Roundup was the only tested compound that significantly changed the rate of metamorphosis. The mean TTM and the modeled ET_{50} of Roundup were significantly accelerated compared to Control 2, which was estuarine water. Most other studies of crab metamorphosis used offshore seawater as the negative control. Those studies have not tested any compounds that have been able to significantly accelerate metamorphosis beyond estuarine water (positive control) (Forward et al., 1994).

Roundup was also the only test compound that was as the commercial formulation. This formulation included tallow amine surfactants that are acutely toxic to some invertebrates (Giesy et al., 2000; Howe et al., 2004; Wang et al., 2005; Brausch et al., 2007; Brausch and Smith, 2007). Surfactants have been shown to increase the level and persistence of sweetness of chocolate drinks to humans (Birch and Ogunmoyela, 1980). Those authors suggest two possible modes of action. The first is that surfactants lower surface tension and increases diffusion rates around sensory receptors. The second is that the formation of molecular complexes with surfactants could increase the lipophilicity of natural agents. We suggest that the tallow amine surfactant in Roundup lowers the surface tension around megalopal sensory receptors making it easier for natural chemical cues in the estuarine seawater, such as humic acids, to induce metamorphosis. If natural metamorphic cue molecules complex with the tallow amine surfactants, their lipophilicity could increase making their contact with receptor surfaces more favorable. Humic acids themselves can act as surfactants (Klavins and Purmalis,

2009). Perhaps it is their surfactant nature that makes humic acids reduce TTM in blue crabs. TTM assays should be performed with the Roundup active ingredient, glyphosate, and its tallow amine surfactant alone and in combination to confirm accelerated TTM and determine its mechanism of action.

Treatment with imidacloprid and λ-cyhalothrin resulted in a significantly reduced frequency of molting (Fig. 4.6a). Only 56.67% of imidacloprid and 58.62% of λ-cyhalothrin treated megalopae molted compared to all others above 80%. This might be explained by error in the original toxicity model from which the LC_{20} was calculated. It could also be due to natural variation in the susceptibility of megalopae collected at different times of the year. Both of these pesticides showed similar trends in death and molting. Most of the megalopae that died under both treatments occurred within the first two days and before any megalopae molted. The timing of metamorphosis and death under the other treatments were much more interspersed. Where imidacloprid and λ-cyhalothrin differ is survival of the molted juveniles (Fig. 4.6b). All of the molted juveniles treated with λ-cyhalothrin were found alive while 41.18% of imidacloprid treated juveniles were found dead. Most striking was the case of aldicarb which resulted in a molting rate of 82.14%, but 91.3% of those were found dead as juveniles. It is possible that much of the λ-cyhalothrin had become adsorbed to the glass test tube (Ali and Baugh, 2003) after a couple days and therefore was not bioavailable to affect molting megalopae.

Imidacloprid, λ-cyhalothrin, and aldicarb all affect the nervous system and induce neuron excitation but belong to different chemical classes and operate by different modes of action (PAN, 2008). Imidacloprid has a hydrolysis half life of 30 days and is a chloro-nicotinyl insecticide that mimics acetylcholine and activates post-synaptic neurotransmitter receptors. Lambda-cyhalothrin has a hydrolysis half life of 233 days and is a pyrethroid that binds to voltage gated channels on neuron axons. Aldicarb is a carbamate acetylcholinesterase inhibitor with a hydrolysis half life of 28 days. Only aldicarb is a suspected endocrine disruptor, while the others are unknown. Blue crab molting is controlled by both the nervous and hormonal systems. The ability of aldicarb to affect both systems could be the reason it is so toxic to molting blue crabs. The long hydrolysis half lives of these three suggests that if direct overspray or drift into water bodies could lead to a prolonged period of danger for molting blue crab megalopae and juveniles.

Molting is an especially sensitive time for crustaceans (McCahon and Pascoe, 1988; Mortimer and Connell, 1994; Rebach and French, 1996). The crabs that molted during acute toxicity testing had a much higher rate of mortality than crabs that did not molt (pers. obs.) This makes the megalops and juvenile stages of crustaceans particularly sensitive to environmental toxicants since they molt often. Blue crab megalopae usually molt in submerged vegetation soon after entering the estuaries using tidal stream transport (Forward et al., 1994; Welch et al., 1997). It is the juvenile stages that then

disperse throughout estuaries, into tidal creeks and up-river (Reyns and Eggleston, 2004). Early stage juveniles can molt as often as once a week (personal observation) resulting in increased sensitivity to pesticide toxicity. Molting has been the physiological target of other classes of insecticides but not for the ones tested here. Some insecticides on the market target ecdysone, the hormone that controls molting in arthropods, and consequently slow or stop the molting and metamorphosis of juvenile arthropods (Clare et al., 1992). Intermolt adults were not tested in this experiment but we anticipate that they will show less sensitivity than juveniles to all of the pesticides assayed here. This would continue the trend that successive life stages are less sensitive to toxins and is consistent with developmental assays of blue crab zoeae and megalopae exposed to other pesticides (Bookhout and Costlow, 1975; 1980)

The trend of decreasing sensitivity to toxins with increasing age or ontogenetic stage has been seen in other species. *Daphnia laevis* 1–3 day old juveniles are more sensitive to aldicarb than mature females in 48 hr acute toxicity assays (Foran et al., 1985). The opposite is seen with brine shrimp *Artemia salina*. Barahona and Sanchez-Fortun (1999) report 24 hr aldicarb LC_{50} values decreasing from 500,383 µg/L for 1 day olds to 213,090 µg/L for 2 day olds to 60,122 µg/L in 3 day old shrimp. *Americamysis bahia* sensitivity to Temik shows a similar trend with 1 day old instars less sensitive than juveniles in 96 hr assays. The reasons behind the differences in sensitive trends are unknown.

Many pesticides and pesticide classes are known inducers of oxidative stress by directly producing reactive oxygen species and/or inhibiting antioxidant defenses. Most of the studies of pesticide-induced oxidative stress used mouse, rat, or hepatic cell lines as their subjects; much fewer have used marine invertebrates. Of the pesticides assayed in this study acephate and Roundup showed the highest levels of lipid peroxidation and the most reduction in total glutathione level, although no pesticides showed significant changes from controls in either biomarker. El-Shenawy (2009) reported that injected Roundup at 269.9 mg/kg for two weeks depleted reduced glutathione and increased LPO levels in rat liver tissue. Lushchak et al. (2009) reported suppressed activities of SOD, glutathione S-transferase, and glutathione reductase but no change in LPO levels in brain and liver tissues of goldfish does for 96 hr between 2.5 and 20 mg/L Roundup.

Acephate is an organophosphate, a class of pesticides that has long been shown to effect cellular redox levels. Organophosphates tend to be metabolized by conjugation with glutathione more than are carbamate pesticides, such as aldicarb (Banerjee, 1999). Therefore it makes sense for an organophosphate pesticide to show more of a reduction in total glutathione levels than a carbamate since the glutathione would be conjugated to the organophosphate more than the carbamate. Conjugated glutathione does not participate in redox cycling antioxidant defense and is not measured in the glutathione recycling assay used in this study.

The general inverse relationship between pesticide toxicity and oxidative stress biomarkers might be related to the amount of pesticide used in the oxidative stress experiments. Less toxic pesticides have higher LC_{20}s than more toxic ones. Since all pesticide trials were run at LC_{20} levels, the less toxic pesticides had more of the chemical present.

Most pesticides including herbicides and insecticides are applied as foliar sprays either at ground level by tractor or by plane. Of those tested here only Temik 15G (aldicarb) is used exclusively as a systemic at-planting granular application. Roundup®, Karate, Orthene, and Trimax are applied as foliar sprays. Both ground and aerial spraying is subject to spray drift from on-target application and from direct accidental off-target application. Since shallow tidal drainage creeks lie adjacent to field being sprayed it is nearly impossible to prevent spray drift from landing in them directly. The first significant rain event after application can carry water soluble compounds from acres of residential and agricultural fields into relatively shallow blue crab environments.

Lambda-cyhalothrin is the only tested compound that is not water soluble and so should have a lower risk of run-off. The adsorption and desorption of pesticides with dissolved or particulate organic matter is a complex matter controlled by several physical and chemical factors (Schwarzenbach et al., 1993). None of those factors were explicitly tested in this project. All of the experiments presented here were performed in

sediment free environments with filtered seawater. Pesticides such as λ-cyhalothrin that have low water solubility and strongly adsorb to sediment (Ali and Baugh, 2003) can still impact blue crabs and their prey. It has been shown that the concentration of freely dissolved λ-cyhalothrin in water is the fraction that is toxic (Maund et al., 1998; Hamer et al., 1999; Amweg et al., 2005). The process of adsorption can also inhibit the breakdown of the pesticide, thus prolonging its persistence in the environment (Schwarzenbach et al., 1993). Creek sediments can be repositories of hydrophobic pesticides for years beyond their original application. Blue crab juveniles and adults bury in the sediment to hide and to prevent the growth of fouling organisms on parts of their carapace they can not reach. Buried blue crabs and other benthic infauna are exposed to the pore water, and thus are exposed to low levels for extended periods of time. The prolonged phase of desorption of pesticides into the pore water makes this habitat particularly vulnerable to chronic toxicity and sub-lethal effects. Maul et al. (2008) reported significant reductions in growth of larval *Chironomus tenans* at λ-cyhalothrin concentrations 4.3 times lower than their LC_{50} when exposed in 10 day sediment-water experiments. If blue crabs respond similarly, decreased growth of blue crabs could have significant ecological and reproductive effects. Rasmussen et al. (2008) reported changed ecosystem function and decreased mobility and feeding in *Gamarrus pulex* (amphipods) and *Leuctra nigra* (stone fly) when treated with 0.1 mg/L λ-cyhalothrin in experimental stream channels. Alternatively, Steuckle et al. (2008)

reported no acute toxicity in fiddler crabs *Uca pugnax* exposed to sediment spiked with the pyrethroid permethrin. They did however show changes in hepatopancreas antioxidant enzyme levels. Future studies of λ-cyhalothrin toxicity in blue crabs should focus on sub-lethal effects of chronic low level exposures from spiked sediment.

Assuming a ditch, stream or other body of water near agriculture has vertical sides, it can be modeled simply as a cuboid (3D rectangle). The depth a water body would need to be for a direct overspray of the tested compounds at the 2007 average rate for cotton (NCDA&CS, 2007) to be equal to the juvenile 24 hr LC_{50} is easily calculated. Since application rates are in the units g/m^2, the depth in meters can be calculated by dividing the LC_{50}, converted to units of g/m^3, by the application rate. Lambda-cyhalothrin and Karate are the most potent insecticide assayed and Karate was applied at a rate of 0.028 lbs a.i./A in 2007. A body of water would need to be greater than 87.7 cm (95% CI: 42 – 267 cm) deep for Karate application and greater than 1.15 m (0.95 – 1.41 m) deep for lambda-cyhalothrin application to ensure the concentration remains below juvenile blue crab 24 hr LC_{50}s (Fig. 4.10). This does not take into account any adsorption of lambda-cyhalothrin to suspended particles, which would likely be significant. Leistra et al. (2004) reported 24 – 40% of λ-cyhalothrin remained in the water column in experimental vegetated ditches after 1 day. If this range of adsorption rates is taken into account those experimental ditches would need to be greater than 21.1 to 35.5 cm deep for Karate application and greater than 27.8 to 46.4 cm deep for λ-cyhalothrin

application. Since many ditches and creeks are often less than 20 cm deep (Rittschof personal obs.), many direct oversprays with Karate or λ-cyhalothrin would likely kill over 50% of the blue crab juveniles in the ditch in the first 24 hr. Direct overspray by imidacloprid or Trimax at the manufacturer's recommended 0.046 lbs a.i./A would require a water body deeper than 4.1 cm (3.1 – 5.4 cm) or 5.6 cm (4.7 – 6.6 cm), respectively, to maintain lower than LC_{50} concentrations (Fig. 4.10). These depths are commonly seen in eastern NC, so direct overspray with Trimax or imidacloprid has a good chance to be acutely toxic to blue crabs. Aldicarb is usually applied as a palletized seed treatment so a direct overspray is less likely, but nonetheless would require a ditch 29.1 cm deep to avoid acute toxicity. Overspray with acephate, Orthene, or Roundup at average NC rates would require a ditch less than 1 mm deep to surpass the juvenile LC_{50} and so are unlikely to acutely impact blue crabs by direct overspray.

Figure 4.10 can also be used as a real world proxy of a pesticide's toxic potential. Since the calculations presented in the figure include juvenile LC_{50} values and application rates they help to place the laboratory results in a real world context. Those pesticides with high depth values (Fig. 4.10) are more likely to cause acute toxicity in blue crab juveniles than pesticides with lower depth values. Since LC_{50} values of the tested pesticides ranged over seven orders of magnitude while the application rates ranged over two orders of magnitude, the LC_{50} values dominated the calculations.

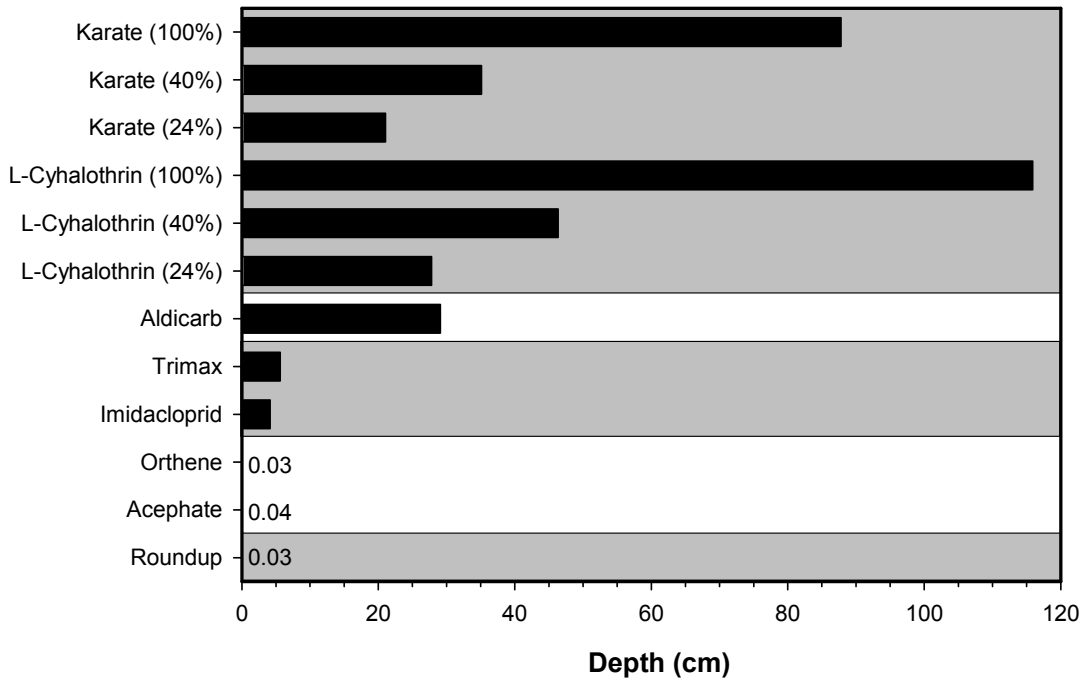

Figure 4.10. Depth a cuboid ditch would need to be so that application rate of each pesticide equals its 24 hr LC_{50} for juvenile blue crabs. Karate and λ-cyhalothrin are listed with 100%, 40%, or 24% free in solution based on Leistra et al. (2004). Shaded regions group commercial product with active ingredient.

Lambda-cyhalothrin- and aldicarb-based pesticides have similar potential toxicities if much of the λ-cyhalothrin is assumed to adsorb to sediment (Leistra et al., 2004). Acephate-based pesticides and Roundup show little potential toxicity to juvenile blue crabs.

Blue crabs can be predators, opportunistic scavengers, as well as detritivores. As such they can be impacted by direct exposure, by eating prey recently killed by pesticides, by sifting through or burying in pesticide-laden sediment, or by starvation

due to lack of prey. The frequency of molting of juvenile blue crab in ditches and creeks could increase their sensitivity above what the LC_{50}s here suggest. Future studies of pesticide interactions with blue crabs should focus on sub-lethal effects of low concentrations of pesticides in blue crabs, the effects of sequential dosing through out a season, and pesticide effects on blue crab prey within and near drainage ditches.

We do not expect the choice of which pesticides or which application method to use will be influenced by this report. Certain actions could reduce the impact of pesticides on commercial crabbing activities. Improved communication of the timing and identity of pesticide application with crabbers and the public could allow for better decision making by the latter to minimize potential impacts to their livelihoods. We recommend blue crab shedders or others with circulating seawater systems not intake water after nearby aerial spraying of these insecticides or after significant rain events within 24 hr of pesticide application. A method of limiting or filtering out particulate matter should help reduce introduction of particle-bound lambda-cyhalothrin.

The problem of agricultural and residential pesticide runoff into ditches, rivers, and estuaries is one that will continue to grow around the world. As pests become resistant to common treatments, new pesticides will be developed and their impacts on the ecologically and economically important estuary ecosystems must be evaluated. The increasing use of crops that are genetically modified for resistance to certain herbicides will promote the increased use and inevitable increased runoff of those herbicides into

estuarine systems. The significant amounts of "inactive" surfactants and adjuvants used in pesticide and herbicide applications might turn out to be more ecologically significant than acute toxicity from the active ingredients.

Conclusions

This investigation was triggered by the experiences of a shedder with high peeler mortality. The timing and location of pesticide application suggests peelers and late stage juvenile blue crabs are the most likely stage to come into contact with pesticides. Peelers proved to be poor test subjects due to high mortality during controls, high price, and large size. We therefore focused on using the ontogenetic stages more amenable to laboratory testing; the megalops and early juvenile stages.

Pesticides were acutely toxic to blue crab megalopae and juveniles in the order of Lambda-cyhalothrin > imidacloprid ≈ aldicarb > acephate ≈ Roundup (glyphosate). Megalopae were almost always more sensitive to pesticides than early stage juveniles. Commercial formations of pesticides generally showed similar toxicity to technical active ingredients alone. Exposure to LC_{20} levels of acephate, aldicarb, imidacloprid and Roundup significantly increased the frequency of juvenile mortality after molting. Roundup significantly reduced the time to metamorphosis as determined by ANOVAs of mean TTM and of modeled ET_{50}. We suggest that the surfactants in the commercial Roundup formulation might have reduced surface tension and increased diffusion rates

of natural metamorphic cues present in the estuarine water in which the megalopae were tested. There was no significant change in total glutathione or lipid peroxidation of exposed megalopae. Lambda-cyhalothrin-, aldicarb-, and imidacloprid-based products have the potential to cause acute toxicity and molting-related mortality in shallow creeks and ditches.

Chapter 5: Summary and Future Directions

This dissertation investigated antioxidant and oxidative stress biomarkers in invertebrates from two natural extreme environments and one anthropogenic extreme environment. The major findings from each chapter are summarized below.

Antioxidant and Oxidative Stress Biomarkers in Vent Organisms

The antioxidant and oxidative stress biomarkers catalase, cytosolic superoxide dismutase (CSOD), total glutathione, and lipid peroxidation were measured in tissues of *Alviniconcha* sp., *Ifremeria nautilei*, *Bathymodiolus brevior* and *Austinograea alayseae* collected from vents sites in the Lau and North Fiji Basins. Species- and tissue-specific biomarker levels were generally similar across chemically distinct vent sites with some exceptions. *Alviniconcha* sp. showed high activities of catalase and CSOD in mantle tissue as well as high total glutathione levels in gill tissue. *Ifremeria* showed high mantle catalase and CSOD like *Alviniconcha* but also showed high CSOD in foot tissue. The catalase tissue pattern of *Bathymodiolus brevior* was the opposite of the two gastropods with low mantle activity and high foot and gill activity. Its CSOD tissue pattern resembled *Alviniconcha* while its total glutathione patterns matched *Ifremeria*. Lipid peroxidation in all three was similar in most tissues. *Austinograea alayseae* catalase activity in all tissues was about 8 fold lower than mollusc activity. All other biomarkers were similar to molluscs.

Catalase and CSOD activities in mollusc gills were about half those in *B. azoricus* from the Mid-Atlantic Ridge (Bebianno et al., 2005), but activities in mantle tissue were similar. Lipid peroxidation, a biomarker of oxidative stress, was at least an order of magnitude higher in Lau and NFB molluscs than *B. azoricus* (Bebianno et al., 2005).

Principal component analysis of all mollusc biomarker measurements grouped individuals by species rather than by site. PCA showed very little overlap among species. The microhabitats from which the specimens within a species were collected could have been very similar across vents, no matter the fluid composition.

Future Directions

The work presented here was hampered by low sample sizes, unbalanced sampling, and lack of associated chemical analysis of low temperature fluid. Measurement of metal concentrations within the tissues used in Chapter 2 will allow for correlational analysis with reported biomarkers. A comprehensive and balanced sampling plan for the three vent mollusc species from three well studied vents within the Ridge2000 Lau Basin bulls eye would allow for more correlations between habitat and oxidative stress.

A return to Mussel Hill to record the extent of the Van Dover et al. (2007) fungal infection is warranted. Determination of whether lipid peroxidation and oxidative stress

is a cause or consequence of the fungal infection of *B. brevior* will help elucidate the vulnerabilities and antioxidant capacity of vent species.

Antioxidant and Oxidative Stress Biomarkers in Seep Mussels

The antioxidant and oxidative stress biomarkers catalase, CSOD, total glutathione, and lipid peroxidation were measured in gill, mantle, and foot tissues of three seep mussels from two seep sites. *Bathymodiolus childressi* and *B. brooksi* were collected from a brine seep at Mississippi Canyon-640 (MC-640) and *B. brooksi* and *B. heckerae* were collected from a non-brine seep at the base of the Alaminos Canyon (AC-645). Data presented here represent the first report of basal antioxidant biomarker levels from three Bathymodiolinae species from Gulf of Mexico seeps.

Biomarker levels in the seep mussels *Bathymodiolus childressi*, *B. brooksi*, and *B. heckerae* were similar across species except for elevated foot and gill cytosolic SOD in mussels from MC-640 compared to those from AC-645. Gill and foot CSOD activity in MC-640 mussels were 3 – 7 fold higher than seep mussels from AC-645, 11 – 23 fold higher than *B. brevior* from Lau and NFB vents, and 2 – 10 fold higher than *B. azoricus* from the Mid-Atlantic Ridge (Bebianno et al., 2005; Company et al., 2006b). All seep and vent mussels consistently showed very low levels of catalase, CSOD, and lipid peroxidation in their mantle tissue. There were no differences in total glutathione levels across all seep and Lau and NFB vent mussels.

The PCA performed on standardized biomarker data of only the three seep species differentiated by species with *B. childressi* isolated from *B. brooksi* and *B. heckerae*. Tight clustering by *B. brooksi* from AC-645 contrasts with the considerably higher degree of scatter by *B. brooksi* from MC-640. Inclusion of biomarker data from the vent mussel *B. brevior* into a new PCA places *B. brevior* individuals clustered around *B. brooksi* from AC-645. *B. childressi* shows considerable variation on component 2 score and is somewhat isolated from the other mussel species. Whether this reflects *B. childressi*'s more distant relationship to the other mussel species or is perhaps due to its lack of a thiotrophic endosymbiont can not be determined.

Future Direction

As mentioned with the vent molluscs biomarker study, increased sample size and habitat characterization would improve this study. Analysis of tissue hydrocarbon and PAH burdens will help deduce the site characteristics.

Acute Toxicity and Sub-lethal Effects of Pesticides to Blue Crabs

Juvenile and adult blue crabs inhabit shallow creeks and ditches that often drain agricultural or residential land that can be treated with pesticides (insecticides and herbicides). Due to the close proximity of this fishery with applied pesticides we tested the 24 hour acute toxicity of four insecticides and one herbicide with blue crab

megalopae and juveniles. It is no surprise that insecticides are very toxic to crabs due to the close relatedness between crustaceans and insects. We tested commercially available formulations, which often include surfactants and other "inactive" (but toxic) ingredients, as well as tested the active ingredient alone.

Blue crab megalopae were always significantly more sensitive to pesticides than juveniles, except for aldicarb to which they showed equal sensitivity. Sensitivity to commercial formulations was usually on the same scale as sensitivity to technical active ingredient. The acute toxicity of active ingredient insecticides to blue crabs followed the order: lambda-cyhalothrin > imidacloprid ≈ aldicarb > acephate. ≈ Roundup. There was a wide range of LC_{50} values from 0.22 µg/L for megalopae exposed to lambda-cyhalothrin to 316,000 µg/L for juveniles exposed to Roundup. Direct overspray of λ-cyhalothrin and aldicarb, especially, could be acutely toxic to crabs in very shallow (<10 in.) ditches. Lambda-cyhalothrin quickly and strongly binds to sediments decreasing the dissolved concentration, but prolonging exposure to sediment-bound and low level pore-water concentrations.

Roundup was the only tested pesticide that significantly changed the time to metamorphosis of blue crab megalopae exposed to LC_{20} levels. Contrary to what was hypothesized, Roundup significantly reduced the time to metamorphosis. The surfactant in Roundup could have reduced the water tension around receptors and increased the ability of natural chemical metamorphosis cues to bind.

Treatment with LC_{20} aldicarb killed 93% of newly metamorphosed juvenile while 42% died when treated with imidacloprid. Molting is an especially vulnerable time for crustaceans. The presence of frequently molting juvenile stage blue crabs in drainage ditches could increase the danger of pesticides beyond what is predicted based on acute toxicity assays.

Acute 24 hr exposure of megalopae to LC_{20} levels of pesticides resulted in increased lipid peroxidation and decreased total glutathione, but none of the differences were significant. Total glutathione and lipid peroxidation levels in adult crabs collected from a drainage creek showed no difference from lab-reared crabs.

Lambda-cyhalothrin-, imidacloprid-, and aldicarb-based products have the potential to cause acute toxicity and/or molting related mortality in shallow creeks and ditches following direct overspray or significant drift. We recommend blue crab shedders or others with circulating seawater systems not intake water after nearby aerial spraying of these insecticides or after significant rain events within 24 hr of pesticide application. A method of limiting or filtering out particulate matter should help reduce introduction of particle-bound lambda-cyhalothrin.

Future Directions

The continued and increasing use of novel pesticides to treat crops in coastal areas will require more laboratory and field work on effects to major fauna. Assessment

of chemical loads in drainage ditches, creaks, and rivers will help illustrate how far downstream certain pesticides are being carried. Lambda-cyhalothrin was the most acutely toxic insecticide tested. Its low water solubility means it should adsorb to sediments. The toxicity of sediment-bound lambda-cyhalothrin to burying blue crabs should be assessed. The mechanism behind the decrease in time to metamorphosis in response to Roundup® exposure should be studied.

Conclusions

Through this dissertation I have explored adaptations to extreme environments. Organisms that have evolved to survive or even thrive under natural "extreme" conditions have recentered their "normal" and therefore no longer live in extreme environments. Those in anthropogenic extreme environments have not had the time to adapt and recenter. By determining the mechanisms used, changed, or developed by these organisms to affect their adaptation we can clarify the forces and processes that structure these ecosystems. At both vents and seeps, principal component analyses group individuals more by species than by site. Species are distributed based upon their physiological adaptations. Similar antioxidant biomarker levels across several sites suggest the oxidative regimes among suitable habitats are comparable. Agricultural drainage ditches have highly episodic input. Neither the ditches nor common inputs induce oxidative stress. Some inputs, however, are acutely lethal to blue crabs.

The addition of agricultural, industrial, and residential chemicals to the world's estuaries, oceans, land, and air will continue to create anthropogenic extreme environments. Understanding how organisms and ecosystems cope with and adapt to these insults is essential to determining the steps needed to mitigate damage. Much has been learned by studying laboratory and field anthropogenic extreme environments. Much can be learned by studying organisms endemic to natural extreme environments.

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Publications

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Osterberg, J.S. The effects of deep-sea sewage dumping on the growth of the urchin *Echinus affinus.* Undergraduate honors thesis. The College of William & Mary, Williamsburg, VA.

Teaching Experience

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2007 DUML McCurdy Derrickson Fellowship. ($2,574)

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2004 NCSG, (D. Rittschof, PI; written and partially performed by JSO). "Ontogenetic Changes in Attraction of Blue Crab Megalopae and Juveniles to Chemical Cues Associated with Habitat." ($27,062)
2004 DUML Robert Safrit Fellowship. ($4,244)
2003 NCSG, (D. Rittschof, PI; written and performed by JSO). "Fishing Baits from Poultry Production Wastes." ($28,957)
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Awards

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