MAGNITUDE OF THE OXIDATIVE STRESS RESPONSE INFLUENCES SPECIES DISTRIBUTIONS

By

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MAGNITUDE OF THE OXIDATIVE STRESS RESPONSE INFLUENCES SPECIES DISTRIBUTIONS

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Animals must employ diverse physiological strategies to survive in aquatic habitats with extreme or fluctuating abiotic factors. Whether these physiological strategies include the oxidative stress response, and whether ability to maintain an oxidative stress response influences species distribution, is not fully understood. In the research projects described in this dissertation, I take three approaches to addressing this topic. I first test whether abiotic factors typical of aquatic habitats cause a cellular stress response consistent with free radical production in the marine bivalve *Donax variabilis*. These results demonstrate that exposure to hydrogen sulfide causes an oxidative stress response in a non-sulfide-adapted bivalve and highlight the importance of examining seasonal variation in stress physiology. I next test whether organisms can mitigate the cellular-level damage associated with exposure to single or multiple stressful abiotic factors. This study was conducted in the estuarine bivalve, *Mercenaria mercenaria.* These results demonstrate that physiological strategies at higher levels of organization buffer the need for a cellular-level oxidative stress response in a stress-tolerant organism. Finally, I test whether the capacity to produce an oxidative stress response affects species distribution. This combined field and laboratory study of the freshwater bivalve *Sphaerium* sp. demonstrates that individuals in a population that overlies an environmental gradient show a variation in their ability to maintain a

cellular stress response that reflects position along the gradient. These three interrelated studies demonstrate that abiotic factors found in aquatic habitats do cause oxidative stress and that ability to respond to the abiotic factors correlates with distribution.

CHAPTER 1
INTRODUCTION

> Much of the work that is done under the name of ecology is not ecology at all, but either pure physiology – *i.e.* finding out how animals work internally – or pure geology or meteorology…In solving ecological problems we are concerned with *what animals do* in their capacity as whole, living animals…We have next to study the circumstances under which they do these things, and, most important of all, the limiting factors which prevent them from doing certain other things. By solving these questions it is possible to discover the reasons for the distribution and numbers of different animals in nature. (Elton, 1927, p. 33-34)

The examination of how physical attributes of the environment interact with physiology to influence the distribution of organisms was an early goal of ecology, as described above in the introduction to one of the first animal ecology textbooks. During the development of the field of ecology in the first half of the twentieth century, many investigators used physiological techniques to address fundamental ecological topics including "the distribution and abundance of organisms" (Spicer and Gaston, 1999; McNab, 2002). Decades later, the two fields split and 'ecologists' began focusing on biotic interactions and demographic processes as the causative agents of distribution and abundance patterns (Andrewartha and Birch, 1984), while 'physiologists' focused on mechanisms occurring at lower levels of biological organization (Hochachka and Somero, 2002). The relatively recent re-integration of these two approaches in the field of ecological physiology utilizes newly developed molecular and biochemical tools to examine how environmental variables limit reproduction and survival and therefore impact distribution and abundance (Spicer and Gaston, 1999; Chown and Storey, 2006).

The set of environmental variables that limits reproduction and survival of a species is the species' niche (Brown et al., 1996). Traditionally, studies that have attempted to define a species' niche involved either *in situ* manipulations combining biotic interactions with environmental variables (exemplified by Connell, 1961) or laboratory studies of physiological responses to abiotic factors. Unlike *in situ* manipulations, which allow the experimental subjects to be

impacted by all aspects of a complex environment, laboratory studies typically involve acclimation to one select environmental variable. However, the limitations of extrapolating laboratory studies of single variables to population-level processes have long been identified (e.g., Hall, 1964), including the tendency of laboratory studies to over-estimate physiological tolerances (Nordlie, 2006). What is most likely to be successful, therefore, is a combined field and laboratory approach that utilizes biochemical and molecular tools to investigate biotic interactions and species distributions.

A combined field/laboratory approach has been successfully applied to the study of species distributions and physiological adaptations of invertebrates in rocky intertidal habitats along the Pacific coast of the United States. This habitat has well-characterized communities and steep gradients of multiple abiotic factors that exhibit diel and seasonal patterns. It has become a model system for ecological physiology, and has been the setting for much of the recent progress in linking population distribution with cellular-level physiology. For example, latitudinal and vertical zonation patterns of snails, mussels, and crabs reflect the thermal tolerances of the individual species, which themselves rely upon a complex web of responses including gene expression, protein repair and degradation processes, mitochondrial energetics, and heart function (for review, Hofmann et al., 2002; Somero, 2002; Stillman, 2002; Tomanek, 2002). These physiological parameters and the resultant patterns in mortality, in turn, influence such biotic processes as predation (Dahlhoff et al., 2001) and competition (Menge and Sutherland, 1987; Menge and Olson, 1990).

Most studies that could be termed ‘ecological physiology’ or ‘comparative physiology,’ as exemplified by the work noted above in rocky intertidal habitats, take a common approach to understanding how organisms respond to a given abiotic factor. Many of these studies are built

upon the assumption that the abiotic factor imposes a stress on the organism, resulting in a perturbation to homeostasis at either the cellular or organismic level (Hoffmann and Parsons, 1991). Suites of physiological responses to this abiotic factor are assumed to be energetically costly, and if the cost of maintenance of these responses grows too high, the animal cannot survive (Parsons, 1991).

To understand the effects of an abiotic factor, therefore, the optimal study organism is typically considered to be one that tolerates extremes of the abiotic factor (Hoffmann and Parsons, 1991; Spicer and Gaston, 1999), because these organisms are most likely to employ protective physiological strategies that can be detected in laboratory studies. However, the usefulness of such organisms may be limited when the goal is to understand the mechanisms by which the stressor exerts its effects, since these same protective physiological strategies will minimize the stressor's impact. In such cases, an alternate and under-utilized approach is to study the effects of an abiotic factor on an organism that is not adapted to the factor. In this 'vulnerable' or 'naive' organism one could be more successful at detecting the consequences of the abiotic factor in the absence of protective responses. Both of these approaches, the study of stress-tolerant and of 'vulnerable' organisms, are employed in the work described in this dissertation, which addresses how abiotic factors in aquatic habitats affect the physiology of bivalves from coastal and inland aquatic habitats.

Abiotic Factors in Aquatic Habitats and Oxidative Damage

Coastal and inland aquatic habitats vary widely in their characterization as stressful or benign. Potentially stressful abiotic factors in aquatic habitats include low dissolved O_2 availability (termed hypoxia), elevated O_2 availability (termed hyperoxia), hydrogen sulfide, thermal extremes, and salinity fluctuations. These factors can occur singly, but more often are found in combination, including the widespread combination of low dissolved O_2 and hydrogen

sulfide in marine sediments (Fenchel and Riedl, 1970) and the combination of high temperature, hypoxia, and salinity extremes in estuaries in summertime (Millie et al., 2004; Caccia and Boyer, 2005).

Free radicals have been suggested as a mechanism by which many abiotic factors, including hypoxia, hyperoxia, hydrogen sulfide, high temperature, and salinity extremes, cause cellular damage and thus influence the distribution of marine invertebrates in extreme or otherwise stressful habitats. Free radicals are atoms or molecules that contain one or more unpaired electrons and are therefore highly reactive (Halliwell and Gutteridge, 1999). During aerobic metabolism, the mitochondria of nearly all eukaryotic cells convert 0.1% (Fridovich, 2004) to 3% (Boveris and Chance, 1973) of O_2 into free radicals such as superoxide. This endogenous free radical production can be increased by many environmental stressors, particularly fluctuations in O_2 availability (Boveris and Chance, 1973; Li and Jackson, 2002). Free radicals spontaneously react in a number of ways, one of which is to strip electrons from cellular macromolecules, particularly proteins, lipids, and nucleic acids, causing oxidative damage. For example, oxidative damage from free radical attacks on nucleic acids include strand breaks and oxidation of nitrogenous bases (Shigenaga et al., 1989; Evans and Cooke, 2004). If free radical production overwhelms a cell's ability to detoxify the free radicals, oxidative damage occurs (Halliwell and Gutteridge, 1999). Cellular oxidative damage, if not repaired, leads to cell death, disease, and aging (Halliwell and Gutteridge, 1999).

To minimize oxidative damage, eukaryotic cells use a variety of protective mechanisms, including the expression of stress proteins. These proteins fall into three general categories: (1) *antioxidants* such as manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/Zn SOD), glutathione, glutathione peroxidase (GPx) and catalase, that convert

free radicals into less toxic or nontoxic forms; (2) *chaperone proteins* such as ubiquitin, the heat shock proteins (e.g., Hsp60, Hsp70 and Grp75) and small heat shock protein (sHsp), that aid the folding or removal of damaged proteins; and (3) *oxidative repair enzymes,* such as OGG1, that repair oxidatively damaged DNA (Halliwell and Gutteridge, 1999).

Hypoxia

Conditions of low dissolved O_2 are widespread and occur naturally in aquatic habitats, particularly in coastal areas affected by upwelling, rock pools in the intertidal zone, all marine sediments, and in standing freshwater bodies (Grieshaber et al., 1994; Diaz and Rosenberg, 1995). Hypoxia results from a variety of processes, including elevated water temperature, low mixing, dense animal populations, and high organic decomposition (Grieshaber et al., 1994). The importance of maintaining adequate access to O_2 centers on its function as the final electron acceptor in the mitochondrial electron transport chain. In the absence of O_2, mitochondrial production of ATP via oxidative phosphorylation ceases, and cells must rely upon the reduced ATP production available from glycolysis and other pathways of anaerobic metabolism (for review of pathways in invertebrates, see Grieshaber et al., 1994). Hypoxia affects a wide variety of ecological processes in aquatic organisms, including recruitment (Marinelli and Woodin, 2002), distribution (Rosenberger and Chapman, 2000), seasonal migration along a vertical gradient (Pihl et al., 1991), predator-prey interactions (Breitburg et al., 1997), the use of refuges (Chapman et al., 2002), and larval settlement and growth rates (Baker and Mann, 1992). Most of the well-studied instances of hypoxia tolerance are found in the invertebrates, particularly the molluscs, annelids, nematodes, and platyhelminths (Hochachka and Somero, 2002).

Whether hypoxia can induce free radical production directly, or whether reoxygenation following a period of hypoxia is necessary for free radical production, is not yet understood (Kukreja and Janin, 1997; Hermes-Lima et al., 1998; Halliwell and Gutteridge, 1999; Semenza,

2000; Hermes-Lima and Zenteno-Savin, 2002; Li and Jackson, 2002). During hypoxia, the absence of O_2 as the final electron acceptor causes accumulation of electrons in mitochondrial electron transport chains (i.e., the chains are reduced). A sudden return of O_2 can cause the production of superoxide due to nearly instantaneous reactions between O_2 and the free electrons that accumulated in proteins of the electron transport chain (Du et al., 1998; Li and Jackson, 2002). Such a scenario might occur during tidal ebb and flow for intertidal animals. However, several recent studies have also shown free radical production during hypoxia without subsequent reoxygenation. The evidence for heightened free radical production during hypoxia comes from direct measurement of free radicals (Vanden Hoek et al., 1997; Becker et al., 1999) and measurement of oxidative DNA damage in mammalian cells (Englander et al., 1999), and changes in antioxidant expression and/or activity in goldfish (Lushchak et al., 2001) and an estuarine crab (de Oliveira et al., 2005). However, several studies of both vertebrates and invertebrates have noted decreases or a lack of changes in antioxidant expression or activity during hypoxia that are consistent with an overall metabolic depression during hypoxia (Hass and Massaro, 1988; Willmore and Storey, 1997; Joanisse and Storey, 1998; Larade and Storey, 2002).

Hyperoxia

While less common in marine habitats than hypoxic conditions, hyperoxic conditions include rocky intertidal pools with photosynthetically active algae (Truchot and Duhamel-Jouve, 1980), boundary layers of intertidal seaweed (Irwin and Davenport, 2002) and brown algae (Pohn et al., 2001), the cold seawater of polar regions (Viarengo et al., 1995; Abele and Puntarulo, 2004), and within the tissues of some algal-cnidarian symbioses (Dykens et al., 1992; Richier et al., 2003, 2005). The physiological effects of hyperoxia are not well-characterized, but it is evident that hyperoxia causes mitochondrial-induced cellular death (Chandel and Budinger,

2007) and decreased cellular metabolism, which is likely due to the inactivation of the citric acid cycle enzyme aconitase (Gardner et al., 1994). The effects of hyperoxia on biotic interactions have been characterized in only a few systems. For example, cnidarian hosts of intracellular algal symbionts utilize protective strategies to minimize damage from hyperoxic conditions induced by excessive algal photosynthesis during periods of elevated temperature (Dykens et al., 1992; Nii and Muscatine, 1997; Richier et al., 2003).

Elevated cellular O_2 levels induce mitochondrial free radical production (Boveris and Chance, 1973; Akbar et al., 2004) and oxidative damage (Dennog et al., 1999). Exposure to hyperoxia has been linked to elevated antioxidant responses in tissues of both vertebrates (O'Donovan et al., 2002; Cho et al., 2005) and invertebrates (Dykens et al., 1992; Viarengo et al., 1995; Abele and Puntarulo, 2004). However, this relationship is not consistent, as demonstrated by a variety of *in vitro* and *in vivo* studies in invertebrates and vertebrates (Abele et al., 1998b; Dennog et al., 1999; Allen and Balin, 2003; Freiberger et al., 2004).

Hydrogen Sulfide

Hydrogen sulfide (referred to here simply as "sulfide") occurs in a variety of aquatic habitats, including mudflats, mangrove swamps, deep-sea hydrothermal vents, hydrocarbon seeps, and anoxic basins, where animals may be periodically or continuously exposed to sulfide at levels up to 12 mM (for review, Fenchel and Riedl, 1970; Somero et al., 1989; Bagarinao, 1992). Sulfide is a highly reactive toxin, and the H_2S form diffuses freely across respiratory surfaces and therefore cannot be excluded from tissues (Denis and Reed, 1927; Julian and Arp, 1992). Sulfide has several mechanisms of toxicity, including the reversible inhibition of cytochrome *c* oxidase, the final enzyme of the mitochondrial electron transport chain (Lovatt Evans, 1967; Nicholls, 1975), reduction in hemoglobin oxygen affinity (Carrico et al., 1978) and inhibition of approximately 20 enzymes (Bagarinao, 1992). Invertebrates inhabiting sulfidic

environments employ a variety of strategies to detoxify sulfide, of which the most widely demonstrated is the oxidation of sulfide to thiosulfate or other compounds (for review, Lovatt Evans, 1967; Vismann, 1991; Grieshaber and Völkel, 1998). Marine invertebrates that are not found in sulfidic habitats and do not employ sulfide detoxification strategies typically show increased mortality upon exposure to hydrogen sulfide (Grieshaber and Völkel, 1998). A number of interspecific interactions are associated with sulfidic habitats, the best-studied of which is the widespread relationship between invertebrate hosts and chemoautotrophic bacterial symbionts that use the chemical energy from sulfide oxidation to fix carbon dioxide into carbohydrates (Felbeck et al., 1981; Ruby et al., 1981; Cavanaugh, 1983). Additionally, sulfide structures coastal communities (Gamenick et al., 1996) and methane seep communities (Levin et al., 2006) by influencing recruitment and spatial patterns, and sulfide gradients influence food web dynamics in deep sea hydrothermal vent communities (Levesque et al., 2006).

Hydrogen sulfide oxidizes spontaneously in the presence of divalent metals (both dissolved and in metalloenzymes), generating oxygen-centered radicals (likely superoxide) and sulfur-centered radicals in aqueous solutions (Chen and Morris, 1972; Tapley et al., 1999) and in animal tissues (Tapley, 1993; Abele-Oeschger and Oeschger, 1995; Eghbal et al., 2004; Julian et al., 2005). Several studies have specifically addressed the link between oxidative damage and sulfide exposure at the organismal level. For example, antioxidant enzyme activities were proportional to the sulfide tolerances of thiobiotic meiofauna (Morrill et al., 1988). Similarly, MnSOD activity increased in response to sulfide exposure in the chemoautotrophic symbiotic bivalve *Solemya velum* but not in the related nonsymbiotic *Yoldia limatula* (Tapley, 1993). In contrast, a relationship between sulfide tolerance and antioxidant activity was not found in a survey of sulfide-tolerant polychaetes and bivalves (Abele-Oeschger, 1996). A relationship

between sulfide and free radical production also has been investigated at the cellular level. In isolated erythrocytes from the marine polychaete *Glycera dibranchiata*, sulfide exposure causes mitochondrial depolarization, increased cellular oxidative stress, and increased mitochondrial superoxide production (Julian et al., 2005). These studies suggest that increased oxidative stress is an additional mechanism by which sulfide exposure could cause toxicity (Morrill et al., 1988; Abele-Oeschger et al., 1994; Abele-Oeschger, 1996).

Elevated Temperature

The importance of temperature in regulating nearly all physiological processes and the resulting impacts on species distribution and abundance is highlighted by extensive discussions of this topic in recent books describing biochemical adaptations (160 pages, Hochachka and Somero, 2002) and physiological ecology (100 pages, McNab, 2002). Although aquatic organisms encounter extremes of high and low temperature, only consequences of, and adaptations to, high temperature will be addressed in this dissertation. Distribution is tightly linked with tolerance to heat stress across a wide variety of aquatic organisms (Gilchrist, 1995; Pörtner, 2002; Chown and Storey, 2006; Nordlie, 2006). This relationship is dependent upon an array of cellular and biochemical processes, including changes in protein structure, enzyme activity and cell membrane composition (Hochachka and Somero, 2002). For example, in ectothermic organisms, the expression levels and activities of enzymes such as lactate dehydrogenase are temperature adaptive, as exhibited by correlations between environmental temperature and activation energy, and conservation of catalytic rate constants and substrate binding ability, over a broad range of temperatures (but not at upper lethal temperatures; for review, Somero, 2004). Temperatures much higher than the environmental or acclimation temperature of an organism cause damage at the cellular level, including mitochondrial swelling and distortion (Cole and Armour, 1988) and structural damage to proteins and cell membranes

(Hochachka and Somero, 2002). Heat stress and the ability to respond to heat stress affect functional responses including burial ability in bivalves (Savage, 1976), development (Mahroof et al., 2005; McMillan et al., 2005), heart function (Stillman, 2002), and the ability to respond to other abiotic factors such as hypoxia (Chang et al., 2000) and low salinity (Cain, 1973). Ability to respond to thermal stresses may affect a variety of biotic processes in addition to distribution, including recruitment success (Menge, 2000), facultative interspecific interactions (Burnaford, 2004), and resistance to predation (Mesa et al., 2002; Pauwels et al., 2005).

A mechanistic link between exposure to high temperature and free radical production likely results from alterations in mitochondrial respiration, as demonstrated in both vertebrates (Zuo et al., 2000; Zhang et al., 2003; Mujahid et al., 2006) and invertebrates (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). Heat stress affects the ability of organisms to minimize or repair oxidative damage, typically by enhancing this ability by preconditioning the cells, as demonstrated in studies of cardioprotection in vertebrates (Arnaud et al., 2002; Joyeux-Faure et al., 2003).

Salinity

The influence of extremes of salinity on physiological processes, particularly when external salinity is not equivalent to intracellular osmotic concentration, is nearly as pervasive as that of temperature (Hochachka and Somero, 2002). Internal osmotic concentrations of aquatic organisms are typically regulated with extensive arrays of 'compatible osmolytes' such as free amino acids, sugars, polyhydric alcohols, and urea (Yancey et al., 1982; Yancey, 2001). Insufficient cellular-level responses to fluctuating or extreme salinity result in changes in cell volume (Neufeld and Wright, 1996), structural and functional damage to mitochondria (Suresh and Jayaraman, 1983) and lysosomes (Pipe and Moore, 1985; Hauton et al., 1998), decreased ability to regulate O_2 consumption during hypoxia (Hawkins et al., 1987), and decreased ability

to respond to elevated temperatures (Cain, 1973; Hauton et al., 1998; Werner, 2004). Ability to tolerate salinity extremes or fluctuations and the energetic costs associated with osmoregulatory strategies affect a wide variety of ecological processes, including foraging behavior (Webster and Dill, 2007), predation and recruitment (Witman and Grange, 1998), and species distributions (Nordlie, 2006; Lowe et al., 2007).

Several studies of stress protein expression (particularly heat shock protein expression) in estuarine invertebrates exposed to hyposalinity or hyposalinity/high temperature treatments have produced conflicting results (Kultz, 1996; Clark et al., 2000; Werner and Hinton, 2000; Spees et al., 2002; Werner, 2004; Blank et al., 2006). A causative relationship between hypersalinity and free radical production is well-established in plant physiology and biomedical fields such as nephrology and immunology (e.g., Hernández et al., 1993; Qin et al., 1999; Hizoh and Haller, 2002). However, whether hyposalinity is linked to alterations in free radical metabolism and oxidative damage is not understood. A recent study of hyposalinity responses of a marine alga detected an elevation in glutathione but not in antioxidant enzymes such as catalase and superoxide dismutase (Jahnke and White, 2003).

Stress Proteins as Indicators of Organism Health

Several characteristics of stress proteins make them useful for assessing the effects of abiotic factors. Many stress proteins are constitutively expressed at low levels, but this expression is upregulated in response to conditions that result in elevated free radical production (Feder and Hofmann, 1999; Downs et al., 2001a; Kultz, 2005) and even to biotic interactions such as predation (Pauwels et al., 2005). Patterns of expression of multiple stress proteins can indicate the stressor to which the organism is exposed (Downs et al., 2000, 2001a, 2001b, 2002b). However, the interpretation and applicability of stress protein expression is hampered by our poor understanding of the temporal profiles of stress protein induction and how this

induction affects the long-term health of the organism. Very few studies have coupled physiological condition assays with stress protein expression levels (Brown et al., 1995; Hamza-Chaffai et al., 2003; Roméo et al., 2003b), and even these studies treated the condition assays only as indicators of overall health rather than investigating whether the protein expression patterns were accurate predictors of the functional assays and vice versa. Additionally, some stress proteins like Hsp70 have a large but short-term response to a stressor (Tomanek and Sanford, 2003), which limits the practical application of monitoring expression of this protein in the field because timing of sampling is crucial to detecting a stress protein response. Finally, there are conditions under which *down*regulation (rather than upregulation) of stress protein expression indicates physiological stress (Werner and Hinton, 1999), particularly if the organism is stressed to such an extreme that all metabolic processes are decreasing and death is imminent (Bierkens, 2000).

Overview of Dissertation Research

Animals that must employ diverse physiological strategies to survive in habitats with extreme or fluctuating abiotic factors tend to be generalists with broad tolerance ranges (Lynch and Gabriel, 1987). Whether these physiological strategies include the oxidative stress response, and whether animals from benign versus extreme habitats exhibit differences in the magnitude of their oxidative stress responses is not fully understood. As noted above, accumulating evidence suggests that free radical metabolism and oxidative stress are linked to many of the abiotic factors typically faced by invertebrates in coastal and inland aquatic habitats. However, whether the ability of these organisms to respond to oxidative stress influences their tolerance to these abiotic factors, and ultimately influences species distribution, is unknown.

In the research projects described in this dissertation, I address three interrelated questions, each of which is examined in a different bivalve species. In the selection of species for these

projects I followed what is typically termed the August Krogh Principle, which Krogh articulated in his opening address on "The Progress of Physiology" to the Thirteenth International Physiological Congress with the comment, "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh, 1929, p. 202).

In Chapter 2 I test whether a subset of the abiotic factors discussed above triggers an oxidative stress response in the marine bivalve *Donax variabilis*. I examine the effects of 24-hour exposures to hypoxia, hyperoxia, and hydrogen sulfide on the seasonal patterns of survival and stress protein expression in *D. variabilis*. Since these clams do not encounter these abiotic factors in the high-energy sandy beaches they inhabit, these clams are unlikely to possess protective mechanisms that would minimize the impact of the stressor and reduce my ability to detect a cellular-level oxidative stress response. This study therefore follows the alternate approach discussed above: examining a 'vulnerable' species for evidence of oxidative stress resulting from an abiotic factor. Results from Chapter 2 were published in the following paper: J. Joyner-Matos, C.A. Downs, and D. Julian, Increased expression of stress proteins in the surf clam *Donax variabilis* following hydrogen sulfide exposure, *Comparative Biochemistry and Physiology* 145:245-257, 2006.

In Chapter 3 I test whether the ability to initiate an oxidative stress response following exposure to single or multiple abiotic factors correlates with changes in whole-organism, functional, metrics of condition. The effects on the cellular- and organismal-level responses of hypoxia, high temperature, and the combination of high temperature and hyposalinity were examined in the estuarine bivalve *Mercenaria mercenaria*. These clams exemplify the generalist strategy described by Lynch (1987), with demonstrated broad tolerances for extremes of and

fluctuations in temperature, dissolved O_2 level, salinity, and pH that reflect the species' widespread distribution in intertidal and subtidal coastal habitats. With the selection of such a stress-tolerant species, I am able to examine whether physiological strategies at higher levels of organization buffer the need for a cellular-level oxidative stress response or correlate with the cellular-level responses. The results from Chapter 3 are in preparation for submission, likely to the *Journal of Shellfish Research*.

In Chapter 4 I take the tools developed from the two previous chapters, which were in essence purely physiological studies, and in a combined field and laboratory study I address how the capacity to produce an oxidative stress response affects species distribution. This study specifically examines whether the distribution of a freshwater clam over a complex and stable gradient of several abiotic factors is related to the physiological condition and oxidative stress response of the clams. The clams used in this project, *Sphaerium* sp., live in a swamp-river system in Uganda and cannot be classified into either the 'vulnerable' or 'tolerant' categories. They clearly are not vulnerable to abiotic factors like *D. variabilis* since they tolerate a range of pH and dissolved O_2 concentrations; however, it is not known whether these clams can tolerate the extremely broad ranges of multiple abiotic factors in the manner demonstrated by *M. mercenaria*. Therefore, I could not form *a priori* predictions about whether clams experiencing different conditions along the environmental gradient would exhibit differences in their oxidative stress responses or levels of oxidative damage. The results from Chapter 4 are in press as the following manuscript: J. Joyner-Matos, L.J. Chapman, C.A. Downs, T. Hofer, C. Leeuwenburgh, and D. Julian, Stress response of an African freshwater clam along a natural abiotic gradient: Too much oxygen can be a limiting factor in aquatic environments, *Functional Ecology*, 21:344-355, 2007.

CHAPTER 2
INCREASED EXPRESSION OF STRESS PROTEINS IN THE SURF CLAM *Donax variabilis* FOLLOWING HYDROGEN SULFIDE EXPOSURE

Introduction

Abiotic factors in marine habitats include thermal extremes, salinity fluctuations, hypoxia, hyperoxia, and sulfide (sum of H_2S, HS^- and S^{2-}). Such factors may influence species distribution by stressing organisms to their physiological limits (Parsons, 1991). However, the mechanisms by which many abiotic factors cause stress are not completely understood. Free radicals have been suggested as a mechanism by which some abiotic factors, including temperature (Abele et al., 1998a, 2002; Downs et al., 2002a; Heise et al., 2003), hypoxia (Greenway and Storey, 1999), hyperoxia (Dykens et al., 1992; Viarengo et al., 1995), and sulfide (Morrill et al., 1988; Abele-Oeschger et al., 1994, 1996; Tapley et al., 1999), cause cellular damage and thus influence the distribution of marine invertebrates in extreme or otherwise stressful habitats. Free radicals, which are atoms or molecules that contain one or more unpaired electrons (Halliwell and Gutteridge, 1999), cause cellular damage, termed oxidative damage, by stripping electrons from cellular macromolecules.

In the mitochondria of nearly all eukaryotic cells, a fraction of O_2 consumption is converted into the free radical superoxide during aerobic metabolism; estimates range from 0.1% (Fridovich, 2004) to 3% (Boveris and Chance, 1973) of O_2 consumption. To minimize oxidative damage, eukaryotic cells utilize a variety of protective mechanisms, including the expression of an assortment of proteins which, for the purposes of this paper, are collectively referred to as "stress proteins." These fall into three general categories: (1) *antioxidants*, such as manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/Zn SOD), glutathione, glutathione peroxidase (GPx) and catalase, which convert free radicals into less toxic or nontoxic forms; (2) *proteins involved in protein rescue and/or degradation,* such as the heat shock

proteins (e.g., Hsp60, Hsp70 and Grp75) and small heat shock protein (sHsp), and the protein ubiquitin, which aid in the folding or removal of damaged proteins (Downs et al., 2005); and (3) *oxidative repair enzymes,* such as 8-oxoguanine DNA glycosylase (OGG1), which repair oxidatively-damaged DNA (Halliwell and Gutteridge, 1999). Expression of some stress proteins is upregulated in response to conditions that result in elevated free radical production (Downs et al., 2001a, 2001b) and to other environmental stressors such as thermal stresses (Hofmann and Somero, 1995). The measurement of stress protein expression levels has served as a cellular-level indicator of elevated free radical production in marine invertebrates (e.g., Abele and Puntarulo, 2004), and in comparative physiological (e.g., Willmore and Storey, 1997) and biomedical studies (e.g., Magalhães et al., 2005).

The aim of this study was to investigate whether sulfide, as well as hypoxia and hyperoxia, have the potential to stimulate a cellular response consistent with increased oxidative stress in a marine invertebrate. To test this, we exposed the marine clam *Donax variabilis* (the coquina clam) to these abiotic stressors in controlled laboratory conditions and assessed the animals' overall tolerance (i.e., survival), stress protein expression, and lipid peroxidation. Additionally, we examined whether the abiotic stressors affect expression of the cytoskeletal protein actin, which has traditionally been measured as a control for sample protein content, but recently has been shown to decrease in vertebrate cells following exposure to hypoxia, hyperoxia, and free-radical generating toxins (Allani et al., 2004; Brown and Davis, 2005; Cho et al., 2005). We conducted identical experiments in fall and spring to investigate seasonal differences (Hofmann and Somero, 1995; Chapple et al., 1998; Sheehan and Power, 1999). Unlike previous studies of oxidative stress in marine invertebrates, we selected our study species based on the high probability that it does *not* encounter hypoxia, hyperoxia or sulfide in its habitat and therefore

likely does not employ additional protective mechanisms, such as those in invertebrates adapted to hypoxia (Grieshaber et al., 1994) and sulfide (Grieshaber and Völkel, 1998). Such adaptations would be expected to minimize the impact of the stressor, reducing our ability to detect a cellular-level oxidative stress response. *D. variabilis* inhabits sandy beaches with moderate to high waves along the southeastern coast of North America (Mikkelsen, 1981; Ellers, 1995). The clams migrate up and down the beach, following the tidal cycle, generally remaining buried in the upper 4 cm of sand. The wave activity and high porosity of sandy beaches likely maintains the seawater surrounding the clams sulfide-free and at or approaching air-saturation. Therefore, we considered it probable that *D. variabilis* is more vulnerable to these stressors than would be expected of invertebrates from habitats such as tide pools, mudflats or marshes (Grieshaber and Völkel, 1998).

Materials and Methods

Clam collection and maintenance. *Donax variabilis* clams were collected at Crescent Beach, FL (approx. 29.7ºN, 81.2ºW), within 30 minutes of high tide during September 2003 ("fall") and March 2004 ("spring"). Water temperatures at all collections were 28 ± 3ºC. All clams were between 1.0-1.5 cm in length. Immediately after collection, the clams were transported to the University of Florida in aerated seawater in an insulated container (*ca*. 90 minutes transport time).

Exposure of Clams to Abiotic Stressors

Flow-through system. Exposure to abiotic stressors was achieved with a constant-temperature flow-through system that used electronic gas flow controllers to regulate water P_{O_2} and sulfide concentration (Fig. 2-1). Seawater in the system was obtained from the University of Florida Whitney Marine Laboratory (Marineland, FL, USA), and was pretreated with

chloramphenicol (2 mg L^{-1}) to prevent the growth of sulfate reducing bacteria. This pretreatment has been shown to markedly increase survival of marine bivalves in respirometry experiments (De Zwaan et al., 2002). The flow-through system consisted of four channels, with each having an animal chamber designed to contain eight clams. Seawater for each of the four channels was continuously equilibrated with air (normoxia) for channel one, N_2 (hypoxia) for channel two, a mixture of O_2 and air (hyperoxia) for channel three, and a mixture of air and hydrogen sulfide gas (from a compressed tank of 2% H_2S, balance N_2) for channel four. The equilibrated water was pulled through the animal chambers at 2 mL min^{-1}. Further details of the apparatus construction and gas equilibration are provided in the legend to Fig. 2-1. Dissolved O_2 was measured twice daily in each channel using a fluorometric dissolved O_2 probe (FOXY probe; Ocean Optics, Inc., Dunedin, FL, USA). The sulfide concentration in the H_2S-channel was measured twice daily using the methylene blue method (Cline, 1969).

Survival during exposure to stressors. *D. variabilis* were exposed to normoxia, hypoxia, hyperoxia and sulfide in each season (fall and spring), during which survival was assessed. Exposures lasted 4-7 days and were conducted with 8 clams. The clams were checked twice daily for mortality, and were presumed to have died when they did not close their valves when disturbed or when obvious tissue degradation had begun (dead clams were immediately removed from the chambers). Surviving clams and tissues from these experiments were not used for any other experiments.

Exposure to normoxia. In a preliminary experiment in the fall, we tested whether maintenance in the flow-through exposure system under normoxic conditions resulted in changes in expression of Hsp70. This protein has shown sensitivity to a variety of stressors, and therefore was used as a general indicator of stress (Feder and Hofmann, 1999). For this experiment, one

additional channel was added to the flow-through system and all channels contained seawater equilibrated with air. Fifty clams were placed in the system initially, and the experiment continued for 9 days. The clams were not fed. Every day, 1 clam was removed from each channel, and tissues from clams removed at days 1, 3 and 5, as well as tissues from control clams collected and frozen in liquid N_2 at the beach collection site, were prepared, stored and assessed for Hsp70 expression, as described below.

Exposure to stressors. For tissues to be used for determination of the remaining stress proteins, clams were exposed to normoxia, hypoxia, hyperoxia and sulfide for 24 hours in each season (fall and spring). A total of 8 clams were exposed to each stressor in each season. At the end of the exposure, the clams were removed and immediately processed, as described below.

Analyses of Stress Protein Expression

Tissue sample processing. Immediately upon removal from the flow-through system, clams were opened by severing their adductor muscles, and their whole tissues were quickly blotted dry and frozen in liquid N_2, followed by storage at –80º C for further processing. Of the eight clams per treatment, four were processed for stress protein expression analysis, as modified from Downs et al. (2002b), and the remaining four clams per treatment were archived at –80º C. For homogenization, whole tissues, frozen and stored as described above, were individually ground in liquid nitrogen to a fine powder in a mortar and pestle pre-cooled by liquid N_2, and then immediately returned to –80º C storage. For resuspension of these homogenates, a small volume of each individual powdered sample was dissolved in denaturing SDS buffer (50 mmol L^{-1} Tris, 15 mmol L^{-1} EDTA, 2% SDS, 15 mmol L^{-1} DTT, 0.5% DMSO, and 0.01% Halt protease inhibitor cocktail from Pierce Biotechnology, Inc. Rockford, IL, USA; pH 7.8). Each suspension was then vortexed for 30 seconds, incubated at 85º C for 3 minutes, vortexed for 15 seconds, incubated again at 85º C for 3 minutes, vortexed for 15 seconds, and centrifuged at

12,000×*g* for 10 minutes. Total soluble protein concentration of each sample was assayed by the method of Ghosh et al. (1988). Tissues from fall and spring experiments were homogenized and resuspended at the same time. The resuspended samples were then aliquotted, frozen in liquid N_2 and stored at –80º C. Unless noted otherwise, all chemicals were obtained from Sigma Chemical Company and were the highest quality available.

Antibodies. Samples were assayed for stress protein expression using mono-specific, ELISA-grade polyclonal antibodies generated by and donated by EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). The antibodies were raised in rabbits against 8-15 amino-acid polypeptides (conjugated to bovine serum albumin) derived from each target protein sequence of the bivalve *Mya arenaria* (Downs et al., 2002b). The following antibodies were used: Cu/Zn SOD (Cat. # AB-SOD-1516), GPx (Cat. # AB-GPX-1433*)*, MnSOD (Cat. #AB-1976), ubiquitin (Cat. #AB-U100), invertebrate small heat shock protein homologues (sHsp; Cat. #AB-H105), heat shock protein 70 (Hsp70; Cat. #AB-Hsp70-1519), mitochondrial Hsp70 (Grp75; log 3219), Hsp60 (Cat. # AB-H100-IN), OGG1-mito (lot 2916), and 4-hydroxy-2*E*-nonenol-adducted protein (HNE; lot 156). Total actin pool was determined with a polyclonal antibody from Stressgen Bioreagents (Victoria, BC, Canada). Specificity of each antibody was verified by SDS-PAGE and western blotting with goat anti-rabbit, alkaline phosphatase-conjugated secondary antibody (Sigma) and a chemiluminescent reporter system (DuoLux Chemiluminescent/Fluorescent Substrate for Alkaline Phosphatase, Vector Laboratories, Burlingame, CA, USA) on samples from both fall and spring exposures (Downs et al., 2002b). Representative antibody verification results for Cu/Zn SOD, GPx, MnSOD, Hsp70, mitochondrial Hsp70, Hsp60, OGG1-mito and actin are presented in Fig. 2-2. Note that

ubiquitin, sHsp and HNE interact with other proteins and therefore typically form "smears" rather than single bands.

After verification of specificity, stress protein expression for each tissue sample was determined by dry-dotting (Cu/Zn SOD, GPx, MnSOD, ubiquitin, Hsp70, Hsp60, Grp75, HNE and actin) or ELISA (sHsp and OGG1-mito), both as described below. All assays were performed on 16 clams from each season (four per treatment), and tissues from both seasons were assayed at the same time to minimize experimental variation.

Dry-dotting. The following dry-dotting technique was developed for this study. Samples were diluted to 30 ng or 100 ng of total soluble protein (TSP) in 50 µl TBS (50 mmol L^{-1} Tris, 10 mmol L^{-1} NaCl, pH 7.5) and triplicate 1 µL volumes were dotted onto dry nitrocellulose membrane (Fisher Scientific, Fairlawn, NJ, USA) with a multichannel micropipette (0.5-10 µL, Eppendorf, Westbury, NY, USA). On each membrane, an eight-fold serial dilution from one sample was dotted in triplicate to allow subsequent confirmation that sample concentrations were within the linear detection range and semi-quantitative comparisons of samples across membranes (see below). Approximately 48 samples could typically be dotted onto a 7 cm by 11 cm membrane. Once dry, the membranes were subsequently treated identically to a western blot. Specifically, each membrane was blocked in TBS-T (TBS with 0.05% Tween-50) with 5% milk (Carnation instant dry milk) or 5% acid-hydrolyzed casein for 30 minutes and then incubated with primary antibody at 1:10,000 in TBS-T for 1 hour at room temperature. Each membrane was then washed with TBS-T, incubated with secondary antibody at 1:10,000 in TBS-T with 5% milk or 5% casein for 1 hour at room temperature, and finally washed three times with TBS-T followed by TBS and then Tris solution (100 mmol L^{-1}, pH 9.5). Chemiluminescence substrate (as above) was then added as per the manufacturer's instructions and each membrane was

visualized on a GeneGnome Chemiluminescent Detection System (Syngene, Frederick, MD, USA). Images were analyzed using GeneTools application software (Syngene). The serial dilution of one sample on each membrane was used to determine the correlation between concentration and luminescence intensity within a membrane using one site, saturation ligand-binding regression curve fits (performed by SigmaPlot 8.02, Systat Software, Inc., Point Richmond, CA, USA). Dry-dotting of tissues from fall and spring experiments were performed at the same time and on the same piece of membrane; allowing direct comparisons among samples.

ELISA. OGG1-m and sHsp expression levels were determined by ELISA at EnVirtue Biotechnologies, Inc. A Biomek 2000 robotic workstation (Beckman Coulter, Inc., Fullerton, CA, USA) was used to conduct the ELISA assays using 384-well microplates. Samples were assayed in triplicate with intra-specific variation of less than 7.5% for all samples combined for each assay. An eight-point calibrant curve using a protein standard relevant to each antibody was added in sextuplicate for each plate (Downs et al., 2002b). ELISA assays of tissues from fall and spring experiments were performed at the same time and on the same plates, allowing direct comparisons among samples.

Data Analysis and Statistics

All stress protein expression levels were standardized by the standard curves run with each dry-dotting or ELISA assay. Data generated by dry-dotting are expressed as relative units per nanogram of total soluble protein (RU · ng TSP^{-1}). Data generated by ELISA are expressed as fmol · mg TSP^{-1} or fmol · ng TSP^{-1}. The data were not normally distributed, as confirmed by the Shapiro-Wilk W test for non-normality (p value < 0.05 for at least one season for each protein), and therefore they were analyzed by the nonparametric Kruskal-Wallis one-way ANOVA. T values for each ANOVA are listed in Table 2-1. Significantly different groups were then

analyzed using the Conover-Inman *post hoc* comparison test, which is a form of the Fisher's least significant difference (LSD) method performed on ranks (Conover, 1999). This analysis was conducted pairwise for each stress protein and individually for each season. We did not analyze the data with two-way ANOVAs with season and treatment as factors because we expected to find extensive seasonal differences in expression patterns within and among proteins and we considered the samples collected in fall and spring to be truly independent. *P* values less than 0.05 were considered significant and are listed in Table 2-1. All statistical analyses were performed with StatsDirect version 2.4.4 (Cheshire, UK). Stress protein data are presented as scatterplots, with the data symbol signifying the median, and vertical asymmetrical error bars denoting minimum and maximum values.

Results

Experimental Conditions.

The conditions of the four chambers in the flow-through exposure system for experiments with the three environmental stressors were: 1) normoxia, 21.6 ± 1.9 kPa P_{O_2} (mean ± s.d.; n = 2 readings per 24 hour period); 2) hypoxia, 12.3 ± 1.4 kPa P_{O_2}; 3) hyperoxia, 36.6 ± 3.0 kPa P_{O_2}; and 4) sulfide, 98 ± 2.9 μmol l^{-1} total sulfide and 24.0 ± 1.8 kPa P_{O_2}. Average pH in both the gas equilibration chambers and the outflow from the animal chambers for the four treatments was 8.11 ± 0.14 with no significant differences between treatments.

Survival Analysis.

To determine the survival tolerance of *D. variabilis* to each exposure condition in each season, we exposed 8 clams per treatment to normoxia, hypoxia, hyperoxia and sulfide for up to 7 d. Clams collected in the fall showed 100% mortality during hypoxia exposure, with 6 of 8 clams dying on the third day and the remaining 2 clams dying on the fourth day (Fig. 2-3). In the

spring, mortality was also 100%, although the clams survived slightly longer, with 4 of 8 clams dying on the fifth day, 1 clam dying on the sixth day, and the remaining clam dying on the seventh day (Fig. 2-3). Exposure to hyperoxia caused no mortality in the fall (4 d exposure). In the spring, 1 of 8 clams died at day 3 during hyperoxia exposure, with no additional mortality (7 d exposure). Hydrogen sulfide exposure showed similar mortality between seasons; in the fall, 6 of 8 clams died on the second day and the remaining 2 clams died on the third day, whereas in the spring, 1 clam died on the second day, 3 more died on the third day and the remaining 4 clams died on the fourth day. There was no mortality in the normoxia treatments in either season (4 d exposure in fall and 7 d exposure in spring).

Exposure to Normoxia

To confirm that changes in stress protein expression were due to the stressors and not simply a result of the clams being maintained in the flow-through system, we conducted a 9 d normoxia exposure study in the fall. We measured expression levels of Hsp70 in clams from days 1, 3, 5, and compared those to expression in control clams that were collected and frozen at the beach (time 0; see Fig. 2-4). We found no significant changes in Hsp70 expression levels ($p = 0.064$). However, Hsp70 levels were slightly increased in clams sampled at day 5. Based on this, we assume that any changes in stress protein expression in clams from the remaining exposure experiments (which lasted 24 h) are due to the stressor(s) themselves rather than representing an artifact of the clams being maintained in the flow-through system.

Antioxidant Protein, Lipid Peroxidation, and Oxidative Repair Enzyme Expression

To test for evidence that 24 hour exposure to hypoxia, hyperoxia or sulfide induced a cellular-level response consistent with oxidative stress in *D. variabilis* with respect to normoxia exposure, we measured changes in the expression of the antioxidant stress proteins MnSOD,

Cu/Zn SOD and GPx, and the DNA repair enzyme OGG1, as well as the concentration of 4-hydroxy-2*E*-nonenol adducted to protein (HNE), with the results as follows.

MnSOD. MnSOD is typically located in the mitochondrial matrix of eukaryotic cells. It catalyzes the dismutation of superoxide to the less reactive pro-oxidant H_2O_2 (Halliwell and Gutteridge, 1999). The appropriate banding pattern for this antibody is a single dominant band at approximately 25 kD (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2). In clams collected and exposed to stressors in the fall, exposure to hypoxia had no effect on MnSOD expression, whereas exposure to hyperoxia or sulfide resulted in twice the MnSOD expression levels compared to clams exposed to normoxia (Fig. 2-5A, Table 2-1). Clams collected and exposed to these stressors in the spring showed no significant change in MnSOD expression (Fig. 2-5B, Table 2-1).

Cu/Zn SOD. Cu/Zn SOD is primarily located in the cytoplasm but may also be detected in lysosomes, mitochondria, peroxisomes and the nucleus, but the isoform detected in this study is expressed in the cytoplasm. As with MnSOD, Cu/Zn SOD catalyzes the dismutation of superoxide into H_2O_2 (Halliwell and Gutteridge, 1999). The appropriate banding pattern for this antibody is a single dominant band at approximately 19 kD (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2).There were no significant changes in Cu/Zn SOD expression in clams collected and exposed to any stressors in the fall (Fig. 2-5C, Table 2-1), whereas in clams collected and exposed to stressors in the spring, exposure to sulfide caused significantly elevated Cu/Zn SOD (Fig. 2-5D, Table 2-1), Exposure to hypoxia or hyperoxia had no effect.

GPx. GPx is located primarily in the cytoplasm (60-75%) and to a lesser extent in the mitochondria. This selenoprotein catalyzes the reduction of H_2O_2 to water with the concomitant oxidation of reduced glutathione (Halliwell and Gutteridge, 1999). The appropriate banding

pattern for this antibody is several bands at approximately 20 - 35 kD with additional bands from tetramer formation possible in the 70 – 90 kD range (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2). The response of GPx was similar to that of Cu/Zn SOD. There were no significant changes in expression of GPx in clams collected and exposed to any stressors in the fall (Fig. 2-5E, Table 2-1), whereas in clams collected and exposed to stressors in the spring, exposure to sulfide caused significantly elevated GPx (Fig. 2-5F, Table 2-1) in comparison to clams exposed to normoxia. Exposure to hypoxia or hyperoxia had no significant effect.

HNE. HNE-adducted protein is a peroxidation product of polyunsaturated fatty acids and indicates increased oxidative damage to lipids (Halliwell and Gutteridge, 1999). The antibody detects all HNE-adducted proteins and therefore does not produce a distinct banding pattern. There were no significant changes in HNE-adducted protein in clams collected and exposed to stressors in the fall, regardless of the stressor (Fig. 2-5G, Table 2-1). In the spring, clams exposed to sulfide, but not hypoxia or hyperoxia, had significantly lower HNE levels than did clams in normoxia (Fig. 2-5H, Table 2-1).

OGG1-m. OGG1-m is a DNA repair enzyme located in the mitochondria. It catalyzes the removal of the highly mutagenic 8-hydroxyguanine (8-OH-G) lesion (Boiteux and Radicella, 2000), which can be generated by oxidative stress and ionizing radiation and, if not removed, causes GC to TA transversions upon replication (Boiteux and Radicella, 2000). The appropriate banding pattern for this antibody is one or two bands at approximately 38 - 45 kD (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2). Expression levels of OGG1-m were near the lower detection limit in clams collected and exposed to stressors in the fall, regardless of the stressor (Fig. 2-5I, Table 2-1). In contrast, clams collected and exposed to hyperoxia or sulfide,

but not hypoxia, in the spring had significant increases in OGG1-m expression (Fig. 2-5J, Table 2-1).

Protein Rescue and/or Degradation

To determine whether *D. variabilis* exposed to the stressors for 24 hour showed responses characteristic of increased protein denaturation conditions, we measured the expression levels of ubiquitin, small heat shock protein (sHsp), Hsp60, Hsp70 and Grp75.

Ubiquitin. Ubiquitin is a small (76 amino acids), highly conserved protein that is expressed in the nucleus, cytoplasm, and cell membrane of eukaryotic cells. It facilitates the degradation of proteins damaged by oxidation (or by other processes) by attaching to the target proteins and aiding in their transport to the 26S proteasome (Wilkinson, 2000; Pickart, 2001; Schnell and Hicke, 2003; Herrmann et al., 2004). The antibody detects all ubiquitinated proteins and therefore does not produce a distinct banding pattern. In clams collected and exposed to stressors in the fall, hypoxia and hyperoxia had no effect on ubiquitin expression compared to clams exposed to normoxia, whereas clams exposed to sulfide had significantly increased ubiquitin expression (Fig. 2-6A, Table 2-1). In clams collected and exposed to stressors in the spring, ubiquitin expression was not significantly affected (Fig. 2-6B, Table 2-1).

sHsp. sHsp are a group of proteins found in the cytosol, nucleus, and mitochondria (Downs et al., 1999). They bind denatured proteins, preventing irreversible protein aggregation, and participate in the ubiquitin/proteasome system (Parcellier et al., 2005)..The sHsp are involved in protective responses to a wide range of stressors, including oxidative stress, heat shock and environmental toxins (Downs et al., 2001a, 2001b; Basha et al., 2004; Arrigo et al., 2005). Since the antibody detects all sHsp-adducted proteins, as well as testing for the proteins themselves, which form up to five bands ranging from 10 kD to 45 kD, testing for antibody specificity in the manner shown for the other antibodies is not appropriate. Clams collected and

exposed to stressors in the fall did not show any significant differences in sHsp expression (Fig. 2-6C, Table 2-1). However, clams collected and exposed to sulfide in the spring had elevated sHsp expression, whereas exposure to hypoxia and hyperoxia had no effect (Fig. 2-6D, Table 2-1).

Hsp70. Hsp70 family proteins are present in prokaryotes and in most cellular compartments in eukaryotes. They have numerous roles involving chaperone functions, protein degradation (Chapple et al., 2004) and protein folding (Frydman, 2001; Kregel, 2002). The appropriate banding pattern for this antibody is two bands at approximately 70 kD (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2). In clams collected and exposed to stressors in the fall, exposure to hyperoxia caused significantly higher Hsp70 expression compared to clams exposed to normoxia, whereas exposure to hypoxia and sulfide had no effect (Fig. 2-6E, Table 2-1). There were no significant differences in Hsp70 expression among clams collected and exposed to stressors in the spring (Fig. 2-6F, Table 2-1).

Hsp60 and **Grp75.** Hsp60 is expressed in the mitochondria. It aids in the folding of newly-formed proteins under normal physiological condition and refolds damaged proteins during stress (Hartl, 1996; Kregel, 2002). The appropriate banding pattern for Hsp60 is one band at approximately 60 kD (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2). Grp75, which is also known as mitochondrial hsp70, is primarily expressed in the mitochondria. The appropriate banding pattern for Grp75 is one band at approximately 75 kD (EnVirtue Biotechnologies, Inc. product information; Fig. 2-2).It is involved in several processes, including responses to oxidative stress (Mitsumoto et al., 2002), chaperone functions, and intracellular trafficking (Wadhwa et al., 2002).We did not detect any significant differences in Hsp60 (Fig. 2-

6G and H, Table 2-1) or Grp75 (Fig. 2-6I and J, Table 2-1) expression in clams collected and exposed to stressors in either fall or spring.

Cytoskeletal Protein Content

We also measured the total actin pool to assess whether *D. variabilis* exposed to the stressors experienced cellular damage in the form of disruption of the cytoskeleton or changes in actin production, which could indicate a change in metabolic activity. The appropriate banding pattern for this antibody is one band at approximately 43 kD (Stressgen Bioreagents product information; Fig. 2-2).Clams collected and exposed to stressors in the fall showed no significant differences in total actin pool (Fig. 2-7A, Table 2-1). However, clams collected in the spring and exposed to hypoxia and sulfide, but not hyperoxia, had significantly decreased total actin pool (Fig. 2-7B, Table 2-1).

Discussion

Donax variabilis upregulated expression of some antioxidants, proteins involved in protein rescue and/or degradation, and repair enzymes in response to 24 hour exposure to sulfide and, to a much lesser extent, to hyperoxia. We also found elevated levels of the lipid peroxidation endproduct, HNE, in clams exposed to hyperoxia but not to sulfide. However, there was a marked seasonality in the response to stressors, with clams collected and tested in the spring showing greater expression of many stress proteins and significant decreases in HNE-adducted proteins and actin. Finally, we found that hypoxia and sulfide were lethal stressors for the clams, although clams in the spring experiment tolerated the stressors for a longer duration. In a marine invertebrate that likely does not experience sulfide, hypoxia or hyperoxia in its habitat, these results indicate that 1) exposure to sulfide, and probably hyperoxia, induces increased stress protein expression and lipid peroxidation in a pattern consistent with oxidative stress, and 2)

clams in the spring had an increased stress protein response and decreased evidence of injury (decreased HNE and increased survival) compared to clams in the fall.

Abiotic Factors are Linked to Free Radical Production

Hypoxia. Hypoxia is widespread and occurs naturally in marine habitats, particularly in coastal areas affected by upwelling, rock pools in the intertidal zone, and all marine sediments (Grieshaber et al., 1994; Diaz and Rosenberg, 1995). Hypoxia has been linked to increased free radical production, but whether hypoxia can induce free radical production directly, or whether reoxygenation following a period of hypoxia is necessary for free radical production is not yet understood (Kukreja and Janin, 1997; Hermes-Lima et al., 1998; Halliwell and Gutteridge, 1999; Semenza, 2000; Hermes-Lima and Zenteno-Savin, 2002; Li and Jackson, 2002). During hypoxia, the absence of O_2 as the final electron acceptor causes accumulation of electrons in mitochondrial electron transport chains (i.e., the chains are reduced), with the result that a sudden return of O_2 can cause the production of superoxide due to nearly instantaneous reactions between O_2 and the accumulated free electrons (Du et al., 1998; Li and Jackson, 2002). Such a scenario might occur during tidal flow for intertidal animals. However, several recent studies have also shown free radical production during hypoxia without subsequent reoxygenation. These are based on direct measurement of free radicals (Vanden Hoek et al., 1997; Chandel et al., 1998; Becker et al., 1999), measurement of oxidative DNA damage in mammalian cells (Englander et al., 1999) and yeast cells (Dirmeier et al., 2002), and the indirect measures of changes in antioxidant expression and/or activity in goldfish (Lushchak et al., 2001) and an estuarine crab (de Oliveira et al., 2005). Several studies of both vertebrates and invertebrates have noted decreased or unchanged antioxidant expression or activity during hypoxia, consistent with an overall metabolic depression during hypoxia (Hass and Massaro, 1988; Willmore and Storey, 1997; Joanisse and Storey, 1998; Larade and Storey, 2002). In the current study, clams

exposed to hypoxia did not show significant changes in antioxidant protein expression, regardless of the season in which the experiments were performed. Similarly, we did not detect significant changes in proteins involved in expression levels of proteins involved in protein rescue and/or degradation in hypoxia-exposed clams, consistent with some vertebrate studies (Gupta and Knowlton, 2002) but not others (Currie and Boutilier, 2001; Magalhães et al., 2004, 2005).

We detected a significant decrease in total actin expression in *D. variabilis* exposed to hypoxia in the spring but not the fall. A decrease in total actin protein expression is consistent with previous studies of bovine brain endothelial cells exposed to hypoxia (Brown and Davis, 2005) and human cortical neurons exposed to a free radical generating neurotoxin (Allani et al., 2004). Interestingly, although *D. variabilis* exposed to hypoxia did not show evidence of oxidative damage, which could have included alterations in antioxidant or OGG1-m expression or increases in HNE, hypoxia nonetheless constituted a lethal stress in both fall and spring survival experiments. This is consistent with a previous study of hypoxia exposure in the congener *D. serra* (Laudien et al., 2002). Therefore, *D. variabilis* are vulnerable to moderately hypoxic conditions, which they typically do not encounter in their habitat. The absence of a stress protein response consistent with elevated free radical production suggests that oxidative stress does not play a large role in the mechanism of hypoxic death, or that these clams were so severely stressed that they were unable to appropriately respond to hypoxia-induced oxidative stress (Werner and Hinton, 1999).

Hyperoxia. Hyperoxic conditions are present in a variety of marine habitats, including rocky intertidal pools with photosynthetically active algae (Truchot and Duhamel-Jouve, 1980), boundary layers of intertidal seaweed (Irwin and Davenport, 2002) and brown algae (Pohn et al.,

2001), in the cold seawater of polar regions (Viarengo et al., 1995; Abele and Puntarulo, 2004), and within some algal-cnidarian symbioses (Dykens et al., 1992; Richier et al., 2003, 2005). Elevated cellular O_2 levels increase mitochondrial free radical production (Boveris and Chance, 1973; Akbar et al., 2004), oxidative damage (Dennog et al., 1999), and antioxidant responses in tissues of both vertebrates (O'Donovan et al., 2002; Cho et al., 2005) and invertebrates (Viarengo et al., 1995; Abele and Puntarulo, 2004). Nonetheless, hyperoxic exposure is not linked to changes in SOD activity in the polychaete *Heteromastus filiformis* (Abele et al., 1998b) or in humans exposed to hyperbaric oxygen treatment (Dennog et al., 1999).

In the current study, we found a significant increase in MnSOD expression in *D. variabilis* that were exposed to hyperoxia in the fall experiment. We did not detect significant changes in the expression levels of the other two antioxidants, Cu/Zn SOD (Freiberger et al., 2004) and GPx (Allen and Balin, 2003), or the marker of lipid peroxidation (HNE). However, we did detect significant increases in expression of the mitochondrial DNA repair enzyme OGG1-m in hyperoxia-exposed clams from the spring experiment. One possible explanation for this discrepancy is that MnSOD did not blunt the increased mitochondrial free radical production in clams exposed to hyperoxia in the spring, thereby resulting in DNA damage and a consequent stimulation of increased OGG1-m expression.

Among the proteins involved in protein rescue and/or degradation, the only significant change in expression was an increase in Hsp70 expression in *D. variabilis* exposed to hyperoxia in the fall. A link between Hsp70 expression and hyperoxia is well supported by studies utilizing a number of different organisms and cell types (Wong et al., 1998; Dennog et al., 1999; Akbar et al., 2004; Shyu et al., 2004; Cho et al., 2005) and may have contributed to the clams' increased survival in response to hyperoxia. We did not detect an effect of hyperoxia on total actin

expression. This contrasts with studies of cultured mammalian cells, which show that hyperoxia causes decreased actin gene expression (Cho et al., 2005) and that toxin-induced free radical production (although not necessarily hyperoxia) causes decreased actin protein expression but not decreased gene expression (Allani et al., 2004). While the stress protein results in this study present some evidence that exposure to hyperoxia caused a stress response indicative of increased free radical production, the survival experiments showed that exposure to the hyperoxic condition was a sublethal stressor.

Hydrogen sulfide. Animals in a variety of marine habitats, including mudflats, mangrove swamps, deep-sea hydrothermal vents, hydrocarbon seeps and anoxic basins, are periodically or continuously exposed to sulfide at levels up to 12 mmol L^{-1} (Fenchel and Riedl, 1970; Somero et al., 1989; Bagarinao, 1992). Hydrogen sulfide is a highly reactive toxin that diffuses freely across respiratory surfaces and therefore cannot be excluded from tissues (Denis and Reed, 1927; Julian and Arp, 1992). Hydrogen sulfide has several mechanisms of toxicity, including the reversible inhibition of cytochrome *c* oxidase, the final enzyme of the mitochondrial electron transport chain (Lovatt Evans, 1967; Nicholls, 1975), reduction in hemoglobin oxygen affinity (Carrico et al., 1978) and inhibition of approximately 20 enzymes (Bagarinao, 1992). Hydrogen sulfide oxidizes spontaneously in the presence of divalent metals (both dissolved and in metalloenzymes), generating oxygen-centered (likely superoxide) and sulfur-centered radicals in aqueous solutions (Chen and Morris, 1972; Tapley et al., 1999) and in animal tissues (Tapley, 1993; Abele-Oeschger and Oeschger, 1995; Eghbal et al., 2004; Julian et al., 2005). Organisms inhabiting sulfide-rich environments employ a variety of strategies to detoxify sulfide (Lovatt Evans, 1967; Vismann, 1991; Grieshaber and Völkel, 1998). Marine invertebrates that are not found in sulfide-rich habitats and that do not employ sulfide detoxification strategies typically

show increased mortality upon exposure to sulfide, such as can occur in upwelling events (Grieshaber and Völkel, 1998). We found that 0.1 mmol L^{-1} sulfide was a lethal stressor for *D. variabilis* in both fall and spring survival experiments. These results are consistent with a previous study of sulfide tolerance in juvenile *Donax serra*, which documented a LT_{50} of 80 hour with exposure to 0.1 mmol L^{-1} under hypoxic conditions (Laudien et al., 2002).

Of the three stressors we tested, we found the greatest evidence for a stress protein response, consistent with a cellular response to oxidative stress in *D. variabilis* exposed to sulfide, and the response was strongest in the spring experiment. Specifically, clams exposed to sulfide had elevated expression of MnSOD (fall), Cu/Zn SOD (spring), GPx (spring), and OGG1-m (spring). Two of the proteins involved in protein rescue and/or degradation, ubiquitin (fall) and sHsp (spring), also increased in clams exposed to sulfide. In contrast, the lipid peroxidation marker HNE and total actin expression levels were significantly decreased in sulfide-exposed clams in the spring experiment. These results suggest that cellular response of *D. variabilis* to sulfide is consistent with a response to oxidative stress and that the mortality detected in the sulfide exposure treatment in both fall and spring survival experiments could be linked to oxidative stress in *D. variabilis*, which do not normally encounter sulfide.

Several studies have specifically addressed the link between oxidative damage and sulfide exposure at the organismal level. For example, antioxidant enzyme activities were proportional to the sulfide tolerances of thiobiotic meiofauna (Morrill et al., 1988). Similarly, MnSOD activity increased in response to sulfide exposure in the chemoautotrophic symbiotic bivalve *Solemya velum* but not in the related nonsymbiotic *Yoldia limatula* (Tapley, 1993). In contrast, a relationship between sulfide tolerance and antioxidant activity was not found in a survey of sulfide-tolerant polychaetes and bivalves (Abele-Oeschger, 1996). A relationship between

sulfide and free radical production has also been investigated at the cellular level; in isolated erythrocytes from the marine polychaete *Glycera dibranchiata*, 1 hour sulfide exposure causes mitochondrial depolarization, increased superoxide production and increased cellular oxidative stress (Julian et al. 2005). Recently, Eghbal et al (2004) showed that free radical production in rat hepatocytes was two-to-three times faster when the cells were exposed to 0.5 mmol L^{-1} sulfide than when they were exposed to cyanide or control conditions, and that the addition of ROS scavengers decreased cell death by up to 40% in hepatocytes exposed to 0.5 mmol L^{-1} sulfide for 3 hours. These studies, in conjunction with the results of the current study, support the theory that increased oxidative stress is a mechanism by which sulfide exposure causes toxicity in marine animals (Morrill et al., 1988; Tapley, 1993; Abele-Oeschger et al., 1994, 1996; Julian et al., 2005).

Sources of Variance

A number of factors, both in the collection and treatment of the clams as well as in the tissue processing procedure, could have contributed to the variance detected within treatment groups. We employed several methods to minimize the influence of these factors. These included: 1) To control for effects of daily cycles in stress protein production (Podrabsky and Somero, 2004), we collected the clams within 30 minutes of high tide. 2) We did not determine the ages of the individuals, which in mammals is closely linked to both endogenous free radical production (Barja, 2002) and ability to synthesize functional stress proteins (Szczesny et al., 2003), but we did control for shell length. Populations of *D. variabilis* from sites on the eastern Florida coast reach maturity in spring and fall and most individuals live for one year (Mikkelsen, 1985). Given these patterns in abundance and size-frequency, it is likely that the clams sampled in the current study were adult and of similar age. 3) We selected *D. variabilis* as a study species because it inhabits a habitat that lacks extremes of dissolved O_2 or sulfide, which allowed us

minimize the potential complications of preconditioning from exposure to environmental stressors (Kultz, 2005). 4) An additional method to limit preconditioning effects would have been to acclimate the clams in the laboratory prior to the experiment. However, because we found slightly elevated Hsp70 expression levels in clams maintained in normoxic conditions for several days, laboratory acclimation would likely have introduced additional variables. Given the short survival times demonstrated in the survival experiments and the potential confounding effect of starvation (Morales et al., 2004), we exposed the clams to the stressors for only 24 hours. 5) To minimize the effects of daily variation in tissue processing methodology, we homogenized and suspended all samples on the same day and with the same batch of buffer solution. 6) Stress protein expression levels were determined for all samples at the same time and on the same piece of membrane to minimize inter-membrane staining differences.

We found that variance in the stress treatment groups was elevated in comparison to variance in the normoxia samples in all stress proteins that had significant changes (excluding actin). This relationship between stressful treatments and elevated variance has been documented in ecotoxicological studies (Orlando and Guillette, 2001) and may even be useful as a biomarker (Callaghan and Holloway, 1999). For example, Callaghan and Holloway (1999) found that when weevils (*Sitophilus oryzae*) were transferred to a toxic food source, the mean activity levels of glutathione-*S*-transferase and two naphthyl acetate esterases did not change significantly, but the variances about the means increased up to 5 fold. The elevated variance that we detected in clams exposed to stressors is consistent with the concept that, at the population level, variance in a physiological metric is indicative of stress.

Physiological Responses to Stress Vary by Season

The importance of assessing seasonal changes in the physiological stress response, particularly stress protein expression and activity levels, is well established. A number of factors,

including availability of nutrients, temperature variation, reproductive status and growth cycle, and seasonal patterns in environmental stressors, shape seasonal changes in bivalve stress physiology (Sheehan and Power, 1999). For example, warm summer conditions were linked to higher catalase and glutathione-*S*-transferase activities and higher condition index in *Mytilus galloprovincialis* (Roméo et al., 2003a), higher hsp70 levels in *M. edulis* (Chapple et al., 1998), and higher ubiquitin conjugate and hsp70 levels in *M. trossulus* (Hofmann and Somero, 1995). Seasonal differences in catalase, metallothionein, and gluathione-*S*-transferase levels in *M. edulis* and *Macoma balthica* corresponded with patterns of temperature and food availability as well as reproductive phase (Leiniö and Lehtonen, 2005). *Helix aspersa* snails estivating during the summer have a greater stress protein response, lower lipid peroxidation and lower protein carbonyl levels than those estivating during the winter (Ramos-Vasconcelos et al., 2005). Similarly, we detected greater changes in expression levels of the antioxidant proteins and some of the proteins involved in protein rescue and/or degradation in clams from the spring experiment in comparison to clams from the fall experiment. This increased expression of stress proteins in clams collected in the spring may have contributed to their enhanced survival (Chapple et al., 1998).

Conclusions

Marine invertebrates from sulfidic environments are likely to have physiological or biochemical adaptations to limit their susceptibility to these abiotic stressors (Grieshaber et al., 1994; Grieshaber and Völkel, 1998). Such adaptations would reduce the need for upregulated expression of stress proteins during experimental exposure to sulfide, thereby reducing our ability to detect whether this stressor has the capacity to cause oxidative damage. The surf clam *D. variabilis* does not experience hypoxia, hyperoxia or sulfide in its habitat and therefore is likely to be more sensitive to these stressors. Over the course of the survival experiments,

particularly in the fall experiment, both sulfide exposure and hypoxia exposure were lethal, whereas hyperoxia and normoxia were not. Therefore, increased expression of key antioxidants and repair enzymes following 24 hour exposure to sulfide, particularly during the spring experiment, but not hypoxia, suggests that the expression changes were a specific response. It remains to be determined how this protein expression pattern differs at shorter and longer time points, although substantially longer time points in hypoxia and sulfide treatments resulted in mortality. Consequently, these data are consistent with sulfide, and to a lesser extent hyperoxia, causing oxidative stress. It remains to be determined whether animals evolutionarily adapted to sulfide exposure have increased capacity for stress protein expression to limit oxidative damage, as has been shown for Hsp70 expression in intertidal mussels exposed to thermal stress (Hofmann and Somero, 1995; Hofmann, 1999).

Table 2-1. Summary of statistical results from comparisons between samples from Donax variabilis exposed to normoxia treatment and samples from animals exposed to hypoxia, hyperoxia, and sulfide. Data were analyzed by Kruskal-Wallis ANOVA followed by pairwise Conover-Inman post hoc comparison tests. Significant values ($p < 0.05$) are highlighted with bold text. T values are for data pooled across treatment within each season.

				p value from *post-hoc* pairwise comparison					
		T value (df=3)		Hypoxia		Hyperoxia		Sulfide	
Protein type	Protein	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
Antioxidant	MnSOD	8.112	0.948	0.181	0.389	**0.033**	0.593	**0.003**	0.639
	Cu/Zn SOD	1.743	4.346	0.413	0.26	0.629	0.326	0.253	**0.047**
	GPx	3.331	4.787	0.178	0.316	0.713	0.173	0.158	**0.038**
Lipid peroxidation	HNE	2.978	6.794	0.391	0.132	0.145	0.736	0.828	**0.038**
Oxidative repair	OGG1-m	1.261	11.206	0.44	0.089	0.421	**0.0007**	0.865	**0.0002**
Protein rescue and/or degradation	Ubiquitin	6.728	2.713	0.0621	0.359	0.436	0.359	**0.014**	0.134
	sHsp	2.027	5.581	0.337	0.297	0.321	0.119	0.235	**0.023**
	Hsp70	7.434	2.051	0.468	0.834	**0.027**	0.626	0.648	0.222
	Hsp60	0.618	0.132	0.691	0.794	0.51	>0.999	0.597	>0.999
	Grp75	0.684	0.088	0.509	0.896	0.739	>0.999	0.552	0.896
Cytoskeletal protein	Actin	0.485	9.529	0.44	**0.048**	0.153	0.668	0.401	**0.004**

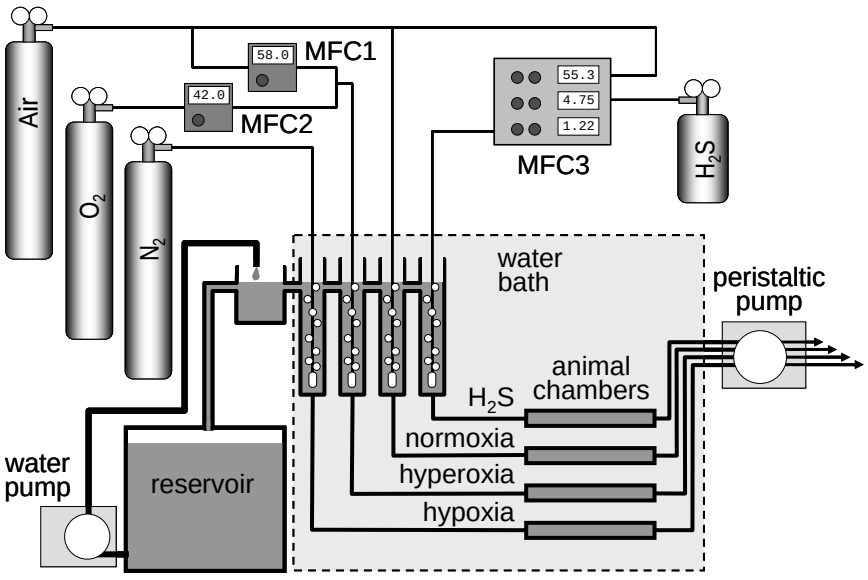


Figure 2-1. Diagram of flow-through system. Filtered, chloramphenicol-treated seawater was pumped from a 20 L reservoir to a 200 ml upper reservoir at 10 ml min^{-1}. Water from this reservoir then drained into each of 4 vertical gas equilibration chambers. These chambers were constructed of 18 mm inner diameter (i.d.) clear, cast acrylic tube, each 25 cm in length. A sintered glass aerator in each chamber was used to bubble either air (normoxia), N_2 (hypoxia), a mixture of O_2 and air (hyperoxia) or a mixture of air and hydrogen sulfide gas (from a compressed tank of 2% H_2S, balance N_2). Gas mixtures were controlled with three digital mass flow controllers, indicated in the figure as MFC1, MFC2 (both being FMA-5400 single-channel controllers, Omega Engineering, Inc., Stamford, CT, USA) and MFC3 (three-channel controller from Cameron Instrument Co., Port Aransas, TX, USA). Controllers that handled hydrogen sulfide gas were customized with corrosion-resistant fittings and O-rings. Gas-equilibrated water from each chamber was continuously pulled into an animal chamber by a 4-channel peristaltic pump (Masterflex cartridge system, Cole Parmer Instrument Co., Vernon Hills, IL, USA) at 2 ml min^{-1} per chamber. These chambers were constructed of 18 mm i.d., 15 cm long clear, cast acrylic tube with one-hole rubber stoppers at end, through which the seawater flowed through 1/8" i.d., 1/16" wall Tygon tubing. All tubing connections were via nylon Luer fittings (Cole Parmer Instrument Co.). Effluent water from the peristaltic pump was monitored periodically for P_{O_2} and pH. The gas equilibration chambers and animal chambers were housed in a polycarbonate water bath maintained at 24 °C. The entire system (except for compressed air, N_2 and O_2 supplies) was housed in a fume hood to minimize the hazards associated with handling H_2S gas.

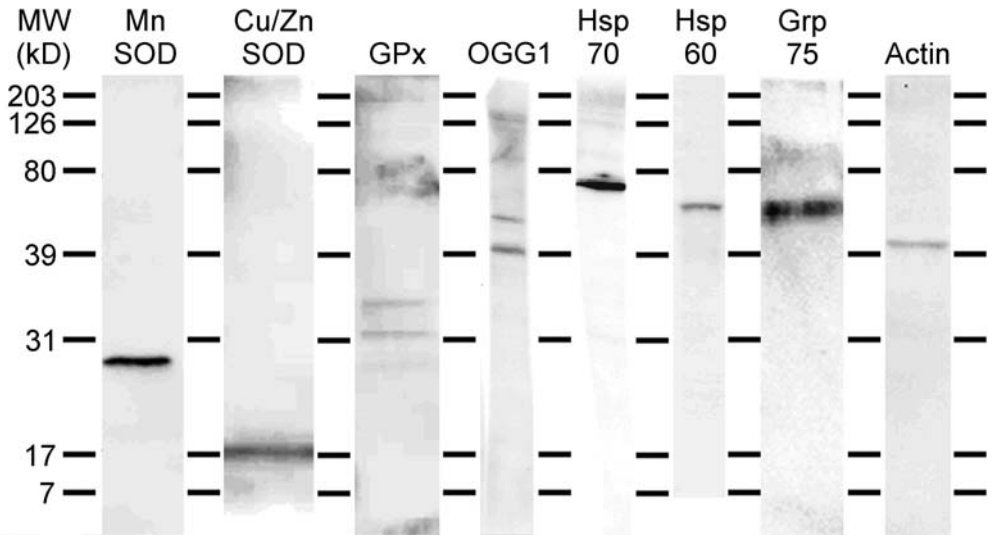


Figure 2-2. Antibody specificity tests. Two random samples of *Donax variabilis* were pooled, subjected to SDS-PAGE, western blotted, and assayed with the antibodies to the following proteins: manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/Zn SOD), glutathione peroxidase (GPx), OGG1-mito (OGG1), heat shock protein 70 (Hsp70), heat shock protein 60 (Hsp60), mitochondrial heat shock protein 70 (Grp75), and actin. The positions of known molecular weights standards are indicated by bars to the left of each individual band image and the molecular weight masses in kilodaltons (kD) are listed at the far left of the figure.

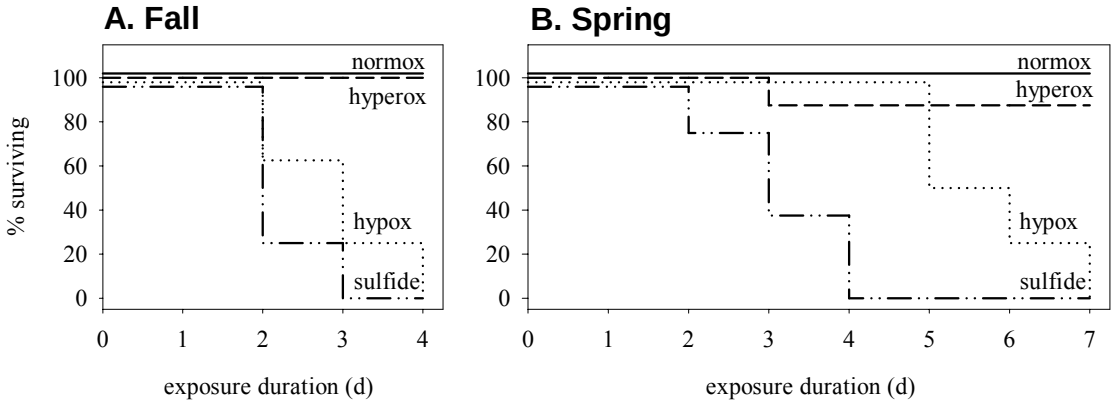


Figure 2-3. Fraction of surviving *Donax variabilis* clams in survival experiments in fall and spring. The fall experiment was conducted for 4 d and the spring experiment for 7 d. The treatments were: normoxia (solid line; 21.6 ± 1.9 kPa P_{O_2}), hypoxia (dotted line; 12.3 ± 1.4 kPa P_{O_2}), hyperoxia (dashed line; 36.6 ± 3.0 kPa P_{O_2}), and normoxic sulfide (alternating dashes and dots; 98 ± 2.9 μmol L^{-1} total sulfide, 24.0 ± 1.8 kPa P_{O_2}). Eight clams were exposed to each condition in each season.

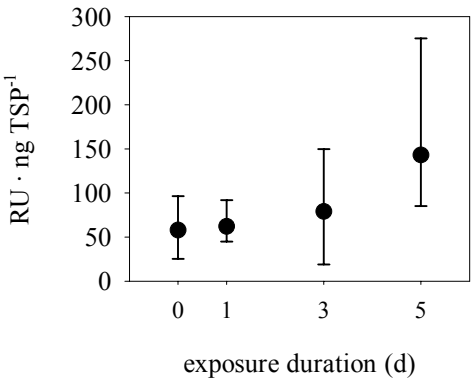


Figure 2-4. Expression levels of Hsp70 in *Donax variabilis* exposed to normoxia for 0, 1, 3, and 5 days. Clams used in the 0 time point were frozen in liquid N_2 immediately after collection at the beach. Data are presented as a scatterplot with asymmetrical error bars denoting minimum and maximum values and central dot signifying the mean of five clams per day. Data are given as relative units per nanogram of total soluble protein (RU · ng TSP^{-1}). Abbreviations: day, d. Data were analyzed by Kruskal-Wallis ANOVA but were not statistically significant.

Figure 2-5. Expression levels of three antioxidant proteins, a lipid peroxidation marker, and an oxidative repair enzyme in *Donax variabilis* exposed to normoxia (normox), hypoxia (hypox), hyperoxia (hyperox), and sulfide. Data are presented as scatterplots with asymmetrical error bars denoting minimum and maximum values and central dot signifying the mean of four clams per treatment in fall (left) and spring (right) experiments. Data for manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/Zn SOD), glutathione peroxidase (GPx), and (4-hydroxy-2*E*-nonenol-adducted protein) HNE are given as relative units per nanogram of total soluble protein (RU · ng TSP^{-1}), and data for OGG1-mitochondria (OGG1-m) are given as fmoles · mg TSP^{-1}. Data were analyzed by Kruskal-Wallis ANOVA and Conover-Inman *post hoc* pairwise comparisons. Similar letters denote statistically indistinguishable samples in data sets with significant ANOVAs. Data sets with no significant differences by ANOVA contain no letters adjacent to the symbols.

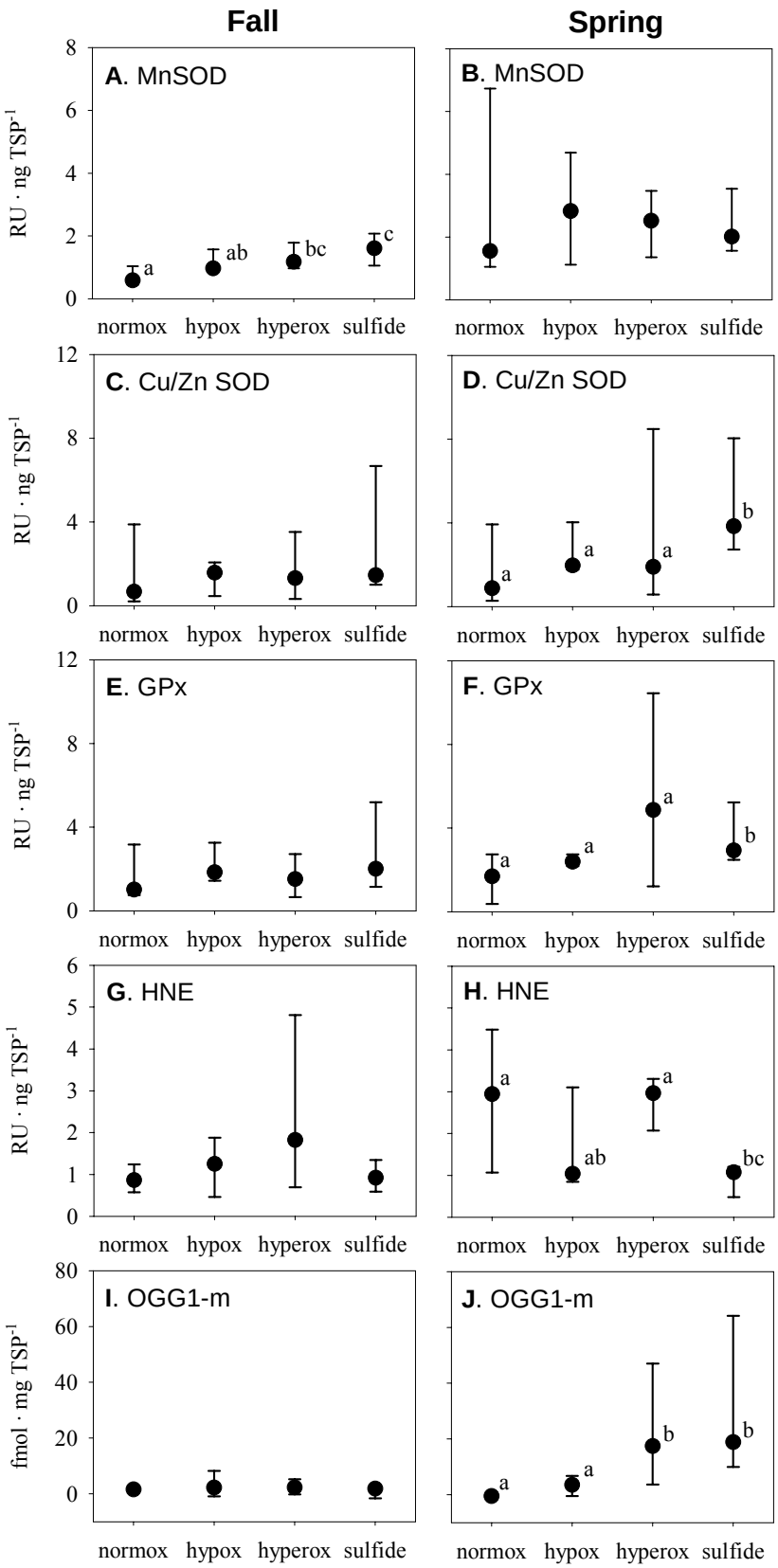
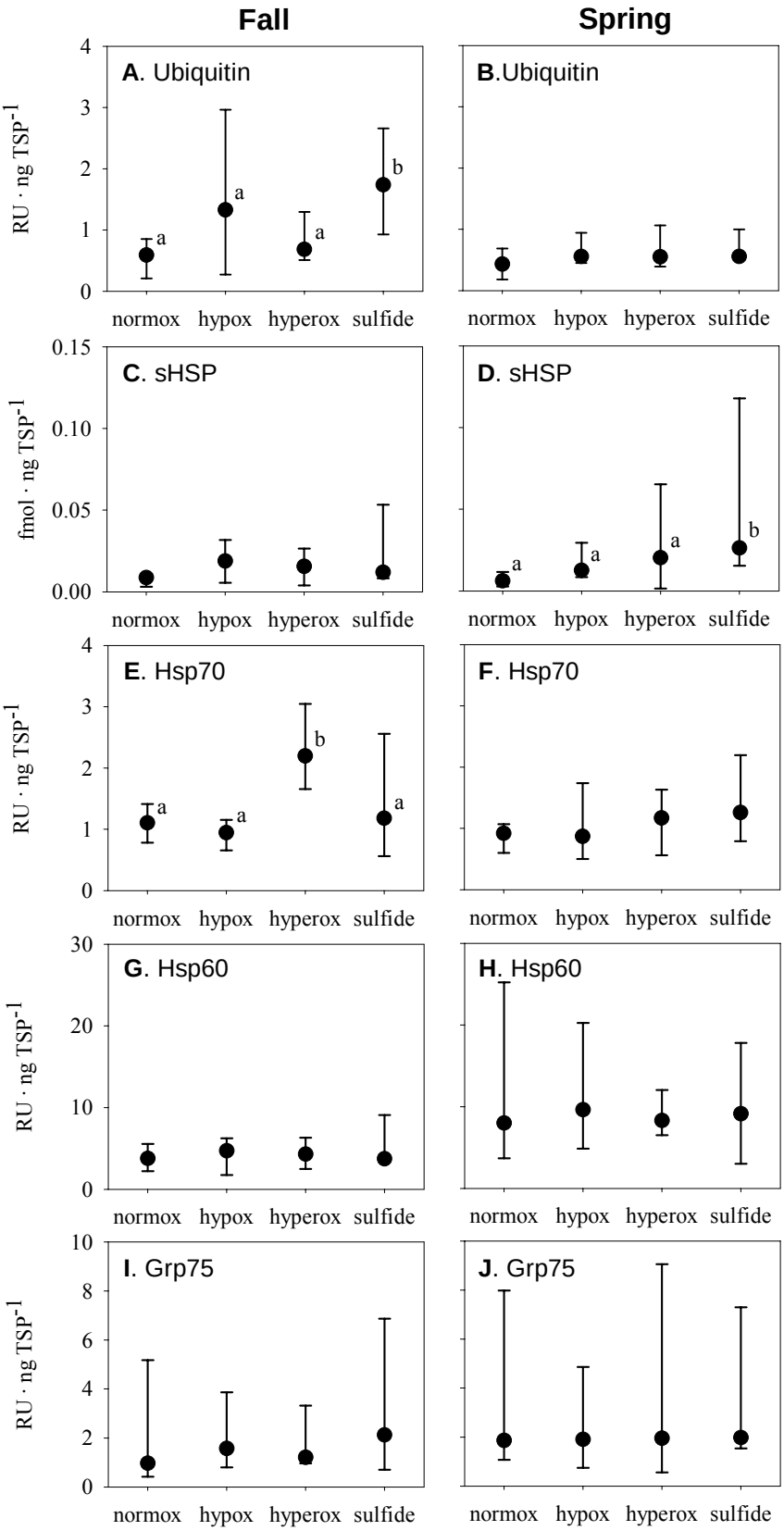

Fall
Spring
A. MnSOD
B. MnSOD
C. Cu/Zn SOD
D. Cu/Zn SOD
E. GPx
F. GPx
G. HNE
H. HNE
I. OGG1-m
J. OGG1-m
RU · ng TSP-1
fmol · mg TSP-1
normox
hypox
hyperox
sulfide
a
ab
bc
c
b

Figure 2-6. Expression levels of five proteins involved in protein rescue and/or degradation in *Donax variabilis* exposed to normoxia (normox), hypoxia (hypox), hyperoxia (hyperox), and sulfide. Data are presented as scatterplots with asymmetrical error bars denoting minimum and maximum values and central dot signifying the mean of four clams per treatment in fall (left) and spring (right) experiments. Data for ubiquitin, heat shock protein 70 (Hsp70), heat shock protein 60 (Hsp60), and mitochondrial heat shock protein 70 (GRP75) are given as relative units per nanogram of total soluble protein ($RU \cdot ng\ TSP^{-1}$). Data for small heat shock protein (sHsp) are given as $fmol \cdot mg\ TSP^{-1}$. Data were analyzed by Kruskal-Wallis ANOVA and Conover-Inman *post hoc* pairwise comparisons. Similar letters denote statistically indistinguishable samples in data sets with significant ANOVAs. Data sets with no significant differences by ANOVA contain no letters adjacent to the symbols.


Fall
Spring
A. Ubiquitin
B.Ubiquitin
C. sHSP
D. sHSP
E. Hsp70
F. Hsp70
G. Hsp60
H. Hsp60
I. Grp75
J. Grp75
RU · ng TSP-1
fmol · ng TSP-1
normox
hypox
hyperox
sulfide
a
b

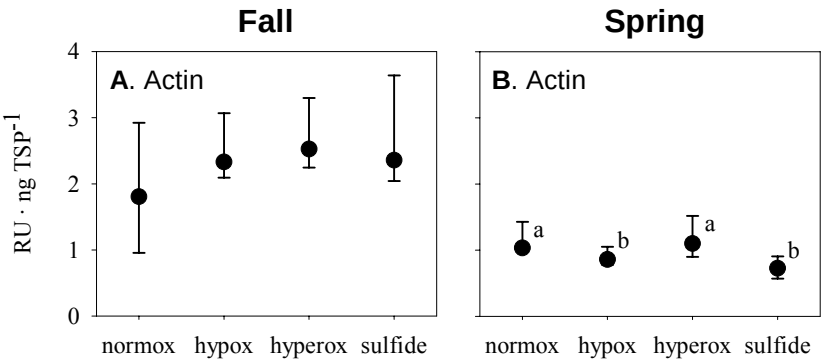


Figure 2-7. Expression levels of total actin in *Donax variabilis* exposed to normoxia (normox), hypoxia (hypox), hyperoxia (hyperox), and sulfide. Data are presented as scatterplots with asymmetrical error bars denoting minimum and maximum values and central dot signifying the mean of four clams per treatment in fall (left) and spring (right) experiments. Data are given as relative units per nanogram of total soluble protein ($RU \cdot ng\ TSP^{-1}$). Data were analyzed by Kruskal-Wallis ANOVA and Conover-Inman *post hoc* pairwise comparisons. Similar letters denote statistically indistinguishable samples in data sets with significant ANOVAs. Data sets with no significant differences by ANOVA contain no letters adjacent to the symbols.

CHAPTER 3
PHYSIOLOGICAL RESPONSES OF *Mercenaria mercenaria* TO SINGLE AND MULTIPLE ABIOTIC FACTORS

Introduction

The examination of how physical conditions in the environment interact with physiology to influence the distribution of organisms is a long-standing goal of ecologists, particularly of physiological ecologists (Brown et al., 1996; Spicer and Gaston, 1999). Numerous studies have attempted to define a species' niche, the set of environmental variables that limits reproduction and survival and therefore impacts distribution and abundance (Brown et al., 1996). Traditionally, these studies involved either *in situ* manipulations combining biotic interactions with environmental variables (exemplified by Connell, 1961) or laboratory studies of physiological responses to abiotic factors. Unlike *in situ* manipulations, which allow the experimental subjects to be impacted by all aspects of a complex environment, laboratory studies involve acclimation to one, or at most two, select environmental variables. Although the limitations of extrapolating laboratory studies of single variables to population-level processes have long been identified (e.g., Hall, 1964), recent reviews continue to emphasize the importance of investigating multiple abiotic factors in the laboratory (e.g., Brown et al., 1996).

The interplay between tolerances of abiotic factors with biotic interactions and species distributions have been rigorously studied in the rocky intertidal habitat (for review, Menge and Olson, 1990; Benson, 2002; Tomanek and Helmuth, 2002). This habitat, with its steep gradients of multiple abiotic factors, diel and seasonal patterns, accessibility, and well-characterized communities, serves as a model system for experimental population and community ecology. Additionally, since most of the study organisms are sessile as adults, distribution patterns are not confounded by behavioral responses to stressors, such as use of refuges. This habitat has

facilitated many recent advances in physiological ecology, such as the linking of population distribution with cellular-level responses including heat shock protein production (Hofmann et al., 2002). The approaches taken and conclusions reached in studies of rocky intertidal invertebrates can be applied to organisms inhabiting other variable aquatic habitats.

Estuaries share many similarities with the rocky intertidal. Estuaries have seasonal and daily fluctuations in a variety of abiotic factors, including temperature, salinity, dissolved O_2 levels, and pH (Hubertz and Cahoon, 1999; Beck and Bruland, 2000). The organisms inhabiting estuaries, like those of the rocky intertidal, must be broadly tolerant of environmental stressors (Fisher, 1977; Parsons, 1994). Although the physiological strategies employed by estuarine organisms are not as well understood as those of rocky intertidal invertebrates, several similarities are apparent. For example, alterations in heat shock protein expression and function in estuarine organisms have been linked to environmentally-relevant variations in temperature (Zippay et al., 2004) and salinity (Blank et al., 2006). Therefore, estuarine organisms, like intertidal invertebrates, could be developed as models for studies of how physiological responses to multiple environmental variables interact to affect distribution and abundance.

One such invertebrate species that is tolerant of multiple environmental stressors, well-studied, broadly distributed in coastal habitats, and accessible year-round is the northern quahog or hard clam, *Mercenaria mercenaria* (Kraeuter and Castagna, 2001). These clams live at intertidal and subtidal depths in bays and estuaries along the Atlantic coast of North America, from the Gulf of St. Lawrence to the southern Florida coastline (Harte, 2001). *Mercenaria mercenaria* is a generalist in its tolerance of temperature extremes, low dissolved O_2 levels (hypoxia), and salinity extremes (Grizzle et al., 2001), as would be expected given its distribution (Lynch and Gabriel, 1987; Gilchrist, 1995). It is therefore an appropriate model

organism for an investigation of how abiotic factors, singly and in combination, affect the physiology of aquatic invertebrates (Grizzle et al., 2001).

The current study examined how hypoxia, high temperature, and hyposalinity (reduced salinity), singly and in combination, affect the physiological responses of *M. mercenaria*. The physiological responses were divided into two categories, traditional functional markers and cellular-level indicators. Traditional functional markers measure long-term growth and fecundity responses to sublethal environmental stressors (Widdows, 1985). These assays measure general, whole-organismal responses, integrating the effects of the environmental stressor over several hierarchical levels of biological organization (Stebbing, 1985). Traditional assays measure behavioral responses such as valve-closure in bivalves (Heinonen et al., 1997), burial ability (Savage, 1976), and metabolic responses such as glycogen content (Hamza-Chaffai et al., 2003), and condition index (Roméo et al., 2003a). In recent years, cellular-level markers, such as stress protein expression (Sanders, 1993; Feder and Hofmann, 1999), oxidative damage markers, and RNA:DNA ratio (Elser et al., 2000), have become increasingly powerful and popular tools in part because they test for evidence of stress at the level of organization primarily affected; the molecular level (Stebbing, 1985; Bierkens, 2000). However, few studies have attempted to investigate both cellular-level indicators and traditional functional techniques (Brown et al., 1995; Hamza-Chaffai et al., 2003; Roméo et al., 2003b). This study attempted to delineate the links between cellular-level and whole-organismal responses to individual and multiple abiotic stressors and detail how an integrated stress profile changes over time.

Materials and Methods

Laboratory Exposures

Hard clams (average 12 mm shell length) were obtained from Southern Cross Sea Farms, Inc. (12170 SR 24 Cedar Key, FL) and maintained in 2.5 gallon aquaria with half water changes daily. The seawater was obtained from the Whitney Marine Lab (Marineland, FL) by the Department of Zoology and diluted to 26 ppt salinity (standard concentration for hypoxia and temperature experiments) or the appropriate salinity (dual stressor experiments) with ultrapure water. The water was pretreated with chloramphenicol (2 mg L^{-1}) to prevent the growth of bacteria. Clams were fed a mixed shellfish diet (1800 formula, Reed Mariculture) at 2-5% of estimated dry weight per day.

The single stressor experiments were designed to examine responses to hypoxia and to elevated temperature. In the hypoxia experiments the clams were exposed to three levels of dissolved oxygen: 1) air saturated (normoxia, approximately 0.25 mmol L^{-1}), 2) mild hypoxia (approx. 0.16 mmol L^{-1}), and 3) moderate hypoxia (approx. 0.07 mmol L^{-1}). Each aquarium was bubbled with air or air/N_2 mixes (one aquarium per treatment). Experimental conditions were monitored daily with a fiber optic O_2 probe (Ocean Optics, Inc., Dunedin, FL). All exposures were conducted at room temperature in all seasons (20-22ºC). Experiments were conducted in three seasons: fall (August 2004), winter (January 2005), and spring (April 2005). Given the lack of a measurable effect of the mild and moderate hypoxia treatments in the fall hypoxia experiment (see results), dissolved oxygen levels were decreased by 50% in both treatments in the winter and spring experiments in comparison to the fall hypoxia experiment.

In the second set of single-stressor experiments, the clams were exposed to three temperature treatments: 1) room temperature (approximately 24°C), 2) mild temperature elevation (approx. 28°C), and 3) moderate temperature elevation (approx. 33°C). The water

temperature was maintained by 15 W or 25W aquarium heaters and was monitored daily. All aquaria were bubbled with air and the dissolved oxygen content was monitored daily. Experiments were conducted during three seasons: fall (September 2004), winter (February 2005), and spring (May 2005). Experimental conditions used in the single-stressor experiments were consistent with environmental values recorded in Florida estuaries (see Discussion for details).

The dual stressor experiments were designed to examine responses to elevated temperature at each of three salinities. The clams were exposed to the following treatments: 1) high temperature/ambient salinity (measurements taken at the hatchery; approximately 33°C/24 ppt), 2) high temperature/mild hyposalinity (approx. 33°C/15 ppt), 3) high temperature/moderate hyposalinity (approx. 33°C/5ppt), 4) room temperature/ambient salinity (approx. 24°C/24ppt), 5) room temperature/mild hyposalinity (approx. 24°C/15 ppt), and 6) room temperature/moderate hyposalinity (approx. 24°C/5 ppt). The water temperature in the high temperature treatment was maintained by 25W aquarium heaters and was monitored in all treatments daily. All aquaria were bubbled with air and the dissolved oxygen content was monitored daily. Salinity was measured daily using a portable refractometer. Experiments were conducted during three seasons: fall (September 2005), winter (January 2006), and spring (May 2006).

In all experiments, two aquaria (one holding, one depuration) and one water jug (to provide fresh water of treatment conditions) were maintained at the experimental conditions for each treatment. Dissolved oxygen content, temperature, and salinity did not differ among the three water containers for any treatment in any of the nine experiments (data not shown). Daily half water changes were conducted using water from the jugs, which minimized abrupt changes in temperature or salinity. In all experiments, levels of ammonia, nitrite, and nitrate were

monitored. The pH of the water was monitored during the first several experiments and averaged 8.17 ± 0.11, which is within the pH range associated with optimal growth for this clam (Calabrese and Davis, 1966). The clams were not provided with burrowing substrate except during timed burial analysis tests, which is consistent with the conditions in which they were acclimated at the hatchery for 1-2 weeks prior to each experiment. Due to space limitations in a shared aquarium room, only one aquarium set was used per treatment. However, the clams could be considered independent experimental units for the purposes of statistical analyses since we maintained a large ratio of water volume (6 L) to clam tissue (maximum 15.7 g), daily half water changes, sufficient feeding, and constant aeration.

Clams were maintained with feeding in experimental aquaria until 24 hours before they were scheduled to be sacrificed (see schedule below), at which time they were transferred to the matching depuration aquaria. The experiments lasted 14 days, with clams sampled on days 1, 5, 9, and 14. The schedule of the experiments is outlined below and was identical for all experiments (actual sample sizes varied with clam availability and effects of the treatments, see details below in the results section):

Schedule.

- Day 0: 20 clams were preserved for Time 0 measurements, < 20 clams were placed into depuration aquaria (for 1 day sampling), the remainder of the clams were placed into the large aquaria and fed daily.
- Day 1: The clams in the three depuration aquaria (< 20 per treatment) were processed.
- Day 4: 20 clams from each large aquarium were transferred to the matching depuration aquarium.
- Day 5: The clams in the three depuration aquaria were processed.
- Day 8: < 20 clams from each large aquarium were transferred to the matching depuration aquarium.
- Day 9: The clams in the three depuration aquaria were processed.

- Day 13: < 20 clams from each large aquarium were transferred to the matching depuration aquarium.
- Day 14: The clams in the three depuration aquaria were processed.

Tissue Processing

Once the burial tests were completed (see below), all clams were removed from the depuration tanks. The clams were opened by severing their adductor muscles were severed. Whole clam tissues were blotted and weighed, then flash-frozen in liquid nitrogen and stored at –80ºC. All of the biochemical assays were conducted on whole clams and/or whole clam homogenates from individual clams. When the sample size was sufficiently large, clams used in the burial tests were not used for glycogen content or stress protein analyses. In several experiments, all available clams were used for burial analyses so the clams were gently rinsed before opened and frozen for the biochemical analyses.

Survival Analyses

In some of the experiments, clams in high temperature treatments died (see appropriate results sections). To maintain consistency, the experiments were continued through fourteen days with the surviving clams. In experiments with treatment-associated mortality, survival in all treatments was analyzed using an extension of the nonparametric Gehan's generalized Wilcoxon test, which assigns a score to each individual's survival time and then calculates and analyzes a Chi-square value for each treatment group (Statistica version 7.1, Tulsa, OK). Pairwise comparisons were made, when appropriate, using the nonparametric Gehan's generalized Wilcoxon test (Statistica).

Analyses:

Ability to bury. Burial rate, which is the proportion of clams that bury themselves in a given period of time when placed on a substrate, can serve as an index of total clam health

(Byrne and O'Halloran, 2000). Sand was utilized as the substrate, since it is a favorable substrate type for burial of *M. mercenaria* (Grizzle et al., 2001). On each sampling day, burial analysis was conducted on up to thirteen of the clams in each depuration chamber. Small containers with sand (approximately 2.5 cm deep) were placed into the depuration aquaria and randomly selected clams were placed flat on top of the sand. In the fall hypoxia experiment (first experiment we conducted) only, the containers of sand were reused throughout the experiment. In all subsequent experiments, the containers were filled with new sand on each sampling day. On days 1, 5, 9, and 14, we analyzed burial rate at 5, 10, 20, 30, and 60 min. Burial rate analyses were not conducted on Time 0 clams. Data collected at the 60 minute time point only were modeled as total number of clams that buried as a function of day and treatment by a generalized linear model with Poisson distribution and log link, Type III likelihood test, with the offset set as the total number of live clams tested on any given day (Statistica). In cases where clam death was high, the model was run with zeros listed for these treatments in both the number of clams buried and the total number of clams, since the analysis is weighted by the total number tested and requires a balanced design across treatment and day.**Glycogen content.** Glycogen content, which is a direct measure of a major energy storage reserve, was measured using standard techniques (Byrne and O'Halloran, 2000). Glycogen analyses were conducted on homogenates of whole clams, with a sample size of 3-5 clams per treatment per day. Briefly, glycogen was extracted from whole clam homogenates and hydrolyzed overnight by amyloglucosidase. Glucose monomers were quantified by a colorimetric assay that utilizes glucose oxidase/peroxidase and the substrate *o*-dianosidine. Sample absorbances were read at 450 nm on a Biotek Synergy-HT plate reader (Biotek, VT). Data from single stressor experiments were analyzed by a general linear model with treatment nested within experiment duration with Fisher's LSD post hoc comparisons for

those experiments for which there was a balanced number of samples (Statistica). Data from dual stressor experiments could not be analyzed using a nested design given the mortality in multiple treatments and therefore were analyzed within each day by one-way ANOVA with Fisher's LSD post hoc comparisons.

RNA oxidative damage. Amounts of total tissue oxidized RNA bases were determined in 5 clams per treatment (same clams as those used for stress protein assays) from dual stressor experiments only using a protocol optimized for bivalves (Joyner-Matos et al., 2007). RNA oxidative damage results from increased production or decreased detoxification of free radicals (Halliwell and Gutteridge, 1999). RNA guanine base oxidation produces 8-oxo-7,8-dihydroguanosine (8-oxoGuo). Data from dual stressor experiments could not be analyzed using a nested design given the mortality in multiple treatments. Data from the 1-day samples are presented and analyzed by 2-factor ANOVA with Fisher's LSD post hoc comparisons. The 1-day time point was the only sampling time in all three experiments that had samples available from every treatment.

Stress protein biomarkers. Five clams from each treatment group in the single stressor experiments were processed according to EnVirtue Biotechnologies Inc. standard bivalve sample preparation protocols (Downs et al., 2002b; Joyner-Matos et al., 2006). The whole clams were individually homogenized in liquid nitrogen. Small amounts (80-100 mg) of homogenized tissue were suspended in suspension buffer [50 mmol L^{-1} Tris, 15 mmol L^{-1} EDTA, 2% sodium dodecyl sulfate (SDS), 15 mmol L^{-1} dithiothreitol (DTT), 0.5% dimethyl sulfoxide (DMSO), and 0.01% Halt protease inhibitor cocktail]. After small amounts of tissue were suspended in suspension buffer, the solutions were vortexed, heated (3 minutes at 85ºC), vortexed, heated (3 minutes at 85ºC), kept at room temperature for 10 minutes, and then centrifuged for 10 minutes

at 12,000 rpm (room temperature). Supernatants were aliquotted, frozen in liquid nitrogen, and stored at –80ºC. Protein concentrations were determined using a modified Ghosh method (Ghosh et al., 1988) prior to freezing.

The samples from the single stressor experiments were assayed in cooperation with C. Downs using high-throughput ELISA analysis. After thawing, samples were diluted so that application of all samples was 25 nanograms of total soluble protein per well (30 μL volume) on 384-well microtiter plates. Sample dilutions, sample application blocking buffer application, primary and secondary antibody application, plate washes, and development solution were all applied to the microtiter plates by a Beckman-Coulter Biomek 2000 liquid handling system. Carousels, plate washers, and plate readers were all integrated with the Biomek 2000 to produce a functional high-throughput system. Standard curves of one sample diluted over an eight-point curve were dispersed across the plates to accommodate minor artifact formation associated with microtiter plate ELISA systems (e.g., edge-effects). Eight stress proteins were analyzed in samples from the single stressor experiments: copper/zinc superoxide dismutase (Cu/ZnSOD), glutathione peroxidase (GPx), manganese superoxide dismutase (MnSOD), heat shock protein 60 (Hsp60), heat shock protein 70 (Hsp70), small heat shock proteins (sHsp), ubiquitin, and 8-oxoguanine DNA glycosylase (OGG1m, mitochondrial isoform). Cross-reactivity of the stress protein antibodies was validated (Figure 3-1) by polyacrylamide gel electrophoresis and western blotting. Given the large variances in all stress protein data sets, data were analyzed by nonparametric Kruskal-Wallis ANOVA with post hoc multiple comparisons of mean ranks when appropriate (Statistica).

Results

Hypoxia Experiments

Experimental conditions. In the fall experiment, the clams were exposed to three levels of dissolved oxygen: 1) air saturated (normoxia; average 0.247 ± 0.024 mmol L^{-1}), 2) mild hypoxia (0.191 ± 0.016 mmol L^{-1}), and 3) moderate hypoxia (0.162 ± 0.021 mmol L^{-1}).

Following the hurricanes in the fall of 2004, the availability of grow-out size (average 12 mm) *M. mercenaria* was severely decreased. For the winter hypoxia experiment, sample size was decreased to nine clams per treatment per sampling day. Since there were no significant effects of the hypoxia treatment in the fall hypoxia experiment (see appropriate sections below), the mean dissolved oxygen levels of the two treatment groups for the remaining experiments were decreased. The clams in the winter experiment were exposed to three levels of dissolved oxygen: normoxia (0.247 ± 0.007 mmol L^{-1}), mild hypoxia (0.161 ± 0.011 mmol L^{-1}), and moderate hypoxia (0.078 ± 0.015 mmol L^{-1}).

In the spring experiment the clams were exposed to three levels of dissolved oxygen: 1) normoxia (0.247 ± 0.011 mmol L^{-1}), 2) mild hypoxia (0.158 ± 0.016 mmol L^{-1}), and 3) moderate hypoxia (0.074 ± 0.009 mmol L^{-1}).

In all three experiments levels of ammonia, nitrite, and nitrate were within acceptable/sublethal levels (data not shown). These experiments were conducted at room temperature and the temperature was monitored daily (data not shown).

Ability to bury. Burial analysis was conducted on a randomly selected subset (n = 6-10) of clams in the fall hypoxia experiment (Figure 3-2A). Burial abilities at the final sampling time (60 minutes) were affected by experiment duration ($p < 0.0001$), but not by treatment ($p = 0.085$). At all time points, more control (normoxia; black bars) clams buried than clams from the mild hypoxia (gray bars) or moderate hypoxia (white bars) treatments, but this difference was

not statistically significant. Surprisingly, burial ability decreased markedly between days 5 and 9, even in the control clams. This might have indicated that even these clams were experiencing some stress. However, additional sand was added to the burial rate testing dishes prior to day 9, and this may have caused or at least contributed to the overall decreased burial on days 9 and 14 because the sand became quite densely packed.

Burial analysis was conducted on all clams (n = 8-9) from each depuration aquarium during the winter hypoxia experiment (Figure 3-2B). Burial abilities at the final sampling time (60 minutes) were affected by experiment duration ($p = 0.023$), but not by treatment ($p = 0.123$). As seen in the fall hypoxia experiment, overall burial in the later two sampling days were lower than in the first two sampling dates.

Burial analysis was conducted on all clams (n = 10-14) from each depuration aquarium during the spring hypoxia experiment (Figure 3-2C). Burial abilities were affected by both experiment duration ($p < 0.0001$) and treatment ($p < 0.0001$). Across all four sampling days, clams in the mild hypoxia treatment buried more ($p < 0.0001$) than clams in the moderate hypoxia treatment. Since none of the clams in the control treatment buried at the 14-day time point, the overall burial of control clams was not significantly different ($p = 0.0755$) from the burial ability of the clams in the moderate hypoxia treatment or the mild hypoxia treatment.

Glycogen content. The glycogen content of whole clams (n = 3 per treatment, per sampling day) from the fall experiment were analyzed (Figure 3-3A). There was no overall effect of treatment on glycogen content ($p = 0.211$) when data were analyzed by a general linear model with treatment nested within experiment duration. In this complete model, experiment duration had a large and significant effect ($p < 0.0001$), with glycogen content decreasing by fourteen days, regardless of treatment. There was also a significant effect of the interaction between

experiment duration and treatment on glycogen content ($p = 0.0324$). Since there were equal numbers of replicates in all samples, post hoc comparisons were conducted. The only significant effect of treatment was detected in the 9-day samples, where clams in the moderate hypoxia treatment had significantly less glycogen than did control clams ($p = 0.042$).

When the glycogen content of whole clams ($n = 3$ per treatment, per sampling day) from the winter hypoxia experiment were analyzed (Figure 3-3B) by a general linear model with treatment nested within experiment duration, there was a significant effect of both experiment duration ($p < 0.0001$) and treatment ($p = 0.047$), as well as a significant interaction term ($p = 0.0024$). Overall, glycogen contents decreased over the duration of the experiment. There were no significant differences among treatments on sampling days 1, 9, and 14. At five days, however, clams in the moderate hypoxia treatment had significantly less glycogen than clams in the other two treatments ($p < 0.0055$).

When the glycogen content of whole clams ($n = 3$ per treatment, per sampling day) from the spring hypoxia experiment were analyzed (Figure 3-3C) by a general linear model with treatment nested within experiment duration, there was no significant effect of experiment duration ($p = 0.082$) or treatment ($p = 0.847$). Unlike previous experiments, glycogen content in all three treatments tended to increase over time, with no significant differences among treatments at any of the sampling days.

Stress protein expression. Since stress protein expression levels were determined via ELISA, only those proteins with little to no nonspecific cross-reactivity were analyzed. The banding patterns presented in Figure 3-1 are consistent with antibody specificity information provided by the manufacturer of the antibodies, EnVirtue Biotechnologies, Inc. and are consistent with other studies utilizing these antibodies with bivalve tissues (Joyner-Matos et al.,

2006, 2007). It is not necessary to test antibody specificity for ubiquitin or small heat shock protein (Joyner-Matos et al., 2007).

Stress protein expression levels were analyzed in clams (n = 5) from all three treatments on all four sampling days, as well as in clams collected and frozen on the first day of the experiment (0d; Figure 3-4, left column). Since there was high variance in all samples for all eight stress proteins, there were no consistent patterns in the effects of dissolved oxygen levels on stress protein expression in clams from the fall hypoxia experiment. Although overall significant differences were detected by Kruskal-Wallis ANOVA in some data sets (e.g., p = 0.0007 for GPx, Figure 3-4D and p = 0.0165 for Hsp70, Figure 3-4M), there were no biologically relevant significant differences among treatments in any stress protein data set.

A similar lack of treatment effect was found in the eight stress proteins analyzed in clams (n = 5) from the winter and spring hypoxia experiments (Figure 3-4, center and right columns, respectively). In both experiments, there were isolated cases of overall significant ANOVAs for some stress proteins, but either no statistically significant or no biologically relevant post hoc comparisons were detected.

Summary. Hypoxia treatments were sublethal in all seasons. In general, clams exposed to the moderate hypoxia treatment had a lower burial ability than clams in the normoxia or mild hypoxia treatments, but this difference was only significant in the spring experiment. In both the fall and winter experiments, glycogen levels in clams in the moderate hypoxia treatment declined one sampling day before glycogen levels decreased in clams from the other two treatments. There were no treatment-associated changes in glycogen content in the spring experiment. Both functional markers, burial ability and glycogen content, decreased significantly over the duration

of the fall and winter experiments. There were no significant, biologically-relevant changes in stress protein expression in clams from any of the three hypoxia experiments.

Temperature Experiments

Experimental conditions. In the fall temperature experiment, the clams were exposed to three treatments: room temperature (24.2 ± 1.2ºC), mild temperature elevation (28.3 ± 0.8ºC), and moderate temperature elevation (32.7 ± 1.2ºC). In the winter temperature experiment the clams were exposed to three treatments: room temperature (23.2±1.4 ºC), mild temperature elevation (29.6±1.2ºC), and moderate temperature elevation (33.9±0.7ºC). In the spring temperature experiment the clams were exposed to three treatments: room temperature (24.4 ± 1.1 ºC), mild temperature elevation (27.9 ± 1.8ºC), and moderate temperature elevation (34.0 ± 1.1ºC). In all three experiments levels of ammonia, nitrite, and nitrate were within acceptable/sublethal levels (data not shown). Dissolved O_2 levels in the three treatments were monitored daily and averaged 0.235 ± 0.013 mmol L^{-1} (data not shown).

Survival analysis. In the winter temperature experiment, the clams in the moderate temperature elevation treatment (Figure 3-5A, white squares) experienced mortality starting at day 2 and were all dead by day 7 . The mean survival time of clams in the moderate temperature elevation treatment was 3.33 ± 1.6 days. There was no mortality in the room temperature (black squares) or mild temperature elevation treatments (gray squares). Clams in the room temperature and mild temperature elevation treatments survived for an average of 7.25 ± 4.9 days. Survival rates among the three treatments were significantly different ($Chi^2 = 59.588$, df = 2, $p < 0.0001$). In pairwise comparisons, clams in the moderate temperature elevation treatment had significantly lower survival than clams in both the ambient temperature ($z = 5.808$, $p < 0.0001$) and the mild temperature elevation treatments ($z = 5.808$, $p < 0.0001$).

In the spring temperature experiment, the clams in the moderate temperature elevation treatment experienced mortality starting at day 8. Mean survival time of clams in the moderate temperature elevation treatment was 7.31 ± 4.9 days. There was no mortality in the room temperature or mild temperature elevation treatments. Clams in these two treatments survived approximately 7.25 ± 4.9 days. The mean survival time for the moderate temperature elevation treatment is slightly higher than for the other two treatments because a smaller-than-normal number of clams from this treatment were sacrificed on day 9, resulting in a greater-than-normal proportion of clams maintained until 14 days. Survival rates among the three treatments were significantly different ($Chi^2 = 9.693$, df = 2, $p = 0.0079$). In pairwise comparisons, clams in the moderate temperature elevation treatment had significantly lower survival time ($z = 2.261$, $p = 0.0238$) than clams in the other two treatments.

Ability to bury. Burial analysis was conducted on a randomly selected subset (n = 8-10) of clams in the fall temperature experiment (Figure 3-6A). Burial abilities at the final sampling time (60 minutes) were affected by experiment duration ($p < 0.00025$), but not by treatment ($p = 0.993$). As seen in the fall hypoxia experiment, overall burial abilities were lower in later days regardless of treatment.

In the winter temperature experiment burial analysis was conducted on all clams ($n = 11$-14) from each depuration aquarium on sampling days 1 and 5, and on clams from the room temperature (black bars) and mild temperature elevation (gray bars) treatments on days 9 and 14 (Figure 3-6B). Overall burial abilities at the final sampling time (60 minutes) were affected by experiment duration ($p = 0.0142$) and by treatment ($p = 0.022$). Since treatment comparisons can only be made at time points with clams present in all three treatments (1 day and 5 day), significant differences between treatments reflect only the first two sampling days. On these

early sampling days, clams in the mild temperature elevation treatment buried more ($p = 0.0058$) than clams in the moderate temperature elevation treatment (white bars). In contrast, clams in the room temperature treatment did not have elevated burial ($p = 0.287$) in comparison to clams in the moderate temperature elevation treatment. On Days 9 and 14, overall burial abilities of clams in both the room temperature and mild temperature elevation treatment increased, with no differences detected between treatments.

In the spring temperature experiment, burial analysis was conducted on randomly selected clams ($n = 4$-10) from each treatment on all sampling days (Figure 3-6C). Burial ability was significantly affected by treatment ($p = 0.034$) but not by experiment duration ($p = 0.3386$). In contrast to previous experiments, overall burial ability did not decrease in later time points. Clams in the room temperature treatment buried more than clams in the moderate temperature elevation treatment on days 5, 9, and 14 ($p = 0.013$). Burial ability of clams in the mild temperature elevation treatment were similar ($p = 0.069$).

Glycogen content. The glycogen content of whole clams ($n = 1$-3 per treatment, per sampling day) from the fall temperature experiment were analyzed (Figure 3-7A) by a general linear model with treatment nested within experiment duration. We were unable to assess glycogen content in clams from the room temperature (black bars) and moderate temperature elevation (white bars) treatments on the first sampling date due to loss of the samples during sample processing. The statistical model, therefore, includes the data only from days 5, 9, and 14. There was a significant effect of experiment duration ($p = 0.00029$), with glycogen content decreasing with time in clams from all three treatments. There were no significant differences among treatments at any sampling day ($p = 0.968$).

The glycogen content of whole clams (n = 3 per treatment, per sampling day) from all treatments with surviving clams from the winter temperature experiment were analyzed (Figure 3-7B). Since mortality occurred in the moderate temperature elevation treatment (indicated by the number symbol), this data set is unbalanced and could not be analyzed by a nested ANOVA. Individual one-way ANOVAs conducted on the 1-day and 5-day data sets did not show a significant effect of the elevated temperature treatment ($p > 0.05$).

When the glycogen content of clams (n = 3) from each treatment and sampling day of the spring temperature experiment (Figure 3-7C) were analyzed by a general linear model with treatment nested within experiment duration, there was a significant effect of experiment duration ($p = 0.0039$), but not treatment ($p = 0.409$). The significant effect of experiment duration likely was influenced by increases in glycogen content of clams from the room temperature and mild temperature elevation (gray bars) treatments at sampling day 9 in comparison to other days. There were no significant differences among treatments at any sampling day.

Stress protein expression. Stress protein expression levels were analyzed in clams (n = 5) from all three treatments on all sampling days of the fall temperature experiment, as well as in clams collected and frozen on the first day of the experiment (0d; Figure 3-8, left column). Since there was either unequal or high variance in all samples, there were no consistent patterns in the effects of elevated temperature on stress protein expression. Although overall significant differences were detected by Kruskal-Wallis ANOVA in some data sets (e.g., $p = 0.0032$ for MnSOD, Figure 3-8G and $p = 0.0041$ for sHsp, Figure 3-8P), there were no biologically relevant significant differences among treatments in any stress protein data set.

A similar lack of treatment effect was found in the eight stress proteins analyzed in clams (n = 5) from the winter and spring temperature experiments (Figure 3-8, center and right columns, respectively). In both experiments, there were isolated cases of overall significant ANOVAs for some stress proteins, but either no statistically significant or no biologically relevant post hoc comparisons were detected.

Summary. The mild temperature elevation treatment was sublethal in all three experiments, but the moderate temperature elevation treatment caused significant mortality in the winter and spring experiments. In general, clams in the moderate temperature elevation treatment had lower burial ability than clams in the other two treatments, although this difference was not always statistically significant. In contrast, there were no treatment-associated changes in glycogen content. Both burial ability and glycogen content tended to decrease with experiment duration. There were no significant, biologically-relevant changes in stress protein expression in clams from any of the three temperature experiments.

Dual Stressor Experiments

Experimental conditions. In the fall dual stressor experiment, the clams were exposed to three levels of hyposalinity at each of two temperatures: 1) high temperature/ambient salinity (37.1 ± 2.6°C, 24.9 ± 0.9 ppt), 2) high temperature/mild hyposalinity (35.6 ± 0.6°C, 15.9 ± 0.9 ppt), 3) high temperature/moderate hyposalinity (35.0 ± 1.0°C, 5.3 ± 0.6 ppt), 4) room temperature/ambient salinity (26.1 ± 1.2°C, 22.5 ± 1.5 ppt), 5) room temperature/mild hyposalinity (25.9 ± 0.7°C, 15.3 ± 1.2 ppt), and 6) room temperature/moderate hyposalinity (23.7 ± 0.6°C, 4.5 ± 0.7 ppt). Ambient salinity was defined as the salinity at the hatchery where the clams had been maintained. Daily dissolved O_2 levels in the six treatments averaged 0.242 ± 0.016 mmol L^{-1} (data not shown). Levels of ammonia, nitrite, and nitrate were within acceptable/sublethal levels [data not shown; Epifanio and Srna, 1975].

In the winter dual stressor experiment, the clams were exposed to the following six treatments: 1) high temperature/ambient salinity (34.5 ± 0.6°C, 24.1 ± 2.1 ppt), 2) high temperature/mild hyposalinity (33.8 ± 0.8°C, 15.4 ± 2.5 ppt), 3) high temperature/moderate hyposalinity (33.0 ± 1.6°C, 5.8 ± 2.2 ppt), 4) room temperature/ambient salinity (22.5 ± 1.3°C, 22.6 ± 1.5 ppt), 5) room temperature/mild hyposalinity (22.3 ± 1.1°C, 14.2 ± 1.5 ppt), and 6) room temperature/moderate hyposalinity (21.7 ± 1.2°C, 5.2 ± 0.5 ppt).

In the spring dual stressor experiment, the clams were exposed to the following six treatments: 1) high temperature/ambient salinity (36.6 ± 1.5°C, 25.9 ± 1.1 ppt), 2) high temperature/mild hyposalinity (34.1 ± 0.3°C, 15.1 ± 0.6 ppt), 3) high temperature/moderate hyposalinity (33.8 ± 0.8°C, 5.0 ± 0.1 ppt), 4) room temperature/ambient salinity (25.0 ± 0.5°C, 25.6 ± 0.9 ppt), 5) room temperature/mild hyposalinity (24.8 ± 0.6°C, 15.0 ± 0.4 ppt), and 6) room temperature/moderate hyposalinity (22.3 ± 0.5°C, 5.2 ± 0.6 ppt). The high temperature/ambient salinity treatment was terminated after 24 hours due to a heater malfunction.

Survival analysis. In the fall dual stressor experiment (Figure 3-9A), clams in the high temperature/moderate hyposalinity treatment (white triangles) experienced mortality starting at day 4 and were all dead by day 5, resulting in a mean survival time of 2.69 ± 0.9 days. Clams in the high temperature/ambient salinity treatment (black triangles) experienced mortality starting at day 7 and were all dead by day 13 (mean survival time of 7.06 ± 3.9 days). Similarly, clams in the high temperature/mild hyposalinity treatment (gray triangles) experienced mortality starting on day 7, but clams in this treatment did not die before the experiment end (mean survival time of 7.50 ± 4.6 days). Among the clams maintained at room temperature, those in the moderate hyposalinity treatment (white circles) experienced mortality starting at day 7 and were all dead

by day 14 (mean survival times of 7.18 ± 4.0 days), a pattern that was significantly different from that of the other two room temperature treatments ($z = 5.98$, $p < 0.0001$). In contrast, none of the clams in the room temperature/ambient salinity (black circles) or room temperature/mild hyposalinity (gray circles) treatments died (mean survival time 7.25 ± 4.9 days). Overall, the mean survival times of the six treatments were significantly different ($Chi^2 = 291.8$, df = 5, $p < 0.0001$). In pairwise comparisons, clams in the high temperature/moderate hyposalinity treatment had a significantly shorter survival time than clams in any other treatment ($z \geq 10.1$, $p < 0.0001$). Clams in the high temperature/mild hyposalinity treatment had a significantly longer survival time than clams in the high temperature/ambient salinity treatment ($z = 1.97$, $p = 0.0484$).

In the winter dual stressor experiment (Figure 3-9B), clams in the high temperature/moderate hyposalinity treatment and the high temperature/mild hyposalinity treatments experienced mortality starting at day 2 and were all dead by day 5, resulting in mean survival times of 2.81 ± 1.2 days and 2.73 ± 1.2 days, respectively. Clams in the high temperature/ambient salinity treatment experienced mortality starting at day 3 and were all dead by day 7 (mean survival time 4.06 ± 1.9 days). There was some death in the room temperature/moderate hyposalinity treatment after day 11 (mean survival time 7.12 ± 4.7 days). In contrast, none of the clams in the room temperature/ambient salinity or room temperature/mild hyposalinity treatments died (mean survival time 7.25 ± 4.9 days). Overall, mean survival times of the six treatments were significantly different ($Chi^2 = 174.12$, df = 5, $p < 0.0001$). Clams in the high temperature/ambient salinity treatment survived significantly longer than clams in the other two high temperature treatments ($z \geq 6.11$, $p < 0.0001$) and significantly shorter than clams in the room temperature/ambient salinity treatment ($z = 5.69$, $p < 0.0001$). Despite the mortality at day 12, survival times of clams in the room temperature/moderate hyposalinity treatment were

not significantly different than clams in the other room temperature treatments ($z = 1.19$, $p = 0.230$).

In the spring dual stressor experiment (Figure 3-9C), clams in the high temperature/moderate hyposalinity treatment began to die at day 3 and were completely dead by day 5 (mean survival time 3.06 ± 1.3 days). Clams in the high temperature/mild hyposalinity treatment began to die at day 4 and were completely dead by day 10 (mean survival time 4.19 ± 2.4 days). Clams in the room temperature/moderate hyposalinity treatment began to die at day 9 and were all dead by day 12 (mean survival time 6.16 ± 3.5 days). In contrast, none of the clams in the room temperature/ambient salinity or room temperature/mild hyposalinity treatments died (mean survival time 7.25 ± 4.9 days). Overall, survival times were significantly different among treatments ($Chi^2 = 82.663$, df = 4, $p < 0.0001$). Clams in the high temperature/moderate hyposalinity treatment had significantly shorter survival times than clams in all other treatments ($z \geq 3.71$, $p \leq 0.00021$). Clams in the high temperature/mild hyposalinity treatment had significantly shorter survival time than clams in the mild hyposalinity/room temperature treatment ($z = 4.25$, $p = 0.00002$). Clams in the room temperature/moderate hyposalinity experiment had a significantly shorter mean survival time than did clams in the other room temperature treatments ($z = 2.98$, $p = 0.0029$).

Ability to bury. Burial analysis was conducted on clams ($n = 6$-10) from each treatment with surviving clams on each sampling day of the fall dual stressor experiment (Figure 3-10A). There was a significant effect of treatment ($p = 0.0035$) but not of experiment duration ($p = 0.739$) on the ability of the clams to bury. Although the burial ability of the clams in the room temperature/ambient salinity treatment was higher than those of any other treatment at all sampling days, pairwise differences among treatments were not significant ($p > 0.05$). Although

clams in the high temperature/ambient salinity (black bar) showed the same ability to bury as those in the room temperature/ambient salinity treatment (thinly striped bar) on day 1, clams experiencing dual stressors were unable to bury at later time points.

In the winter dual stressor experiment, burial analysis was conducted on all (n = 9-10) surviving clams at all treatment times (figure 3-10B). There was a significant effect of treatment (p = 0.0029) but not of experiment duration (p = 0.741) on burial ability. On all sampling days, clams in the room temperature/ambient salinity treatment exhibited burial ability, which decreased after day 5. On the first sampling day, the only day with clams present in all treatments, clams exposed to high temperature buried less than those at room temperature, regardless of salinity level.

In the spring dual stressor experiment, burial analysis was conducted on a subset (n = 4-8 clams) of surviving clams at all treatment times (Figure 3-10C). There was no significant effect of either experiment duration (p = 0.507) or treatment (p = 0.266), likely because burial was only detected in two of the six treatments.

Glycogen content. The glycogen content of whole clams (n = 5) from all treatments on day 1 and from treatments with a sufficient surviving clams on subsequent days of the fall dual stressor experiment were analyzed (Figure 3-11A). Since this data set is unbalanced (due to mortality in some treatments), each day was analyzed individually by one-way ANOVA with Fisher's LSD where appropriate. There were no significant differences among the six treatment groups on sampling day 1 (p = 0.873). On sampling day 5, there was a significant effect of treatment (p = 0.021). The clams in the high temperature/ambient salinity (black bars) and high temperature/mild hyposalinity (gray bars) treatments had significantly less glycogen than did clams in the room temperature/ambient salinity (thinly striped bars) treatment (p < 0.026;

significances not indicated in graph). Additionally, the clams exposed to room temperature/mild hyposalinity (thickly striped bar) had significantly less glycogen than clams in the other two salinity (and room temperature) treatments ($p < 0.035$). At the day 9 sampling date, treatment again had a significant effect on glycogen content ($p = 0.033$). Clams exposed to high temperature/mild hyposalinity treatment had significantly less glycogen than clams exposed to room temperature/mild hyposalinity ($p = 0.0045$). There were no significant differences in glycogen content of clams surviving to 14 days ($p = 0.061$). Unlike most of the single stressor experiments, overall glycogen levels did not decrease over the duration of the fall dual stressor experiment.

The glycogen content of whole clams ($n = 5$ for all but 14 day room temperature/moderate hyposalinity treatment, for which $n = 2$) from all six treatments on sampling day 1, and from the room temperature treatments on subsequent sampling days of the winter dual stressor experiment were analyzed (Figure 3-11B). We did not find any significant differences among treatments on day 1 ($p = 0.301$), the only sampling day with all six treatments present. Similarly, there were no significant differences in glycogen content among the three room temperature treatments on day 5 ($p = 0.774$). In contrast, we found significant effects of the hyposalinity treatments at both day 9 ($p = 0.0055$; significant differences are not indicated on the graph) and day 14 ($p = 0.0012$). On both days, the clams in the ambient salinity had significantly more glycogen than clams in either hyposalinity treatment (9 day, $p < 0.045$; 14 day, $p < 0.039$). Additionally, the clams in the moderate hyposalinity treatment on sampling day 14 had significantly less glycogen than clams in the mild hyposalinity treatment ($p = 0.0046$). Glycogen content in clams from the room temperature/ambient salinity and room temperature/mild hyposalinity treatments did not decline with experiment duration.

The glycogen content of whole clams (n = 2-3 clams) from all six treatments on sampling day 1 and of the room temperature treatments on subsequent sampling days of the spring dual stressor experiment were analyzed (Figure 3-11C). There was no significant treatment effect on glycogen content of clams on sampling day 1 (p = 0.779) or day 5 (p = 0.72). Glycogen content of clams sampled on days 9 or 14 were not statistically analyzed due to low sample sizes.

RNA oxidation. The levels of oxidatively damaged RNA bases, 8-oxoGuo, were measured in clams (n = 5) from each treatment on day 1 of the fall dual stressors experiment (Figure 3-12A). RNA oxidation was significantly affected by salinity treatment (p = 0.0157) and a marginally affected by temperature (p = 0.0734), but not by the interaction between the two factors (p = 0.809). Several post hoc comparisons were statistically significant; of those only one was biologically significant. Clams exposed to the room temperature/mild hyposalinity treatment (triangle) had significantly more oxidized RNA than did clams in the room temperature/ambient salinity treatment (diamond; p = 0.0211, significances not noted on graph).

Levels of oxidatively damaged RNA were measured in clams (n = 5 for all except room temperature/mild hyposalinity, which had n = 2) from each treatment on day 1 of the winter dual stressors experiment (Figure 3-12B). RNA oxidation was significantly affected by temperature (p < 0.0001) but not by salinity (p = 0.142) or the interaction between temperature and salinity (p = 0.210). In general, RNA oxidation levels were higher in clams in the high temperature treatments in comparison to clams in the room temperature experiments. Specifically, clams exposed to high temperature/ambient salinity (circle) or high temperature/mild hyposalinity (inverted triangle) had significantly more oxidized guanine bases than did clams from any room temperature treatment (p < 0.00091). The clams exposed to high temperature/moderate hyposalinity had more oxidized RNA than did clams in the two room temperature/hyposalinity

treatments ($p < 0.012$), but this difference was only marginally significant ($p = 0.0587$) in comparison to clams from the room temperature/ambient salinity treatment. There were no significant differences among the three room temperature treatments ($p > 0.229$).

Levels of oxidatively damaged RNA were measured in clams ($n = 5$) from each treatment on day 1 of the spring dual stressor experiment (Figure 3-12C). Unlike in previous seasons, there were no significant effects of either treatment factor or the interaction between the factors on RNA oxidation ($p > 0.104$). However, the post hoc multiple comparison test did detect two significant comparisons to the RNA oxidation levels of the clams in the room temperature/ambient salinity (diamond) treatment. Clams exposed to high temperature/ambient salinity had significantly more oxidized RNA bases than did clams exposed to room temperature/ambient salinity ($p = 0.0151$). Similarly, the clams in the two hyposalinity treatments (room temperature) had more oxidized RNA bases than did the room temperature/ambient salinity clams, a increase that was significant in the mild hyposalinity treatment ($p = 0.0429$) and marginally significant in the moderate hyposalinity treatment ($p = 0.0541$).

Summary. The dual stressor treatments resulted in significant mortality in all three seasons. Additionally, the high temperature/ambient salinity treament caused significant mortality, particularly in the winter experiment. Clams exposed to dual stressors had decreased burial in all three experiments, but no significant changes in glycogen content. Although there was no consistent pattern in RNA oxidation across season, in several cases the levels of RNA oxidation were higher in clams exposed to hyposalinity in comparison to clams in ambient salinity.

Discussion

These results support several overall conclusions concerning how one should investigate the ability of an organism to respond to individual and multiple abiotic factors. First, a strong influence of season was detected in all experiments, with an overall decrease in the ability of the clams to tolerate the abiotic stressors evident during the winter experiments. Second, there was no clear relationship between cellular-level and functional markers, with most of the treatment effects detected at the whole-organism level rather than the cellular level. Third, many of the functional responses were negatively impacted by experiment duration, calling into question the utility of long-term laboratory exposures. Fourth, high temperature and hyposalinity were found to have an additive effect on most metrics, particularly whole-organism metrics. Since this combination of abiotic factors occurs in estuarine environments, particularly in the summer, these results highlight the importance of investigating the effects of multiple stressors in controlled laboratory conditions to more accurately determine how tolerance of abiotic factors may influence species distribution.

Hypoxia. Conditions of low dissolved O_2, or hypoxia, are prevalent in estuaries, generally resulting from nutrient enrichments, tidal cycles, and excessive rainfall (Hubertz and Cahoon, 1999; Beck and Bruland, 2000). Since temperature affects O_2 solubility, elevated water temperatures cause both seasonal and diel cycles of hypoxia and normoxia. *Mercenaria mercenaria* thrive in estuarine habitats which, along the Florida coast, have typically have O_2 levels of 0.19 – 0.31 mmol L^{-1} (Millie et al., 2004; Harris et al., 2005) but can be as low as 0.09 mmol L^{-1} (Caccia and Boyer, 2005). Hard clams are hypoxia tolerant and are oxyregulators until dissolved O_2 levels reach the critical P_{O_2} of 0.15 mmol L^{-1}, at which point the clams close their valves and maintain anaerobic respiration for extended periods of time (reviewed in Grizzle et

al., 2001). During these periods of anaerobic metabolism, hard clams, like many bivalves, rely upon glycogen fermentation as a major energy source (Grieshaber et al., 1994; Grizzle et al., 2001). While survival of *M. mercenaria* is not strongly affected by hypoxia (Winn and Knott, 1992; Grizzle et al., 2001; Carmichael et al., 2004), functional responses of hard clams are impacted by hypoxia, but only at very low dissolved O_2 levels. Pumping rate, which directly affects feeding ability, is linearly correlated with dissolved O_2 levels from 0.03 – 0.15 mmol L^{-1} (reviewed in Grizzle et al., 2001). Clam growth is negatively impacted by dissolved O_2 levels below 0.15 mmol L^{-1} (Appleyard and Dealteris, 2002), an effect that may be confounded by decreased feeding under these conditions. Burial time in *M. mercenaria* also is inversely related to dissolved O_2 levels, with burial time doubling over a range of dissolved O_2 levels from 0.21 mmol L^{-1} to 0.03 mmol L^{-1} (Savage, 1976).

In the fall hypoxia experiment, both hypoxia treatments were well above the threshold dissolved O_2 level determined to affect functional responses of hard clams, and it is therefore not surprising that there were no treatment-associated declines in burial ability or glycogen content in clams from this experiment. However, both burial ability and glycogen content decreased over the duration of the experiment, a trend that has been documented for glycogen levels in bivalves in field (Byrne and O'Halloran, 2000) and laboratory (de Zwann and Zandee, 1972) studies.

In the winter and spring experiments, hypoxia treatments were modified to encompass the threshold dissolved O_2 level (mild hypoxia treatment, approximately 0.16 mmol L^{-1}) as well as a level significantly below threshold (moderate hypoxia treatment, approximately 0.08 mmol L^{-1}), both of which are representative of conditions in estuaries with hard clams (Winn and Knott, 1992; Ringwood and Keppler, 2002). Although there was no treatment-associated mortality in the hypoxia experiments, there was some evidence for a decline in functional responses. In most

sampling days of the winter and spring experiments, the burial abilities of clams in the moderate hypoxia treatment were lower than those of the normoxia and mild hypoxia treatments, which is consistent with previous characterizations of hard clam burial abilities (Savage, 1976).

In both the fall and winter experiments, glycogen levels in clams in the moderate hypoxia treatment declined one sampling day before glycogen levels decreased in clams from the other two treatments, a trend that occurred earlier in the winter experiment. In contrast, glycogen levels in the spring experiment increased or showed no change with experiment duration, a pattern repeated in the spring temperature and dual stressor experiments, regardless of treatment. Since the feeding regimen did not differ among experiments, it appears that maintaining the clams in the aquarium system was most detrimental to clam health (assessed by glycogen content) in the winter, somewhat detrimental in the fall, and not detrimental in the spring. Seasonal differences in glycogen storage in bivalves are well-established (e.g., Byrne and O'Halloran, 2000; Hamburger et al., 2000), and are typically linked to reproductive status. *Mercenaria mercenaria* are consecutive hermaphrodites and at the size used for these experiments (12 – 15 mm) are likely in the male juvenile stage. Clams living in estuaries along the Florida coast typically have a semiannual spawning cycle, with spawns in March/April and October/November (Eversole, 2001). The spring experiment was conducted in April, and therefore these clams may have utilized their glycogen stores differently than the clams in the fall and winter experiments.

Short-term exposure to hypoxia has been linked to increased free radical production (Hermes-Lima et al., 1998; Halliwell and Gutteridge, 1999; Hermes-Lima and Zenteno-Savin, 2002; Li and Jackson, 2002). During hypoxia, the absence of O_2 as the final electron acceptor causes accumulation of electrons in mitochondrial electron transport chains (i.e., the chains are

reduced), with the result that a sudden return of O_2 can cause the production of free radicals due to nearly instantaneous reactions between O_2 and the accumulated free electrons (Du et al., 1998; Li and Jackson, 2002). Although most studies of free radical production during hypoxia include a period of reoxygenation, recent studies have documented free radical production occurring during hypoxia without subsequent reoxygenation (Vanden Hoek et al., 1997; Chandel et al., 1998; Becker et al., 1999). Hypoxia-associated free radical production, if it occurs, can be accompanied by oxidative damage (Englander et al., 1999; Dirmeier et al., 2002) and changes in antioxidant expression and/or activity (Lushchak et al., 2001; de Oliveira et al., 2005). However, changes in antioxidant expression or activity are not always detected in animals or cells exposed to hypoxia (Hass and Massaro, 1988; Willmore and Storey, 1997; Joanisse and Storey, 1998; Larade and Storey, 2002).

In the current study, *M. mercenaria* exposed to hypoxia did not show significant changes in stress protein expression, regardless of the season in which the experiments were performed. There are two possible interpretations for these results. First, it is possible, in light of the conflicting literature summarized above, that prolonged hypoxia in the absence of reoxygenation does not induce free radical production or cellular damage. Upregulation of stress protein expression, therefore, would be counterproductive and metabolically expensive (Somero, 2002). Alternatively, if hypoxia exposure can cause oxidative damage, then it is possible that the degree of hypoxia utilized in these studies was not sufficiently severe to incite a stress protein response. These results are not consistent with those suggesting that cellular-level responses are more likely to be detected than organismal responses, which integrate over several levels of biological organization (Stebbing, 1985; Bierkens, 2000). Most of the studies cited above, particularly those that showed cellular damage following hypoxia, were performed on organisms that are not

hypoxia-tolerant. It is possible that even though the severity of the hypoxia stress utilized in the winter and spring experiments was sufficient to cause declines in functional responses, the stress was not severe enough to cause damage or initiate responses at the cellular level. The relationship between the timing and degree of cellular-level responses such as stress protein expression and/or activity and whole-animal tolerance of hypoxia has not been explicitly studied for any organisms. These results suggest that for animals as tolerant of a stressor as *M. mercenaria* is tolerant of hypoxia, cellular-level markers alone are not sufficient to detect detrimental effects of exposure to individual abiotic stressors and should not be used to investigate species distribution.

Temperature. Diel and seasonal temperature fluctuations are typical of estuaries. The estuaries along the Florida coastline experience 'wet' and 'dry' seasons that can differ by 8°C in average temperature (Millie et al., 2004). *Mercenaria mercenaria* are tolerant of a wide range of temperatures, from 0 – 30°C, and typically more tolerant of warm temperatures than cold. Maximum growth and pumping rates of hard clams occur at temperatures ranging from 20 – 26°C, with both functional responses declining dramatically above 32°C and below 7°C (for review, Grizzle et al., 2001). This pattern of thermal tolerance closely matches both their distribution (Wells, 1957; Harte, 2001) and their reproductive activity, which in warmer southern latitudes has a semiannual pattern (Eversole, 2001). Burrowing time in *M. mercenaria* mirrors that of growth and pumping rates, with shortest burrowing times detected at 21 – 29°C and longer burrowing times at 37°C (Savage, 1976).

In the current study, the functional responses of hard clams to temperature elevation varied strongly by season, with the greatest susceptibility to high temperature detected during the winter temperature experiment. Whereas none of the clams died during the fall temperature experiment,

all clams exposed to moderate temperature elevation (34°C) died by day 7 in the winter experiment and some clams in this treatment died on day 9 of the spring experiment. Seasonal differences in susceptibility to thermal extremes has been documented previously and linked to ability to express heat shock proteins (Chapple et al., 1998).

Burial abilities did not follow clear seasonal patterns, which is consistent with a previous study (Savage, 1976). Burial abilities decreased over time in the fall experiment, but this pattern was not repeated in the winter and spring experiments. In the winter experiment, clams in the moderate temperature elevation treatment had very low burial ability on day 1 and did not bury on day 5. However, burial abilities in the room temperature and mild temperature elevation treatments were low on these days, so the lack of burial in the moderate temperature elevation treatment cannot be considered predictive of subsequent mortality.

Patterns in glycogen content did not vary by season and were not significantly affected by elevated temperature. However, in contrast to the hypoxia experiments, there were no *a priori* expectations for changes in glycogen content with exposure to elevated temperature. In the winter experiment, glycogen levels were not significantly different among clams in the three treatments in the first two sampling days, and therefore cannot serve as a predictor of imminent clam death. In the winter and spring experiments, glycogen levels in the room temperature clams increased over time, which for the spring experiment is consistent with the hypoxia experiments.

According to a large body of literature, mild and moderate elevations in temperature result in increased heat shock protein expression and, if cellular proteins are damaged beyond repair, elevations in ubiquitin expression (for review, Feder and Hofmann, 1999; Hofmann et al., 2002; Kregel, 2002; Dahlhoff, 2004). The degree of upregulation and temperature at which expression

is stimulated varies seasonally (e.g., Hofmann and Somero, 1995; Lesser and Kruse, 2004) and is influenced by previous exposure to stressors (Somero, 2002).

Statistically significant treatment- or season-dependent changes in expression levels were not detected for any of the stress proteins measured in this study, including the heat shock proteins. Although these results may seem surprising, they are consistent with recent studies of thermotolerant organisms, such as those demonstrating a temperature-insensitivity of Hsc70 function over the environmentally-relevant range of temperatures for an estuarine fish (Hofmann et al., 2002; Zippay et al., 2004). The extreme thermotolerance exhibited by *M. mercenaria* may result from physiological strategies that buffer the need for a cellular-level response to elevated temperature. The temperatures utilized in this experiment may not have been high enough to trigger a stress protein response, which typically requires thermal denaturation of proteins (Feder and Hofmann, 1999).

Even though the moderate temperature elevation treatment resulted in mortality in the winter experiment, it did not induce a significant stress protein response. Clams in this treatment experienced a substantial (but not significant) increase in sHsp expression between 1 and 5 days and decreases in Hsp60, Hsp70, and ubiquitin expression over the same period. While this pattern could be consistent with extreme cellular stress (Werner and Hinton, 1999; Joyner-Matos et al., 2007), given the lack of statistical significance and lack of detectable response in other experiments, it is unlikely that these changes reflect anything more than random variation. The physiological parameters examined in this study cannot rule out the possibility that the cause of death in the winter experiment clams was a loss of function at a higher level of organization than the cellular level, similar to the “heart failure” documented in intertidal crabs exposed to high temperature (reviewed in Somero, 2002).

Dual stressors. Given the lack of detectable cellular-level response or consistent functional response to hypoxia or high temperature, the ability of *M. mercenaria* to respond to a combination of high temperature and hyposalinity was examined in a second set of experiments. The combined effects of high temperature and hyposalinity are well-studied, likely because this combination is common in coastal habitats, particularly during summer periods of high temperature and substantial rainfall (e.g., Millie et al., 2004; Caccia and Boyer, 2005). While these two factors have been shown to interact in their effects on hard clam pumping rate (for review, Grizzle et al., 2001), it is not clear whether these stressors have an additive effect on bivalve growth, development, or survival (Cain, 1973; Lough and Gonor, 1973).

While *M. mercenaria* are tolerant of a wide range of temperatures, their distribution patterns suggest the clams are not as tolerant of hyposalinity (Wells, 1957; Harte, 2001). *Mercenaria mercenaria* function as osmoconformers, allowing their pallial fluids and hemolymph to be isosmotic with the environment when their valves are open (reviewed in Grizzle et al., 2001). The clams usually close their valves, however, when the environmental salinity drops by 50%, a response that impacts the ability of the clams to feed and grow (Carmichael et al., 2004). Pumping rates, ability to bury, and growth are maximized between 20 – 30 ppt, and decrease dramatically above 32 ppt and below 15 ppt (Grizzle et al., 2001). Survival is significantly decreased at salinities ranging from 5 – 10 ppt (Winn and Knott, 1992). Hard clams are particularly susceptible to rapid decreases in salinity, experiencing substantial mortality following a decrease of approximately 20 ppt over a 24-hour period (Baker et al., 2005). Such rapid fluctuations often occur where hard clams live, due either to rainfall events or anthropogenic activities such as water discharges (Caccia and Boyer, 2005; Wilson et al., 2005). A multiyear survey of salinity at sites close to where the clams utilized for this study are

maintained revealed daily and seasonal variation in salinity (Baker et al., 2005). Across three years, salinities along the Florida Gulf coast ranged from approximately 5 ppt to 35 ppt, with daily fluctuations ranging from 5 to 24 ppt in magnitude (Baker et al., 2005).

In the dual stressor experiments, the effects of three levels of salinity (approximately 25, 15, and 5 ppt) at each of two temperatures, room temperature and high temperature (average 34°C) were examined. A strong additive effect of the two stressors was detected in the pattern of survivorship of clams in all three seasons. Regardless of season, the clams exposed to the most extreme set of conditions (high temperature/moderate hyposalinity) had the lowest survivorship, with all clams in this treatment dead by day 4 or 5. In the winter experiment, a strong effect of temperature on survival was again documented, with clams in all of the high temperature treatments dead by day 7. A similar additive effect of high temperature and hyposalinity has been documented for growth of *M. mercenaria* larvae (discussed in Lough and Gonor, 1973) and survival of larvae of the estuarine clam *Rangia cuneata* (Cain, 1973). Among the room temperature treatments, only the moderate hyposalinity treatment resulted in clam death, with the greatest effect of this treatment detected during the spring experiment, which is consistent with reports of hard clam susceptibility to low salinity.

It is less clear whether salinity alone or salinity in combination with high temperature affected the burial abilities of clams in the dual stressor experiments. In the fall and spring experiments, none of the clams exposed to high temperature and hyposalinity buried after 24 hours of exposure, the only time point with surviving clams in all six treatments. In the winter, one clam in the high temperature/mild hyposalinity treatment buried, and none of the clams in the high temperature/moderate hyposalinity treatment buried. In contrast, some clams exposed to high temperature/ambient salinity in the fall and winter experiments buried on the first day of

sampling, which suggests that high temperature alone was not sufficient to inhibit burial. Interestingly, burial ability was strongly influenced by hyposalinity. Only two of the clams exposed to room temperature/moderate hyposalinity buried in the fall experiment and none of the clams in this treatment buried in the winter or spring experiments, even though they survived to nearly the end of the experiment. In nearly all cases, burial abilities of clams in the room temperature/mild hyposalinity treatments were lower than those of clams in the room temperature/ambient salinity treatments. The few studies that have examined the effects of hyposalinity on burial ability or burial rate of bivalves have produced conflicting results but have the general trend of decreased burial in hyposaline conditions, regardless of temperature (Grizzle et al., 2001; Lardies et al., 2001; Matthews and Fairweather, 2004)

In contrast to the significant effects of the single and dual stressors on survival and burial ability, there were no clear effects of the dual stressor treatments on glycogen content. At day 1, the only sampling day with all treatments present, there were no significant differences in glycogen content in any of the three seasons. Unlike in previous experiments, glycogen content did not decrease over time. A causal relationship between glycogen content and salinity or high temperature has not been established for bivalves, but several studies have reported seasonal correlations between reproductive cycle, glycogen content, and environmental temperatures, salinities and chlorophyll levels (e.g., Li et al., 2006).

A causative relationship between hypersalinity and free radical production is well-established in plant physiology and biomedical fields such as nephrology and immunology (e.g., Hernández et al., 1993; Qin et al., 1999; Hizoh and Haller, 2002). However, whether hyposalinity is liked to alterations in free radical metabolism and oxidative damage is not understood. A recent study of hyposalinity responses of a marine alga detected an elevation in

glutathione but not in antioxidant enzymes such as catalase and superoxide dismutase (Jahnke and White, 2003). Similarly inconsistent results in other studies, all of which have been conducted on plants, have not yet determined whether hyposalinity causes elevated free radical production and oxidative damage. In the current study, some evidence of a link between hyposalinity and oxidative damage to RNA was detected in the fall and spring experiments, but not in the winter experiment. In the fall experiment, clams exposed to hyposalinity had more 8-oxoGuo than clams exposed to ambient salinity, regardless of temperature treatment. In the spring experiment, clams in the room temperature/hyposalinity treatments again had higher 8-oxoGuo levels than clams in the ambient salinity treatment. In neither fall nor spring was there a significant effect of temperature or an interaction between temperature and salinity. These results suggest that clams exposed to hyposalinity experienced greater oxidative damage to their RNA, either due to elevated free radical production or to a diminished ability to repair oxidative damage. In the winter experiment, in contrast, RNA oxidation was closely linked to temperature (higher oxidative damage in clams in the high temperature treatment) but not related to salinity level. These results suggest that the high temperature treatment severely limited the ability of the clams to minimize or repair oxidative damage, but do not give any indication of what may have caused the elevated oxidative damage.

An examination of stress protein expression levels in clams from the dual stressors experiment may provide further evidence for a link between hyposalinity and oxidative damage and also may provide further evidence for an additive effect of high temperature and hyposalinity. The tissue samples for this analysis have been processed but the stress protein analyses are not yet complete. Several studies of stress protein expression (particularly heat shock protein expression) in estuarine invertebrates exposed to hyposalinity or hyposalinity/high

temperature treatments have produced conflicting results (Kultz, 1996; Clark et al., 2000; Werner and Hinton, 2000; Spees et al., 2002; Werner, 2004; Blank et al., 2006). The combination of high temperature and hyposalinity was lethal for *M. mercenaria*, particularly in the winter experiment. While the sublethal hypoxia treatments did not cause detectable stress protein responses and no significant pattern of stress protein expression was detected in the sublethal or lethal temperature treatments, the combination of high temperature and hyposalinity may trigger a stress protein response. The oxidative damage results suggest that the clams in the dual stressor experiments are experiencing cellular-level damage, and this damage may prove to be sufficient to induce changes in stress protein expression.

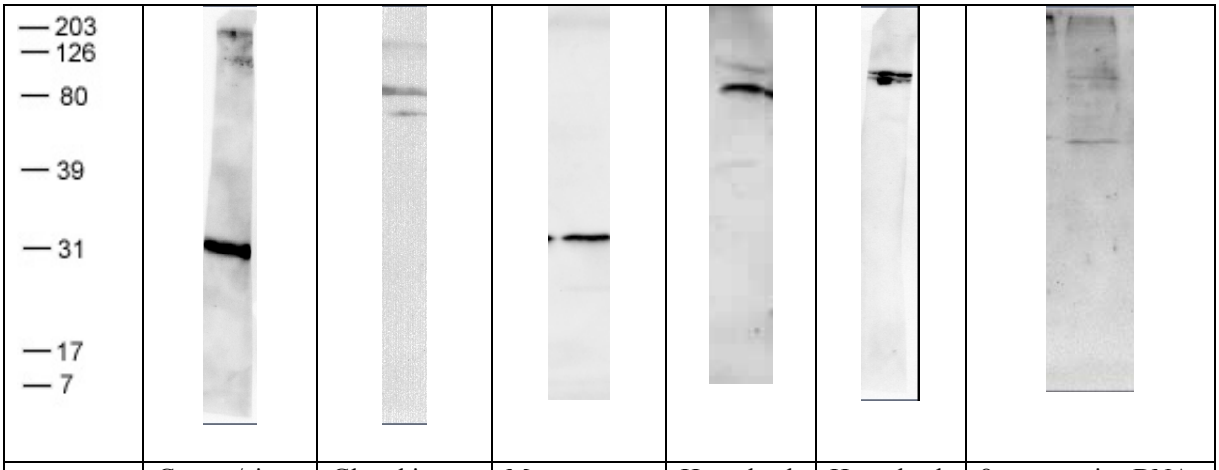
Conclusions. The effects of the single- and dual-stressor treatments on functional and cellular-level responses in *M. mercenaria* illustrate the importance of examining a variety of physiological responses, across several levels of biological organization. For example, based on the extensive studies of heat shock protein expression and thermotolerance in rocky intertidal invertebrates, the seasonal susceptibility to high temperature demonstrated in this study would be predicted to be accompanied by changes in heat shock protein expression. It is unlikely that the high variance in stress protein expression levels masked any treatment-related changes as a similar level of variance (with the same sample size and methodology) existed in the data set presented in Chapter 2, and significant changes were detected in that study. Many of the potential sources of variance in stress protein expression that were discussed in Chapter 2 (page 48), as well as the methodological steps taken to minimize that variance, are applicable to this study as well. The fact that no significant pattern in stress protein expression was detected, particularly during those seasons in which the treatments caused mortality, highlights the

importance of assessing multiple physiological responses, particularly the more sensitive whole-animal responses such as burial ability.

Not surprisingly, a strong effect of season was detected in all experiments and in nearly all measured parameters. Several environmental parameters, including salinity, temperature, food availability, and dissolved O_2 levels, vary seasonally in estuarine habitats and thus influence the growth, reproductive cycle, and stress responsiveness of estuarine organisms.

In all seasons, functional responses tended to decrease over the duration of the experiment, a trend that has been documented previously. It cannot be determined from this study whether these declines resulted from prolonged maintenance in laboratory conditions or whether they would occur in the field. In their habitat the clams likely would not experience prolonged exposure to the equivalent of the most extreme treatments, particularly the high temperature/moderate hyposalinity treatment, since abiotic conditions in estuaries fluctuate daily. However, since the functional declines occurred in the control treatments as well as the experimental treatments, it cannot be concluded that the functional declines represent reduced tolerance of the stressors.

Finally, although an additive effect of high temperature and hyposalinity was apparent in the survival analyses, we were unable to detect additive effects on cellular-level or functional markers. These results suggest that we did not characterize the cause of mortality in the dual stressor experiment. Examination of a wider variety of functional metrics and stress protein expression levels may lead to an understanding of how the dual stressors affect the hard clams in the laboratory and how tolerance of these abiotic factors may influence species distribution.



MWM	Copper/zinc superoxide dismutase	Glutathione peroxidase	Manganese superoxide dismutase	Heat shock protein 60	Heat shock protein 70	8-oxoguanine DNA glycosylase

Figure 3-1. Antibody specificity tests in *M. mercenaria* whole clam homogenates. Molecular weight markers (in kilodaltons) are specified in the first column.

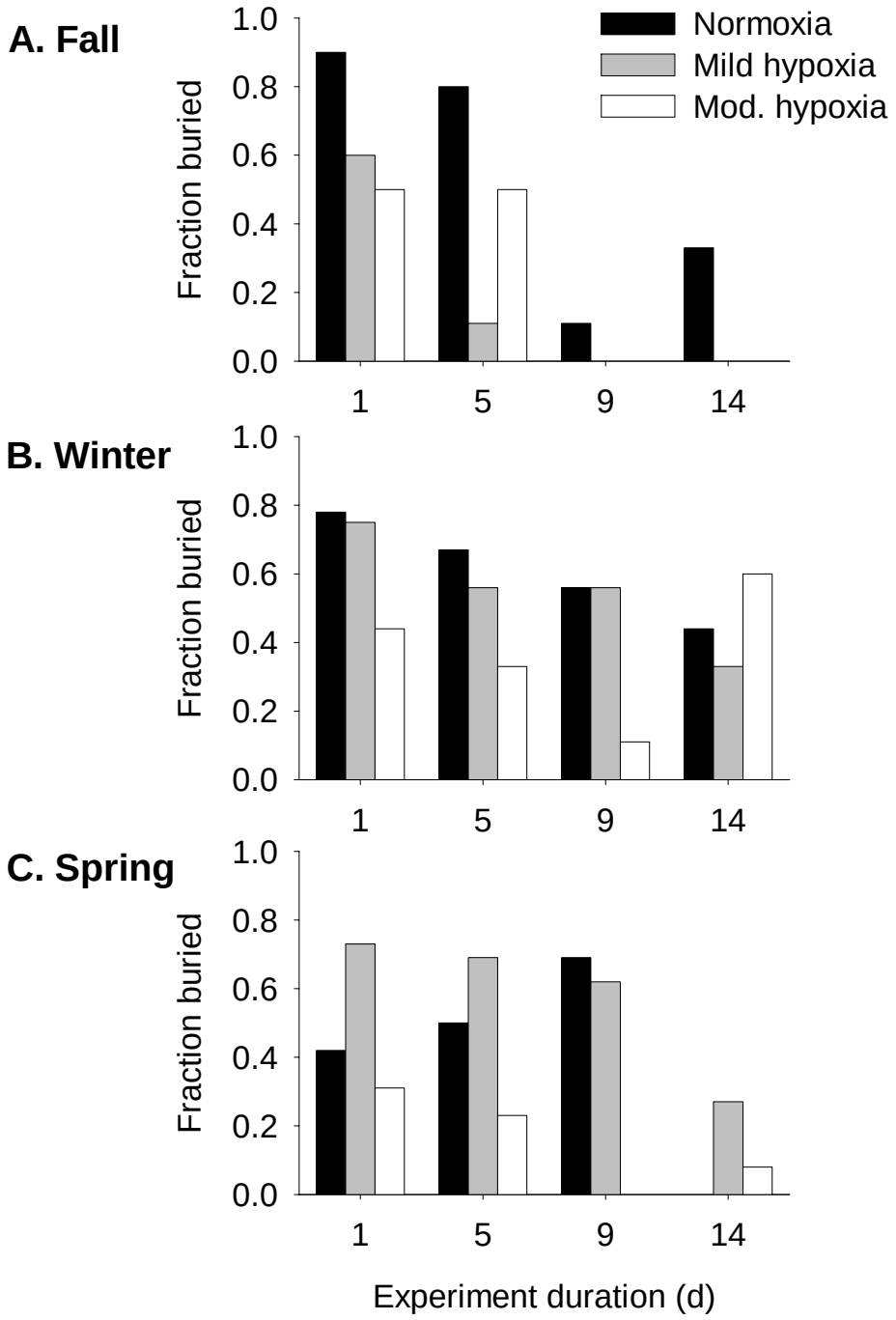


Figure 3-2. Fraction of clams that buried in the three hypoxia experiments. Data are expressed as a summary of fraction buried at the 60 minute time point of each sampling date. Experiment duration is in days.

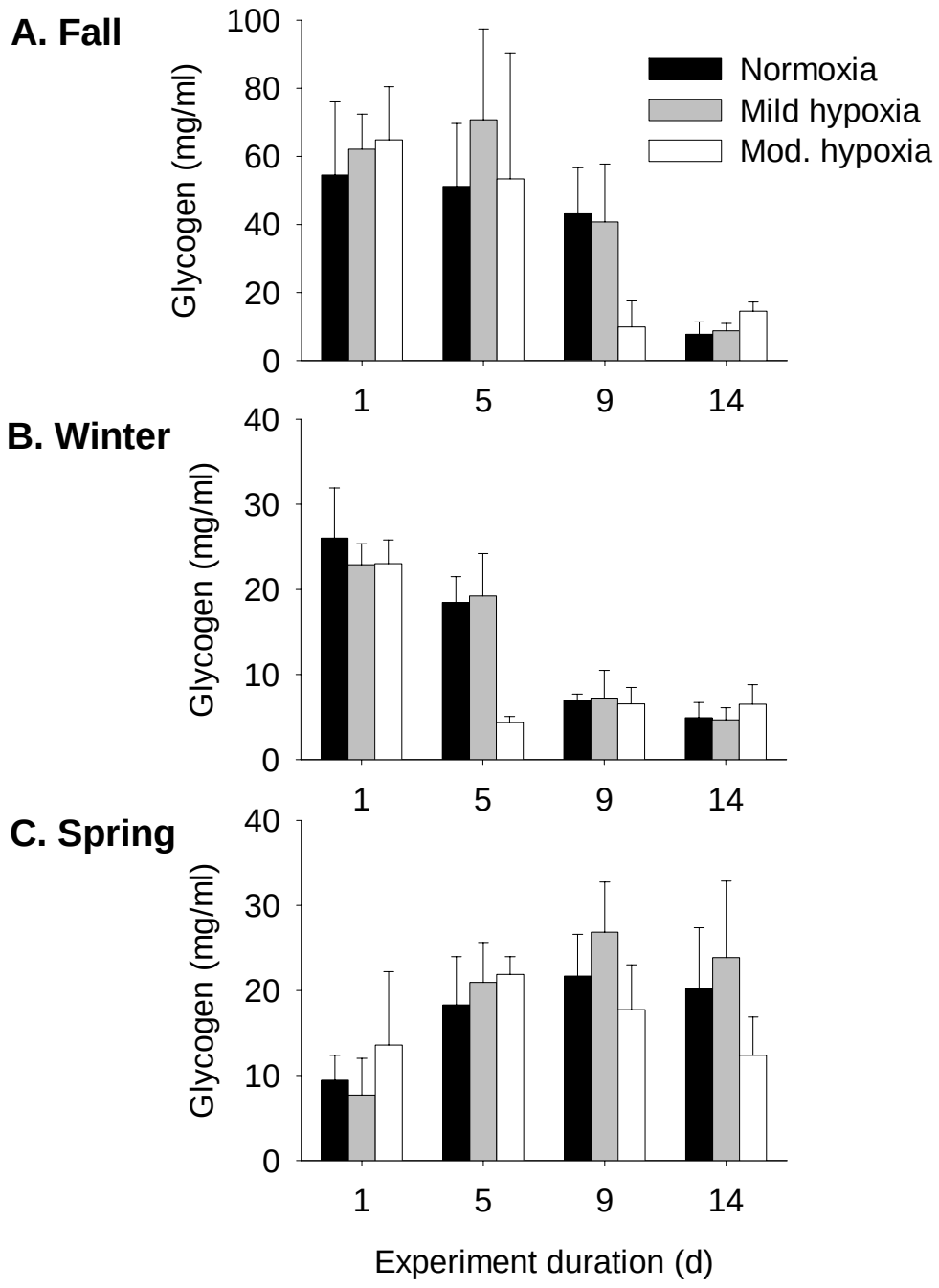


Figure 3-3. Glycogen content of whole clams in the three hypoxia experiments. Data are expressed as mean ± SD. Experiment duration is in days. Abbreviations: mg, milligrams; ml, milliliters.

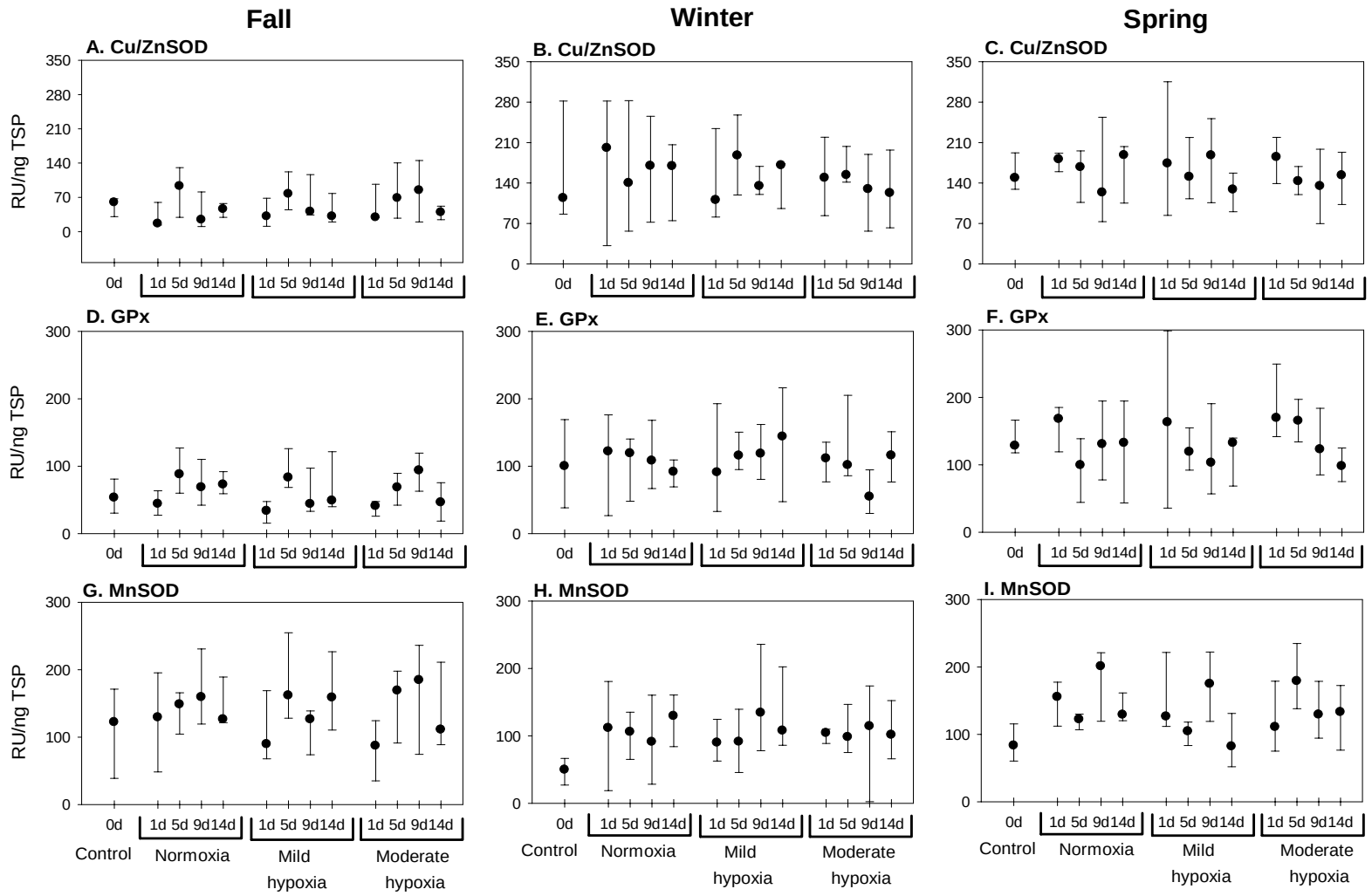


Figure 3-4. Stress protein expression levels in clams from hypoxia experiments. Data are expressed as relative units of expression per nanogram of total soluble protein (RU/ng TSP) and are presented as scatterplots with asymmetrical error bars. The dot represents the median and the error bars define the minimum and maximum. Abbreviations: d, day; mod, moderate; Cu/Zn SOD, copper/zinc superoxide dismutase; GPx, glutathione peroxidase; MnSOD, manganese superoxide dismutase.

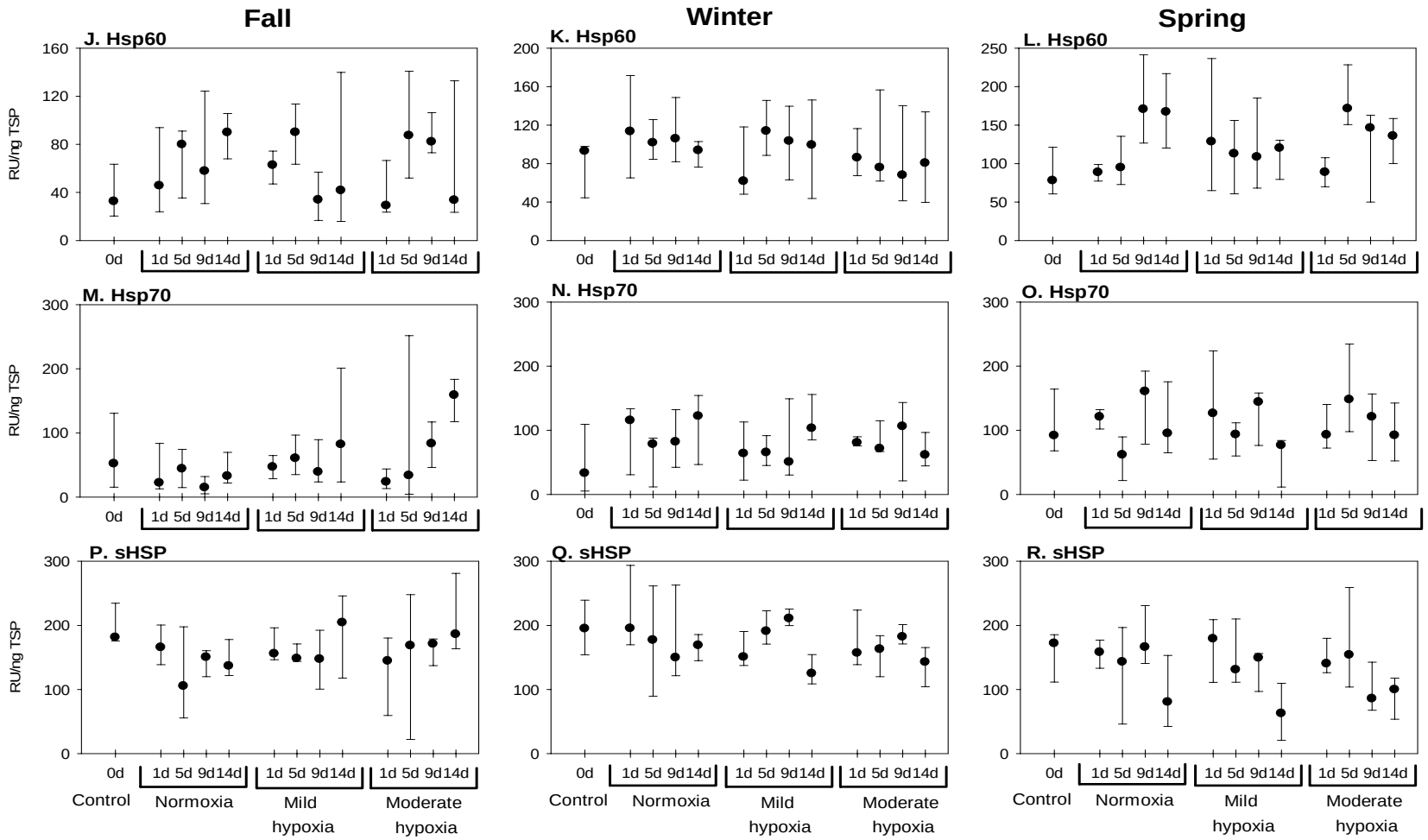


Figure 3-4 continued. Stress protein expression levels in clams from hypoxia experiments. Data are expressed as relative units of expression per nanogram of total soluble protein (RU/ng TSP) and are presented as scatterplots with asymmetrical error bars. The dot represents the median and the error bars define the minimum and maximum. Abbreviations: d, day; mod, moderate; Hsp60, heat shock protein 60; Hsp70, heat shock protein 70; sHsp, small heat shock protein.

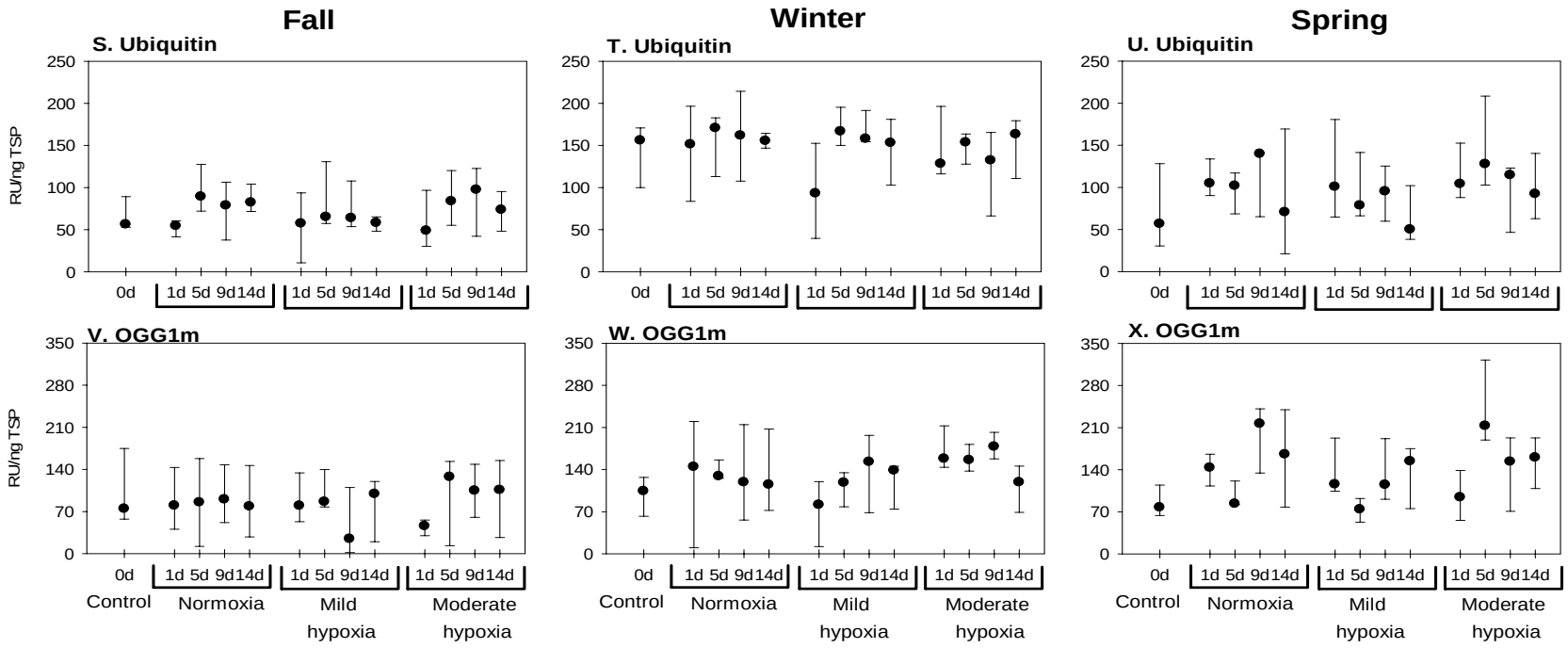


Figure 3-4 continued. Stress protein expression levels in clams from hypoxia experiments. Data are expressed as relative units of expression per nanogram of total soluble protein (RU/ng TSP) and are presented as scatterplots with asymmetrical error bars. The dot represents the median and the error bars define the minimum and maximum. Abbreviations: d, day; mod, moderate; OGG1m, 8-oxoguanine DNA glycosylase.

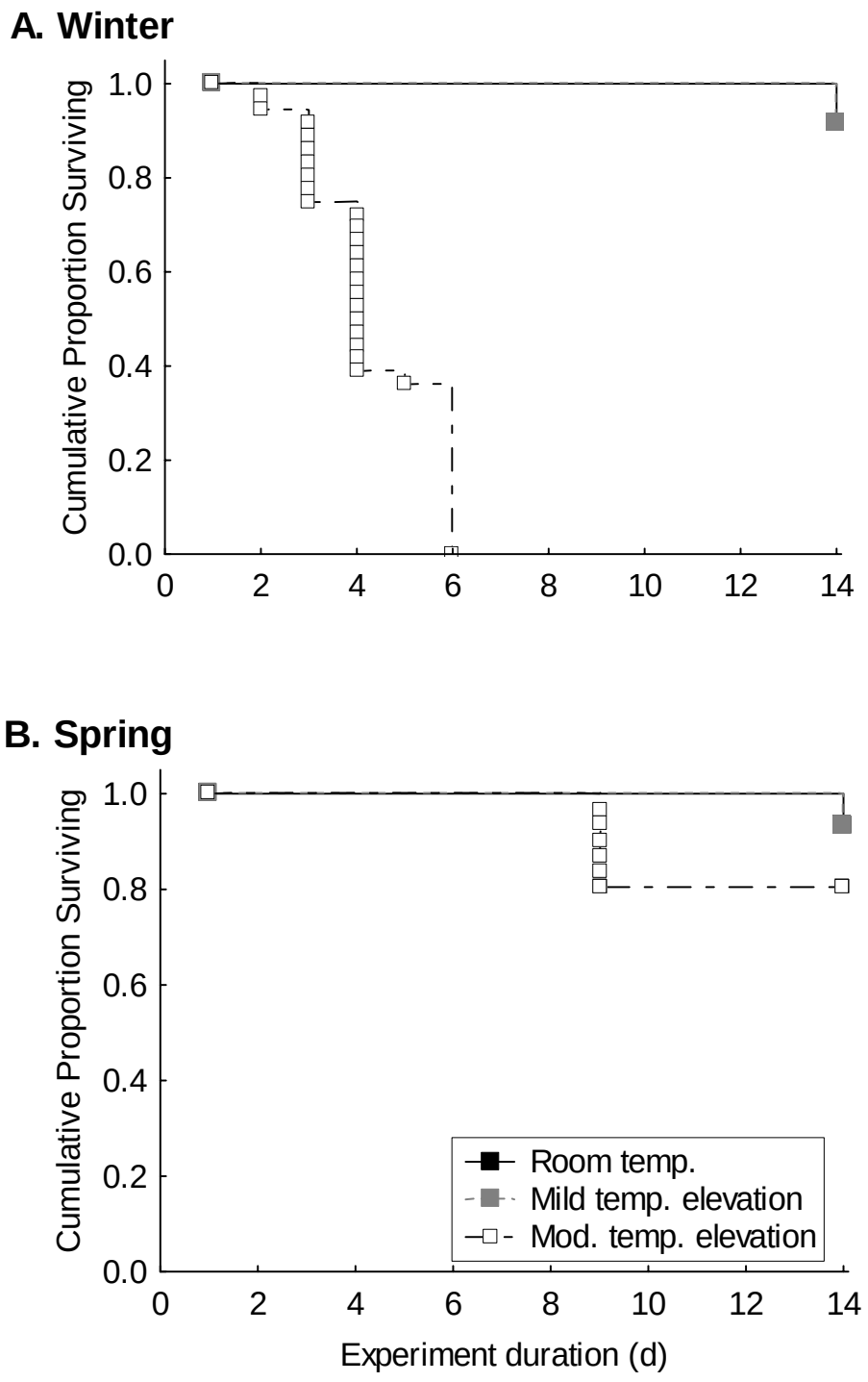


Figure 3-5. Survivorship of clams in the winter (A) and spring (B) temperature experiments. Data are expressed as cumulative proportion of surviving clams. Each symbol represents one clam on the day the clam died. Experiment duration is in days. Symbols for treatments with identical survival patterns (room temperature and mild temperature elevation) are overlaid, with only one treatment visible.

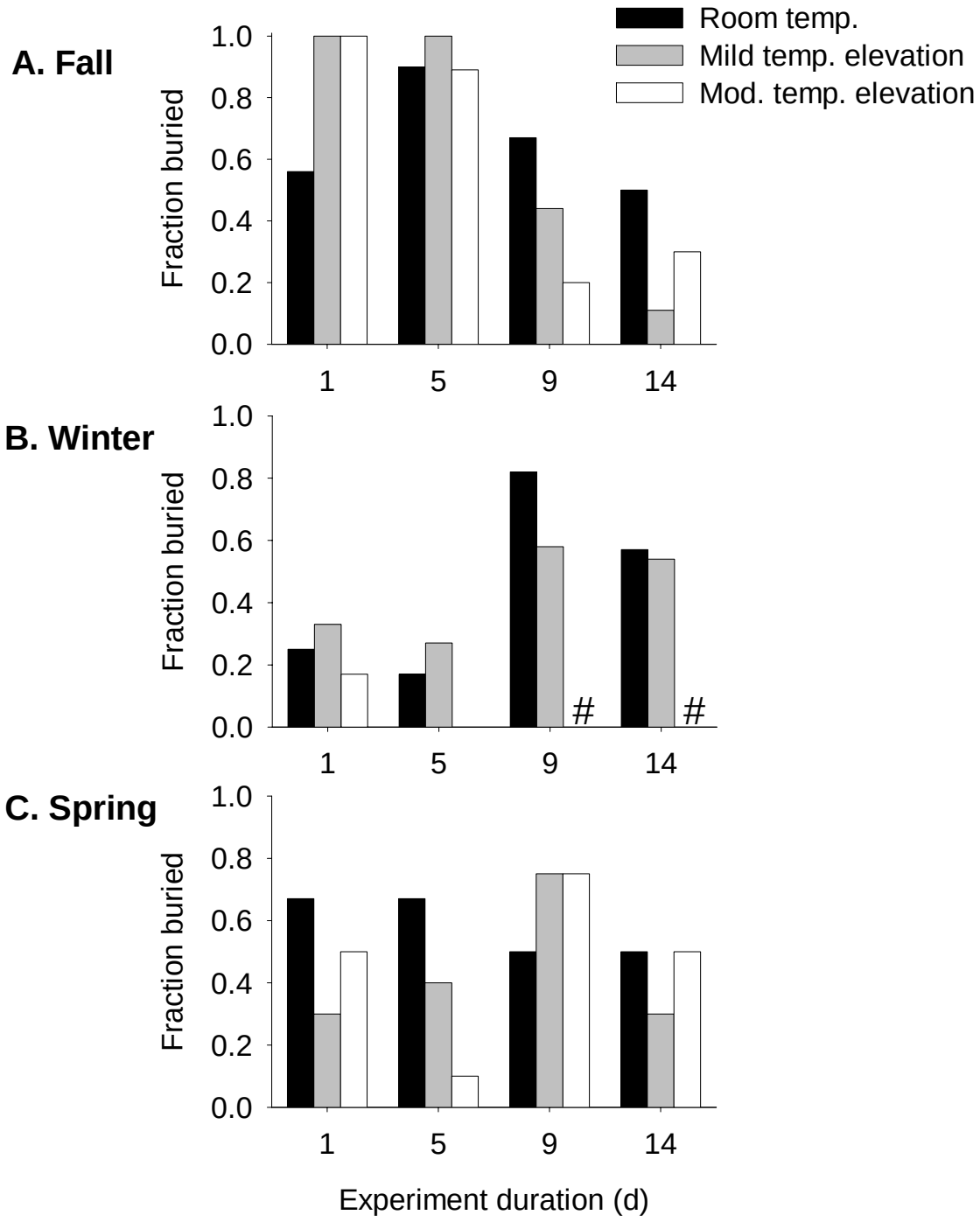


Figure 3-6. Fraction of clams that buried in the three temperature experiments. Data are expressed as a summary of fraction buried at the 60 minute time point of each sampling date. Experiment duration is in days. Number signs indicate that all clams in that treatment were dead.

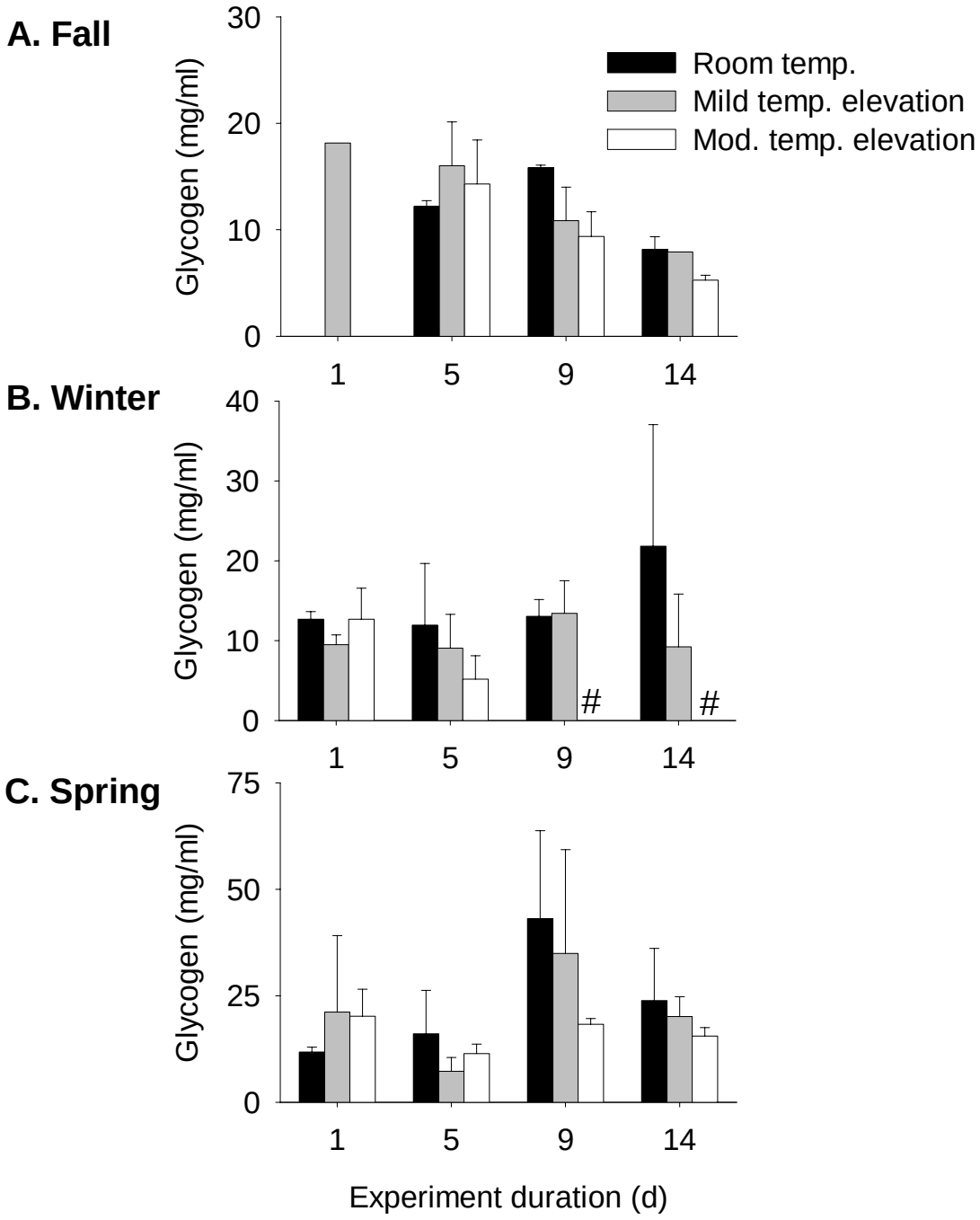


Figure 3-7. Glycogen content of whole clams in the three temperature experiments. Data are expressed as mean ± SD. Experiment duration is in days. Abbreviations: mg, milligrams; ml, milliliters. Number signs indicate that all clams in that treatment were dead.

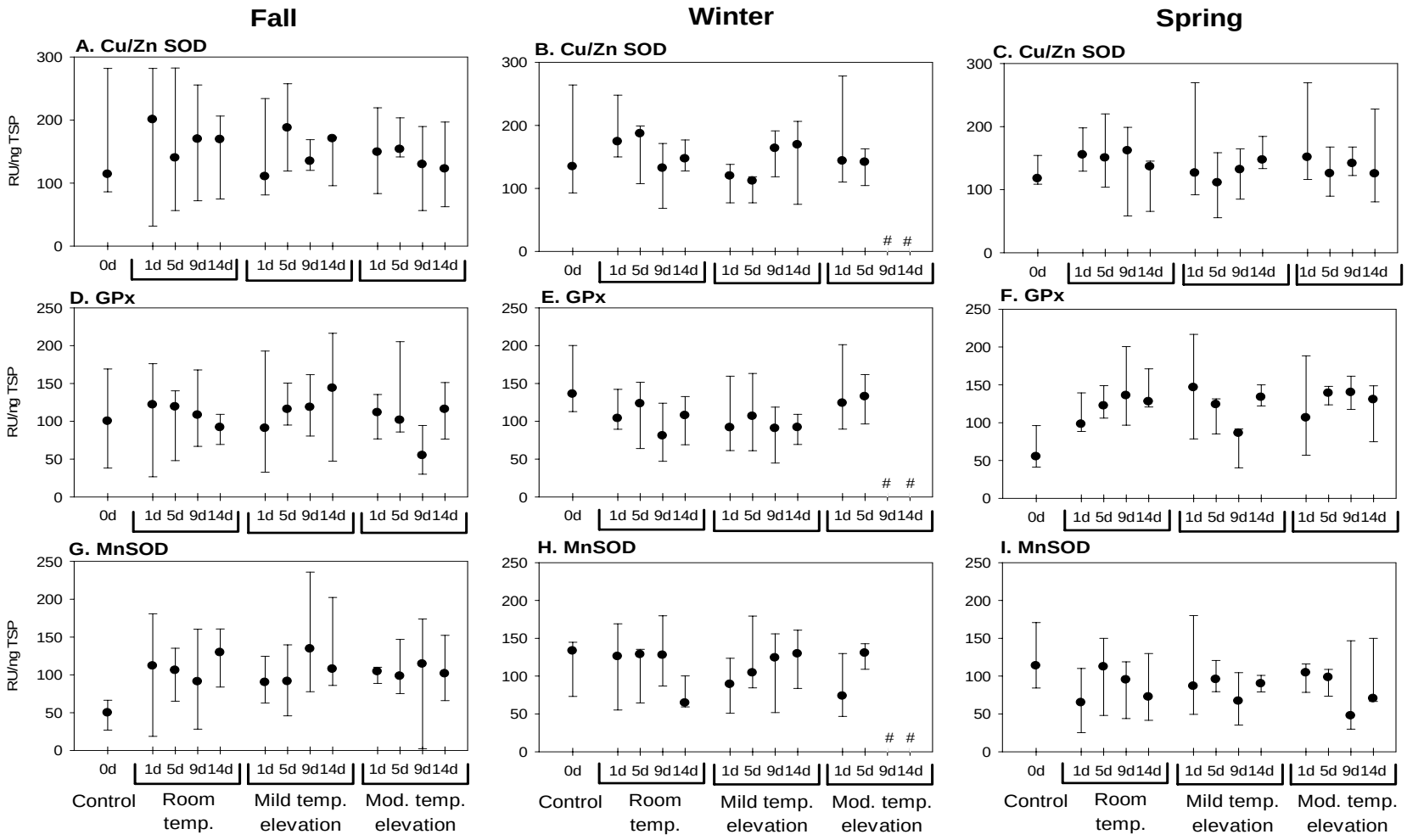


Figure 3-8. Stress protein expression levels in clams from temperature experiments. Data are expressed as relative units of expression per nanogram of total soluble protein (RU/ng TSP) and are presented as scatterplots with asymmetrical error bars. The dot represents the median and the error bars define the minimum and maximum. Abbreviations: d, day; mod, moderate; Cu/Zn SOD, copper/zinc superoxide dismutase; GPx, glutathione peroxidase; MnSOD, manganese superoxide dismutase. Number signs indicate clam death.

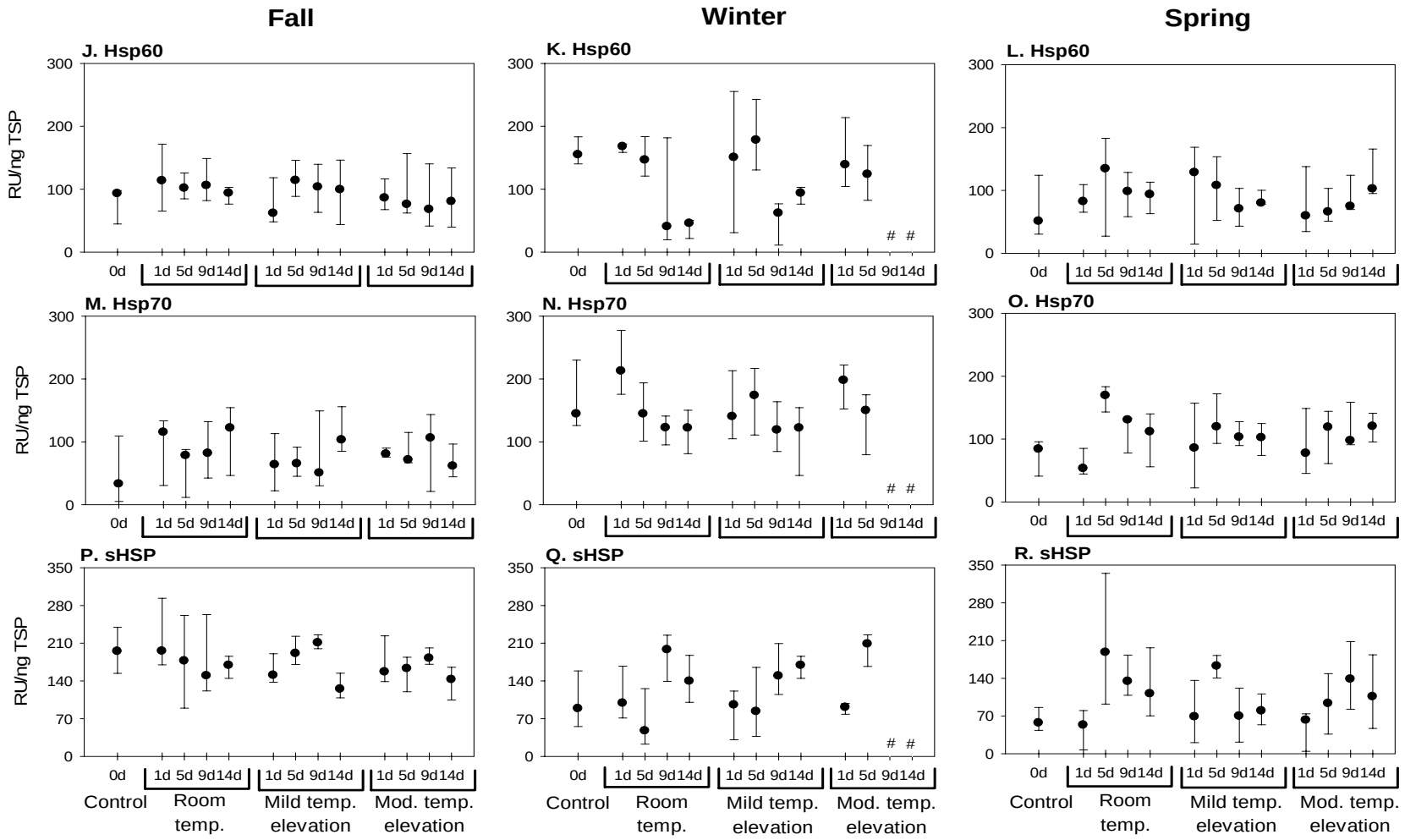


Figure 3-8 continued. Stress protein expression levels in clams from temperature experiments. Data are expressed as relative units of expression per nanogram of total soluble protein (RU/ng TSP) and are presented as scatterplots with asymmetrical error bars. The dot represents the median and the error bars define the minimum and maximum. Abbreviations: d, day; mod, moderate; Hsp60, heat shock protein 60; Hsp70, heat shock protein 70; sHsp, small heat shock protein. Number signs indicate clam death.

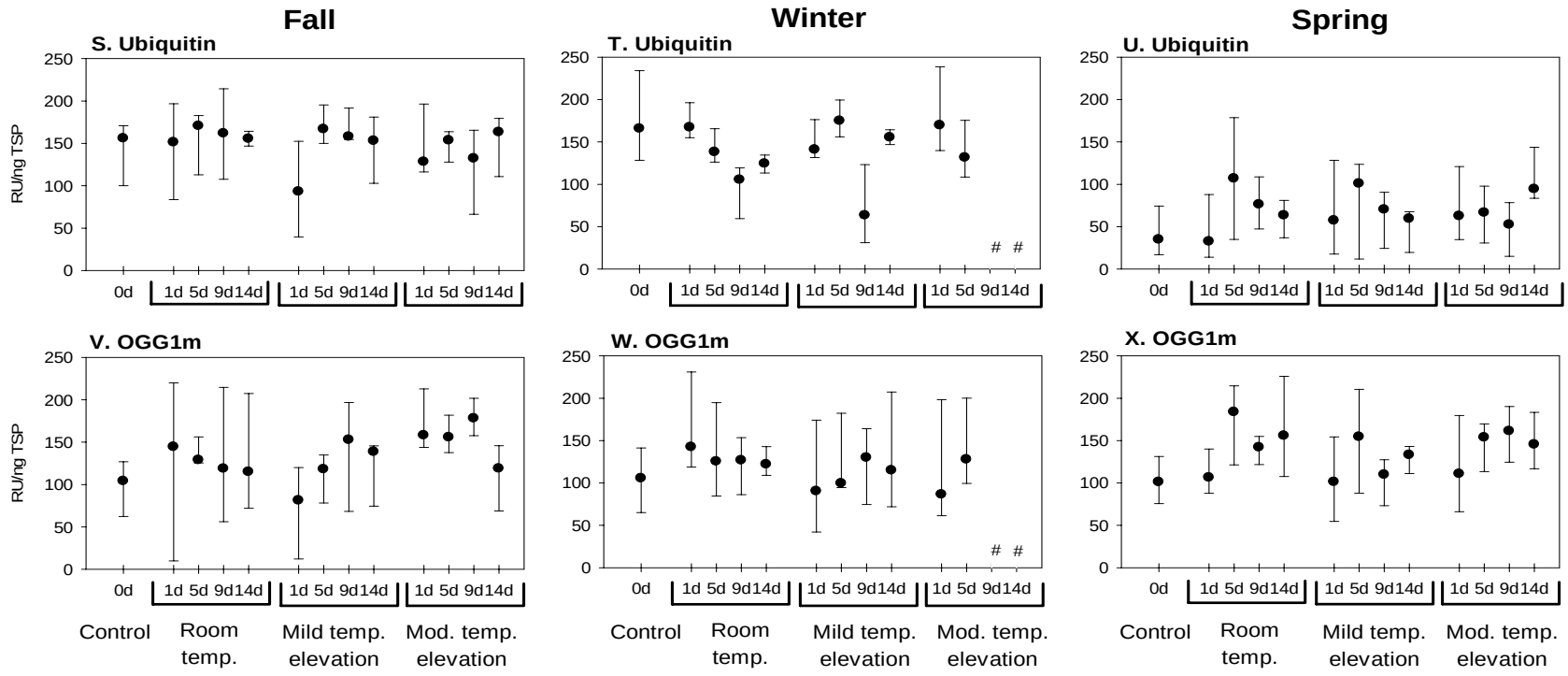


Figure 3-8 continued. Stress protein expression levels in clams from temperature experiments. Data are expressed as relative units of expression per nanogram of total soluble protein (RU/ng TSP) and are presented as scatterplots with asymmetrical error bars. The dot represents the median and the error bars define the minimum and maximum. Abbreviations: d, day; mod, moderate; OGG1m, 8-oxoguanine DNA glycosylase. Number signs indicate clam death.

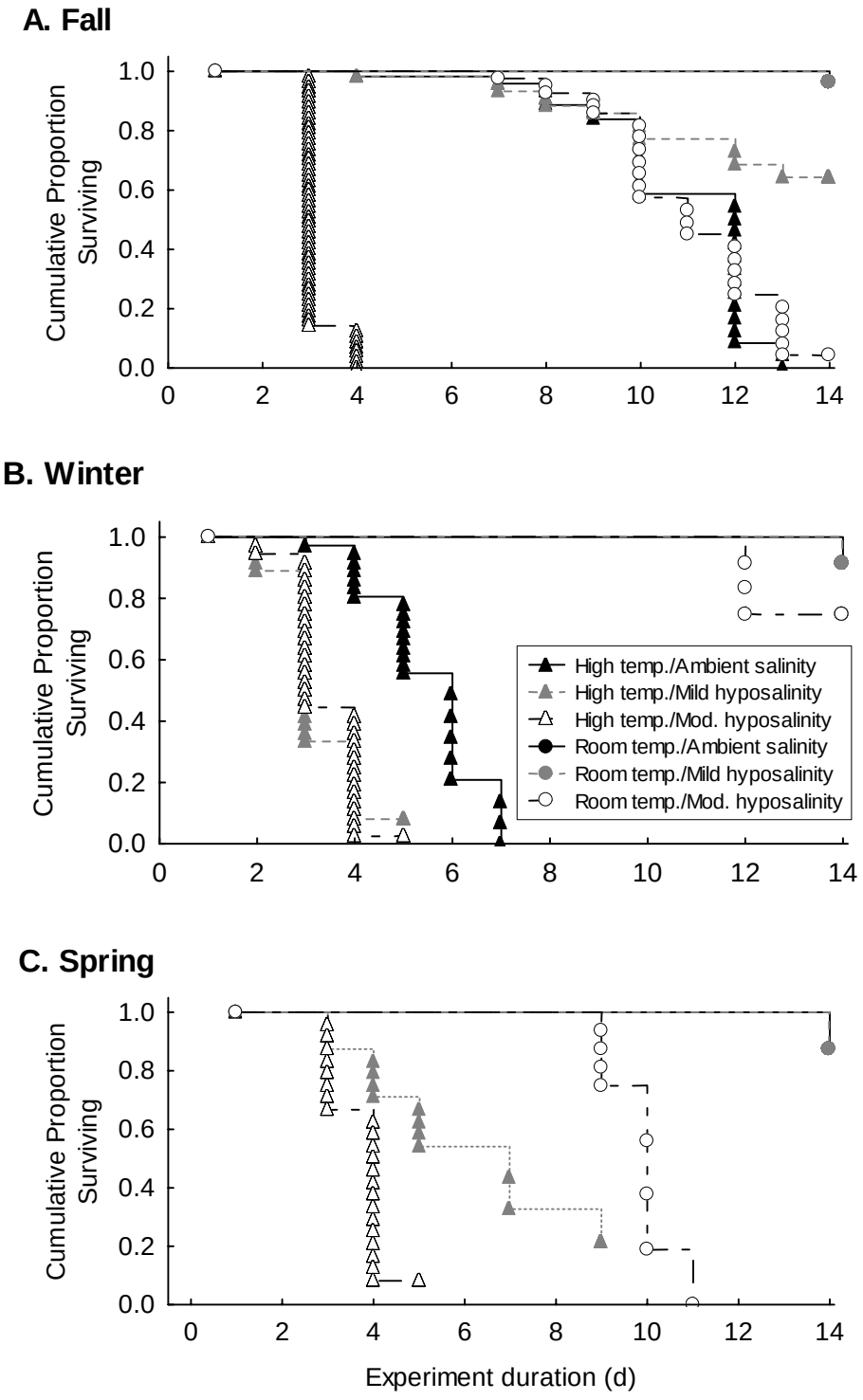


Figure 3-9. Survivorship of clams in dual stressor experiments. Data are expressed as cumulated proportion of surviving clams. Each symbol represents one clam on the day it died. Experiment duration is in days. Treatments with identical survival patterns are overlaid. The high temperature/ambient salinity treatment is missing from the spring.

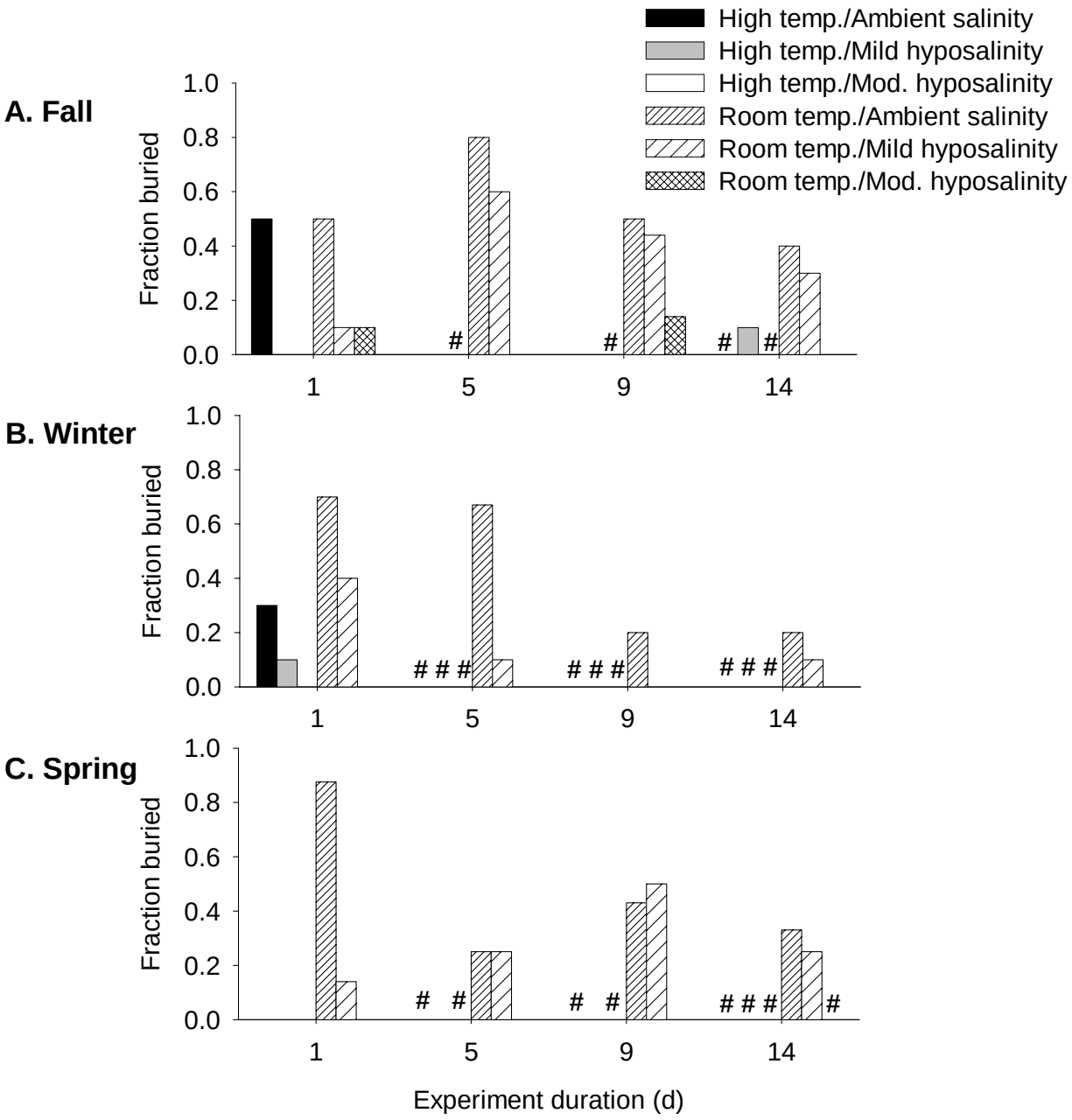


Figure 3-10. Fraction of clams that buried in the dual stressor experiments. Data are expressed as a summary of fraction buried at the 60 minute time point of each sampling date. Experiment duration is in days. Number signs indicate that all clams in that treatment were dead.

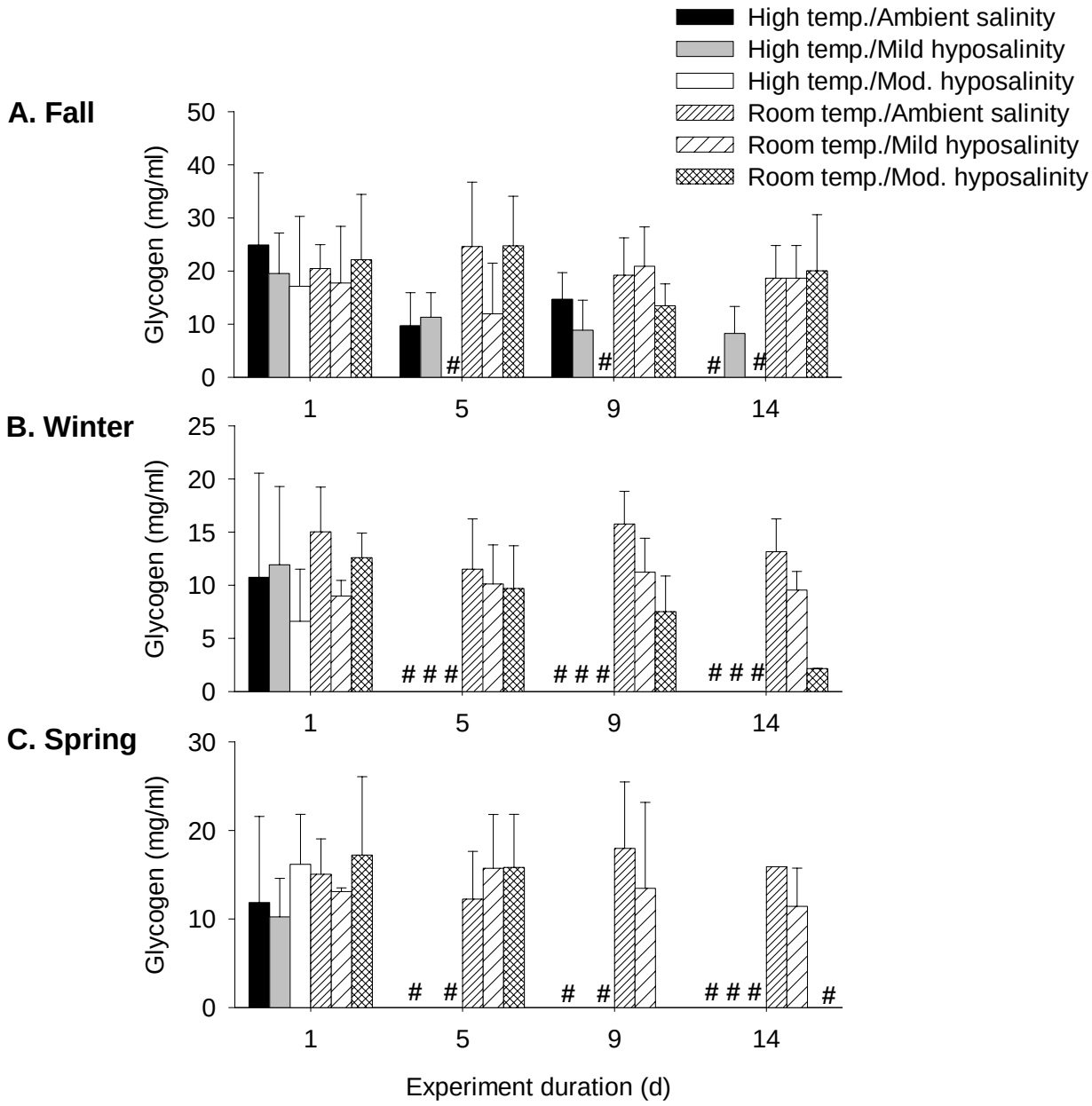


Figure 3-11. Glycogen content of whole clams in the dual stressor experiments. Data are expressed as mean ± SD. Experiment duration is in days. Abbreviations: mg, milligrams; ml, milliliters. Number signs indicate that all clams in that treatment were dead.

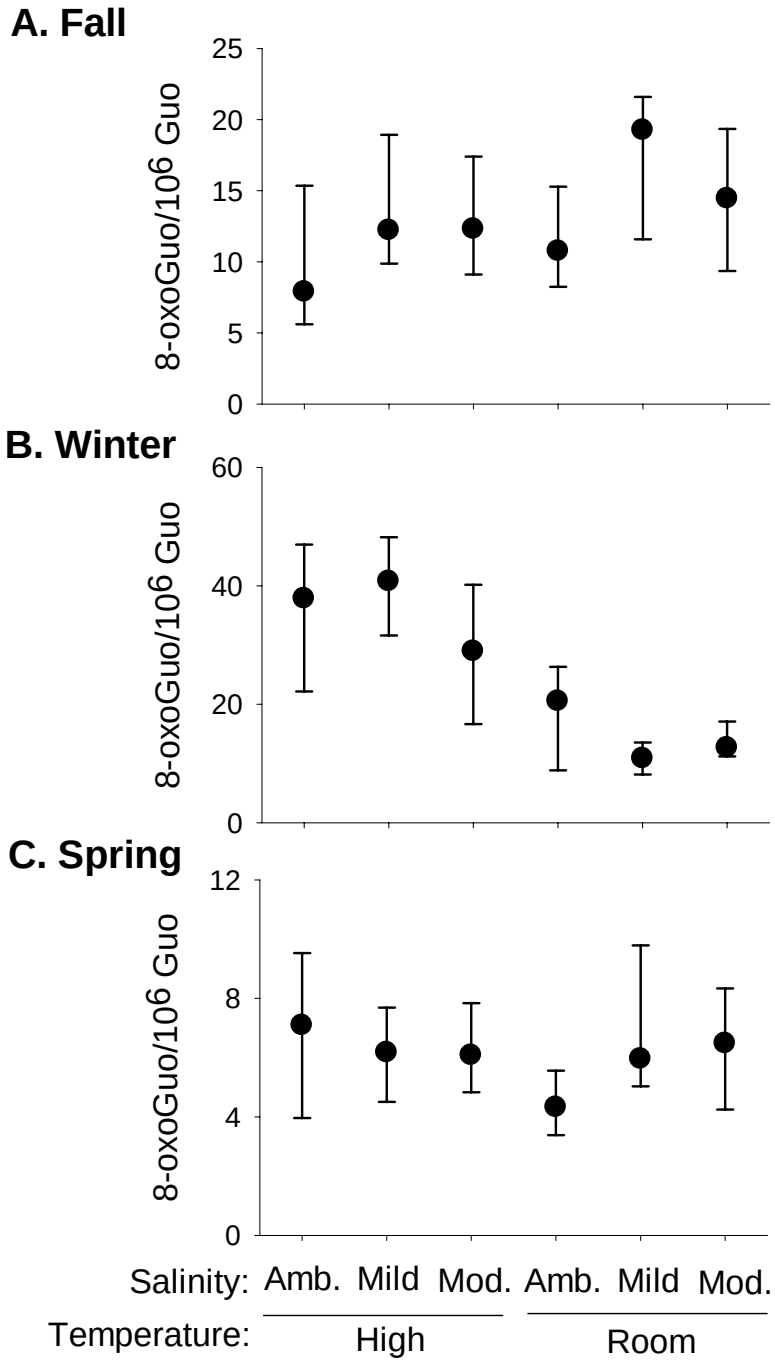


Figure 3-12. RNA oxidation after 24 hour exposure in dual stressor experiments. Data are presented as scatterplot of median with uneven error bars denoting minimum and maximum values. Note different scales on the three graphs. Abbreviations: 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; Guo, guanosine; amb, ambient; mod, moderate.

CHAPTER 4
STRESS RESPONSE OF A FRESHWATER CLAM ALONG AN ABIOTIC GRADIENT: TOO MUCH OXYGEN MAY LIMIT DISTRIBUTION

Introduction

The size and location of a species' range are dynamic and influenced by evolutionary constraints (Holt, 2003), biotic interactions (Case et al., 2005) and abiotic factors (Brown et al., 1996), acting either singly or in combination. Abiotic factors may influence a species' distribution by stressing organisms beyond their physiological limits (Parsons, 1991). Near the edge of a species' distribution, particularly if that edge is influenced by abiotic factors, we expect to find decreased abundance as well as decreased body condition of individuals. Condition has traditionally been assessed using whole-organism characters such as body size and fecundity (Caughley et al., 1988), but cellular-level indicators may be at least equally useful for assessing condition. Such cellular-level indicators include: RNA:DNA ratio as an indicator of protein synthesis and growth (Dahlhoff, 2004); DNA and RNA oxidation as indicators of cellular damage from free radicals (Halliwell and Gutteridge, 1999); and upregulated expression of specific stress proteins, such as heat shock proteins, as indicators of a homeostatic cellular response to a stressor (e.g., Hofmann and Somero, 1995; Downs et al., 2001a; Abele and Puntarulo, 2004). Therefore, these indicators provide information about cellular metabolic and homeostatic responses to stress, and thus may be more sensitive than whole-organism characters for determining how abiotic factors affect the condition and distribution of individuals in a population.

In the current study we used cellular-level indicators to test the following hypothesis: if a population's distribution overlies a stressful environmental gradient, then abundance should decrease near the distribution edge, and edge individuals should have increased stress and lower condition. In selecting the appropriate system, we looked for a stable population of sessile

animals in an environment with a stable gradient of a small number of potentially stressful abiotic factors. For this study, we examined the distribution and physiology of a population of small freshwater clam, *Sphaerium* sp., which inhabits a swamp/stream system in western Uganda. This habitat contains a relatively stable dissolved O_2 (DO) gradient, from freshwater tributary streams with normoxic water (equilibrated with atmospheric O_2 levels) to a papyrus swamp with very hypoxic water (DO less than 10% of fully-aerated water), and a pH gradient from the neutral stream water to acidic swamp water. A 2-year, broad-scale survey of macroinvertebrates in this system (Chapman et al., 2004) revealed that the clams were very abundant in the swamp but largely absent from regions of tributary streams with normoxic water and neutral pH, despite the absence of any apparent physical or biotic barriers limiting access to stream sites. Since sphaeriid clams are ovoviviparous (i.e., they internally brood their young; Mackie, 1978), it appears that these clams do not spend any part of their life cycle in normoxic, neutral pH water. This distribution pattern could arise because hypoxic and acidic conditions are correlated with other factors that make the swamp a habitat of high suitability or, alternatively, because the hypoxic and/or acidic conditions are favorable for these clams. This latter possibility is especially intriguing because it is typically assumed that marine and freshwater invertebrates avoid hypoxic and/or acidic conditions when possible, or utilize physiological mechanisms to compensate for the resulting hypometabolism and acidosis when the conditions cannot be avoided (Burnett, 1997).

To test whether the sphaeriid clams, which are abundant in the hypoxic and acidic swamp, show evidence of physiological stress in the normoxic and neutral pH conditions of the tributary streams, we measured clam distribution and cellular-level indicators along a gradient from the swamp into a tributary stream. In addition, we measured the limnological characters of DO,

conductivity, pH, temperature and transparency, and we quantified the relationship between these characters and clam density. To examine how the abiotic gradient along this transect affects the physiological condition of the clams, we sampled clams from a reference site from the dense swamp interior and from three sites along the transect, including the extreme edge of the population's distribution. We assessed RNA:DNA ratio, oxidized DNA and RNA bases, and stress protein expression levels, including several stress proteins that indicate generalized cellular stress, as well as those that specifically respond to increased free radical production, which has been linked to fluctuations in DO and pH (Boveris and Chance, 1973; Li et al., 2002; Veselá and Wilhelm, 2002). Our goal was to determine whether the patterns of cellular indicators were consistent with increased stress in clams near the edge of their distribution and furthermore whether the pattern was consistent with increased oxidative damage.

Materials and Methods

Study Site

This study was conducted in a swamp/stream system in Kibale National Park in western Uganda (0º13' –0º41'N, 30º19' –30º32'E). The park is an equatorial moist evergreen forest, transitional between lowland rain forest and montane forest, with distinct bimodal wet and dry seasons (Chapman et al., 1997). The Rwembaita Swamp is a large (approximately 6.5 km in length) papyrus (*Cyperus papyrus*) swamp, with dense papyrus stands that can reach 5 m in height and form a closed canopy. The low rates of mixing and low incident light induced by the heavy forest of papyrus sedge, and high rates of organic decomposition produce hypercapnic (elevated dissolved CO_2) and hypoxic water (Chapman et al., 2001). Over a 3-year period, Chapman *et al.* (2000) reported DO in the Rwembaita Swamp averaging 0.0466 ± 0.0072 mmol

L^{-1} across dry and rainy seasons.[1] The swamp is fed by several small streams that have intermediate DO, which can vary among streams from 0.118 ± 0.0056 mmol L^{-1} to 0.222 ± 0.0059 mmol L^{-1} (Chapman et al., 2004). The streams form ecotonal gradients of decreasing DO concentration as they enter the swamp.

In May 2004, at the beginning of the summer dry period, we measured limnological characters and clam abundance along a transect from the Rwembaita Swamp, through an ecotonal region and into a tributary of the swamp, Inlet Stream West (see Figure 4-1). Data from the broad range surveys conducted by Chapman and colleagues (Chapman et al., 2000, 2004) were used to design our sampling regime. Sites on the transect were separated by 10 m. Five sites (sites 1-5) with papyrus canopy were designated as swamp sites; five sites (sites 6-10) with emergent (non-papyrus) vegetation mixed with forest understory vegetation were designated as ecotone sites; and six sites (sites 11-16) were designated as stream sites. Water depth was shallow (10.2 ± 4.3 cm, n = 48) across all 16 sites. The width of the water stream at ecotone and stream sites (n =11), was 1.15 ± 1.33 m. The substrate was mud mixed with vegetation in all sites, with some sand also present in the stream sites.

Sampling Methods

Triplicate readings of all limnological characters were collected between 900 and 1200 hour at each site along the transect, following previous protocols (Chapman and Liem, 1995). DO, water temperature and conductivity readings were collected using YSI meters, and pH was measured using an Oaktron meter. Water transparency was measured using a transparent tube marked off in cm, with a miniature Secchi disc at the bottom; all measurements were taken by the same individual. At each site we recorded maximum water depth and water velocity

[1] To convert mmol O_2 L^{-1} to mg O_2 L^{-1}, multiply by 32 (molecular weight of O_2).

(categorical variables ranked on a scale of 0 to 3; 0 = no current, 1 = low, 2 = medium, 3 = high; Chapman, 1995).

At each site along the transect, clams were sampled using triplicate scoop nets (frame size 30.5 x 40.6 cm, mesh size of 0.32 cm), with triplicates separated by approximately 0.5 m. The samples were taken by disturbing and scooping the bottom substrate. Scoop net samples were sorted directly in the field. We calculated an index of clam density (catch per unit effort; CPUE) as the average number of clams per scoop. All clams collected in the initial survey were used for the size/frequency distribution analysis. For size measurements, shell length was measured from umbo to ventral margin using digital calipers.

Sampling for RNA/DNA, Nucleic Acid Oxidation and Stress Proteins

Clams used for nucleic acid analyses and stress proteins were collected from four sites, three of which were on the transect: a swamp site (site 2), an ecotone site (site 8) with comparable clam abundance to the swamp site but higher DO, and the stream site (site 11) with the highest DO of any site with clams. The fourth site (our reference site) was in the swamp interior, approximately 500 m downstream from stream water input. At this site, multi-year surveys indicated presence of clams in all seasons (Chapman et al., 2004). DO at this site averaged 0.0337 mmol L^{-1} over a 2-year period (Chapman et al., 2004). Clams were collected with a scoop net, briefly rinsed in water from their habitat, pierced with a dissecting needle to release water held in the mantle cavity, and flash-frozen in a liquid N_2 dry shipper (CX100, Taylor Wharton Cryogenics, Theodore, AL, USA). The time from scoop netting to flash-freezing was less than 1 minute. Clams were collected on two consecutive days between 900 and 1200 h. Clams were maintained in the dry shipper during transport back to the University of Florida, where they were stored at -80ºC. All clams used for nucleic acid and stress protein analyses were 5 – 7 mm in shell length.

Amounts of total tissue DNA, RNA, oxidized DNA and RNA bases were determined in 4-5 clams per site. DNA guanine base oxidation produces 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), and RNA guanine base oxidation produces 8-oxo-7,8-dihydroguanosine (8-oxoGuo). Samples consisting of 80 – 100 mg whole clam homogenate were processed as described previously (Hofer et al., 2006). Briefly, we simultaneously extracted both DNA and RNA from whole clam homogenates using guanidine thiocyanate and phenol/chloroform at neutral pH, after which nucleic acids were hydrolyzed with Nuclease P_1 and alkaline phosphatase. Hydrolytic enzymes were then removed by filtration, and the hydrolysate was analyzed by high performance liquid chromatography coupled to electrochemical detection (HPLC-ECD; ESA Inc., Chelmsford, MA, USA).

Stress protein expression levels were determined using mono-specific, ELISA-grade polyclonal antibodies generated by and donated by EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). The following antibodies were used: manganese superoxide dismutase (MnSOD; Cat. #AB-1976), copper/zinc superoxide dismutase (Cu/Zn SOD; Cat. # AB-SOD-1516), glutathione peroxidase (GPx; Cat. # AB-GPX-1433*)*, mitochondrial 8-oxoguanine DNA glycosylase (OGG1-mito; lot 2916), heat shock protein 60 (Hsp60; Cat. # AB-H100-IN), heat shock protein 70 (Hsp70; Cat. #AB-Hsp70-1519), and invertebrate small heat shock protein homologues (sHsp; Cat. #AB-H105). A summary of the functions of these proteins is presented in Table 4-1. The antibodies were raised in rabbits against 8-15 amino-acid polypeptides (conjugated to bovine serum albumin) derived from each target protein sequence of the bivalve *Mya arenaria* (Downs et al., 2002b). Stress proteins were analyzed in whole clam homogenates (Joyner-Matos et al., 2006) from 10 clams from each of the four sites, including those clams used for nucleic acid analyses. Briefly, whole clams (with shells) were homogenized in liquid N_2.

Homogenized tissue from each clam was suspended in protein denaturing buffer, and total soluble protein concentration was determined (Ghosh et al., 1988). Each clam was processed individually, and all clam samples were prepared on the same day using the same buffer. Antibody specificity of seven stress proteins was verified by polyacrylamide gel electrophoresis (PAGE) and western blotting, as described in the Results section. Expression levels were analyzed in triplicate by enzyme-linked-immunosorbent assay (ELISA).

Statistical Analyses

The nonparametric Spearman's rank correlation was used to test for a significant relationship between each limnological variable and distance along the transect, and the index of clam density (CPUE) and distance. Linear regression was used to evaluate the relationship between clam CPUE and the following variables: DO, conductivity, pH and transparency. Multiple regression was used to quantify the relationship between clam CPUE and the same suite of independent variables when entered into the regression model together. RNA oxidation data and stress protein expression levels were analyzed by one-way ANOVA with Fisher's LSD post-hoc multiple comparison test. The DNA oxidation data were not normally distributed (Shapiro-Wilks test $W = 0.77764$ and $p < 0.0007$) and were therefore analyzed using a Kruskal-Wallis one-way ANOVA with Conover-Inman pairwise comparisons, using StatsDirect (v. 2.4.5, www.statsdirect.com). To better understand the relationship between overall stress protein expression pattern and site, a principal components analysis was performed on the stress protein data. Factor scores resulting from an analysis with two unrotated factors were then analyzed by one-way ANOVA as above. Except as noted all statistical analyses were performed with Statistica (v. 7.1, Statsoft Inc., Tulsa OK, USA). Except as indicated, all values are reported as mean ± standard error. Statistical significance was accepted at $\alpha = 0.05$.

Results

Limnological survey. DO in the swamp sites (sites 1-5) averaged 0.023 mmol L^{-1}, which is less than one-tenth that of aerated stream water (0.25 mmol L^{-1}). DO increased along the transect from the swamp into the stream ($r_s = 0.944$, $p < 0.0001$, Figure 4-2a), reaching 0.19 mmol L^{-1} at the most upstream site (site 16). The surface water temperatures averaged 17.5 ± 1.5ºC ($n = 48$; data not shown) across all sites. Conductivity was not related to distance along the transect ($r_s = 0.329$, $p = 0.226$, Figure 4-2d), but pH increased along the transect ($r_s = 0.889$, $p < 0.0001$, Figure 4-2c), as did water transparency ($r_s = 0.743$, $p = 0.0021$, Figure 4-2b). At all sites (with the exception of site 16), water velocity was low with ranks between 0 and 1, and with most sites ranked as 0.5, indicating sluggish water flow (data not shown).

Clam density and size-frequency pattern. The catch per unit effort (CPUE) of clams was high but 'patchy' in the swamp (range from 17–144, corresponding to ca. 140-1200 clams m^{-2}) and decreased slightly in the ecotone sites (Figure 4-3a). CPUE decreased along the transect ($r_s = -0.889$, $p < 0.0001$), with clams absent from stream sites with DO level higher than 0.17 mmol L^{-1} (i.e., sites 13-16). Clams were buried below the sediment surface.

We examined the degree to which DO, conductivity, pH and transparency explained variation in CPUE of clams along the transect. In a multiple regression, only DO was marginally significant (whole model, $R^2 = 0.4168$, $p = 0.0005$; DO, *partial* $r = -0.308$, $p = 0.0596$); however, all four factors exhibited collinearity (VIF ranging from 1.11 to 3.65). We therefore examined the relationship between clam CPUE and each independent variable using least-squares linear regression. DO explained 52.9% of the variance in clam CPUE ($p = 0.0014$), pH explained 48.2% ($p = 0.0041$), and transparency explained 39.8% ($p = 0.0117$). In contrast, the relationship between conductivity and clam abundance was not significant ($p = 0.34$).

The umbo-to-margin length of the clams was quantified to examine variation in size and size frequency among sites. For this analysis we grouped the data according to site type (Figure 4-3b). All size classes from 2 mm to 7 mm were present in samples from the swamp (n = 1209) and the ecotone (n = 545). In the swamp and ecotone, the distribution of clams into size classes roughly matched a normal distribution. In the stream site, the sample population (n = 15) was heavily skewed towards the larger (> 5 mm) size classes, with only one (7%) of the 15 clams having a shell length of less than 5 mm. In the swamp and ecotone, clams with a shell length of less than 5 mm represented 40% and 59% of the samples, respectively.

RNA:DNA ratio. To investigate whether clams near the edge of the distribution exhibited decreased protein synthesis or growth in comparison to clams from the swamp interior, we analyzed RNA:DNA ratio in 4-5 clams per site. The RNA:DNA ratio of the clams in the ecotone and stream was 25% lower than that of clams in the swamp margin (F = 5.283, p = 0.012; swamp margin vs. ecotone, p = 0.049, swamp margin vs. stream, p = 0.054, Figure 4-4a).

Nucleic acid oxidation. To determine whether clams experienced cellular oxidative damage, which results from increased production or decreased detoxification of free radicals, we measured the levels of two forms of oxidatively damaged nucleosides: the DNA oxidation product 8-oxodGuo and the RNA oxidation product 8-oxoGuo. DNA yield was consistent across sites (0.472 ± 0.07 μg/mg tissue; F = 0.246, p = 0.863, data not shown), indicating that nucleoside extraction was equivalent in all samples. Clams in the ecotone and stream sites had significantly more DNA and RNA oxidation products than clams from the swamp interior (8-oxodGuo, Kruskal-Wallis ANOVA H_3 = 8.456, p = 0.037, Conover-Inman pairwise comparisons t_{14} = 2.145, $p \leq$ 0.0077, Figure 4-4b; 8-oxoGuo, F = 5.871, p = 0.008; for all comparisons, $p \leq$

0.036, Figure 4-4c). At each site, RNA oxidation was proportionally more extensive than DNA oxidation.

Stress protein analyses. We analyzed the expression levels of seven stress proteins (Table 4-1), which we categorized according to function as antioxidants (MnSOD, Cu/ZnSOD, and GPx), oxidative repair (OGG1m) and chaperones (Hsp60, Hsp70, sHsp). **Antioxidants:** MnSOD levels were higher in clams from the swamp margin and ecotone sites than in clams from either the swamp interior or stream sites (Figure 4-5a; $n = 40$, $F = 2.83$, $p = 0.052$; swamp margin vs. stream $p = 0.014$, ecotone vs. stream $p = 0.020$). Cu/ZnSOD levels were lower in the clams from the stream site than in clams from the other three sites (Figure 4-5b; $n = 40$, $F = 4.21$, $p = 0.012$; stream vs. other sites $p \leq 0.012$). There were no significant changes in GPx expression levels (Figure 4-5c; $n = 40$; $F = 1.39$, $p = 0.262$). **Oxidative repair:** Clams from the swamp margin expressed more OGG1m than did clams from the swamp interior or stream sites (Figure 4-5d; $n = 40$; $F = 3.82$, $p = 0.018$; swamp interior vs. swamp margin $p = 0.013$, swamp margin vs. stream $p = 0.003$). **Chaperones:** Clams from the swamp margin and ecotone expressed more Hsp60 than clams from the dense interior, with clams from the swamp margin expressing more Hsp60 than clams from the stream (Figure 4-5e; $n = 40$; $F = 5.333$, $p = 0.004$; for all significant comparisons, $p \leq 0.036$). Clams from the stream site had lower Hsp70 levels than clams from the swamp margin or ecotone site, but comparable expression levels to clams from the swamp interior (Figure 4-5f; $n = 40$; $F = 3.647$, $p = 0.021$; stream versus swamp margin and ecotone, $p \leq 0.0125$). Clams from the swamp margin had higher sHsp expression levels than clams from the other three sites (Figure 4-5g; $n = 40$; $F = 3.87$, $p = 0.017$; swamp margin versus other sites $p \leq 0.014$).

We conducted a principal components analysis to investigate whether the stress protein expression showed evidence of a coordinated response. We extracted two factors that explained a total of 63.53% of the variance, with the first factor explaining 45.13% of the total variance. The first factor was composed of four proteins with factor loading scores greater than 0.7: GPx, Hsp60, Hsp70, and MnSOD, all of which had positive loading scores ranging from 0.718 to 0.827. The second factor, consisting only of OGG1m, explained 18.4% of the variance in stress protein expression. OGG1m loaded heavily on the second factor with a score of -0.762. We next examined whether the mean factor scores (assigned to each individual on the basis of factor 1) differed among sites. Factor 1 loading scores were significantly different among sites ($F = 4.034$, $p = 0.0153$, Figure 4-5h), with clams from the swamp margin having a higher score than clams from the dense interior or the stream ($p = 0.035$ and $p = 0.039$, respectively).

Discussion

The "abundant center" distribution pattern (Sagarin and Gaines, 2002) predicts that when a population overlies a strong environmental gradient, abundance will be highest where conditions are the least stressful and/or the most beneficial. Furthermore, those individuals living where abundance is highest are expected to be in better condition than those living where abundance is low (Caughley et al., 1988). Although the *Sphaerium* sp. distribution pattern matches the abundant center pattern, the distribution appears to indicate that the hypoxic, acidic swamp water is less stressful (or more beneficial) than the normoxic, neutral pH stream water.

Limnological Characters and Relationship with Clam Density

A previous characterization of the Rwembaita Swamp showed that the water is extremely hypoxic and mildly acidic relative to the tributary streams feeding into it (Chapman et al., 2000), consistent with abiotic conditions in other papyrus swamp systems in Africa (Carter, 1955; Chapman et al., 2001). The ecotonal region of intermediate pH and DO is always present along

the tributary streams, but the exact location of the ecotone varies with season and other local habitat characteristics (e.g., stream volume), and it can be disturbed by large animals such as elephants (Chapman et al., 2001). We conducted a fine-scale survey of a transect extending from within the Rwembaita Swamp into one of its tributary streams (Figure 4-2a). We found that clams were abundant in the swamp, less abundant along the ecotone, and absent from stream sites with DO above 0.17 mmol L^{-1} and a pH above 6.9. In a multiple regression of our measure of abundance (CPUE) versus DO, pH, transparency and conductivity, DO was the only significant predictor. However, all four factors were collinear and when examined by least-squares linear regression, DO, pH, and transparency each explained significant variation in CPUE.

The population of clams at the distribution edge was skewed towards larger size classes (5 – 7 mm). Since this is the first assessment of a size-frequency pattern in this system, and it was only done at one season, these results must be interpreted with caution. Nonetheless, these data suggest that demographic differences exist along this physicochemical gradient. Sphaeriid lifespans range from less than 1 year to 5 years (Burky, 1983), and all known species internally brood their young, which are released as shelled juveniles (Mackie, 1978). The lack of smaller size classes in stream sites could reflect fluctuations in the position of the ecotone; for example, a period of low rainfall could have led to abiotic conditions in these sites that were transiently similar to the swamp, allowing successful recruitment.

Other physicochemical characters, as well as biotic interactions, likely influence clam distribution and abundance in this swamp-stream system. For example, substrate type typically influences the distribution of burrowing bivalves, although a relationship between substrate type and habitat preference was not supported in several studies of sphaeriid clams (reviewed in

Burky, 1983). In the Rwembaita Swamp-stream system, substrate type (mud mixed with vegetation) was similar across all sites with clams and no clams observed on the surface of the substrate, which is consistent with a previous survey in which clams were sampled from a depth of 2-3 cm (Osborne et al., 2001; Chapman et al., 2004). The extent to which the increased sandiness in the stream might negatively influence burial and thus influence dispersal to these sites is unknown. Currently, we cannot address the potential influence of biotic factors such as competition and predation on clam distribution. However, previous studies (Chapman, 1995; Chapman et al., 1999, 2004; Osborne et al., 2001) have additionally highlighted the importance of considering effects of physicochemical gradients when assessing how biotic interactions shape this community structure (Menge and Sutherland, 1987; Case et al., 2005).

Cellular-level Indicators

To test whether clams at the distribution edge were under more stress and whether the condition of the clams changed along the abiotic gradients, we investigated several cellular-level indicators of stress: RNA:DNA ratio, nucleic acid oxidative damage, and number of different stress proteins. Compared to clams in the swamp interior and swamp margin, we expected clams from the ecotone and stream site (distribution edge) to have lower RNA:DNA ratio, higher oxidative damage, and higher stress protein expression, all consistent with higher stress.

Decreased RNA:DNA ratio indicates reduced levels of protein synthesis, as could occur with stress or decreased growth rate (Elser et al., 2000; Dahlhoff, 2004). We found that clams from the ecotone and stream sites had lower RNA:DNA ratio in comparison to clams from the swamp margin. Although statistically significant, the difference in RNA:DNA ratio was small in comparison to studies examining changes across seasons or following experimental manipulations (Dahlhoff, 2004). Nonetheless, to our knowledge comparable studies of

RNA:DNA ratio across a population do not exist, so it is unknown whether even small differences are functionally significant in natural populations.

Oxidative damage, including damage to DNA and RNA, occurs when free radical production overwhelms a cell's ability to detoxify the free radicals (Halliwell and Gutteridge, 1999). The deleterious effects of DNA oxidation on mutation accumulation and aging are well-established (Hartman et al., 2004; Sanz et al., 2006), but the effects of RNA oxidation have not been well characterized (Seo et al., 2006). We found that clams from the ecotone and stream sites had significantly elevated levels of oxidatively damaged DNA and RNA in comparison to clams from the dense interior of the swamp, and furthermore that RNA oxidative damage was proportionally higher than DNA damage at all sites. To our knowledge, this is the first quantification of DNA and RNA oxidation in a natural population.

Stress proteins are produced by nearly all eukaryotes, and patterns of stress protein expression and activity serve as cellular-level indicators of stressor type (e.g., Willmore and Storey, 1997; Abele and Puntarulo, 2004; Magalhães et al., 2005) and indicators of how stressors influence habitat preferences (e.g., Feder and Hofmann, 1999; Downs et al., 2002b). We found patterns of stress protein expression in clams along the transect indicating that clams in the swamp margin and ecotone were experiencing more stress than those in the dense interior of the swamp. The elevations in chaperone proteins (Hsp60, Hsp70, and sHsp) in clams in the intermediate sites indicate generalized cellular stress, particularly that which results in protein misfolding, protein aggregation, or heightened need for protein degradation (Kregel, 2002). Furthermore, elevations in antioxidants (MnSOD) and the repair enzyme OGG1-m in clams in the swamp margin and ecotone compared to the dense interior suggests that these clams experienced elevated free radical production and oxidative damage (Halliwell and Gutteridge,

1999). Similar relative changes in protein expression were detected in bivalves exposed to abiotic stressors in controlled laboratory conditions (Joyner-Matos et al., 2006) and in bivalves at sites impacted by a crude oil spill (Downs et al., 2002b). However, these data on relative changes do not allow comparisons of absolute stress protein expression levels between different organisms nor of comparisons between expression levels and enzyme activity.

Principal components analysis, which provides a composite view of a coordinated stress response, showed that clams in the swamp margin and ecotone had a larger stress response than did clams in either the dense interior or stream sites, but that the response of clams in the swamp margin did not differ from that of clams in the ecotone. This integrated stress response was dominated by two antioxidants, GPx and MnSOD, and two chaperone proteins, Hsp60 and Hsp70, indicating that clams at the intermediate sites were experiencing stress at the cellular level and that at least some of that stress was due to elevated free radical production.

The Extreme Edge of the Distribution

Comparison of clams in the swamp margin with those in sites closer to the edge of the distribution followed the expected pattern: as abundance decreases, RNA:DNA ratio decreases, oxidative damage increases, and, for the swamp margin and ecotone sites, stress protein expression increases. Surprisingly, however, stress protein expression in clams from the stream site was equivalent to, or lower than, that of clams from the dense interior. One interpretation of this pattern is that clams from the stream site were less stressed than clams from the swamp margin and ecotone, but this is not consistent with the RNA:DNA ratio and nucleotide oxidative damage results, which both indicate that clams in the stream were under more stress. The alternate interpretation is that clams from the stream site were so stressed that they were not capable of maintaining high stress protein expression (Werner and Hinton, 1999). Therefore, the clams at the extreme edge of the distribution show a stress response pattern that might otherwise

be interpreted as pathologic loss of cellular homeostatic stress response and repair mechanisms. It is unknown whether this pattern of extreme physiological stress in organisms at the extreme distribution edge is present in other systems with stable population distributions overlying stressful abiotic gradients.

Relationships Between Limnological Characters and Cellular-level Stress Indicators

The distribution data demonstrated that the swamp is less stressful (or more beneficial) than the stream, consistent with the abundant center distribution pattern. Of the limnological variables measured along the transect, DO, pH and transparency were significant predictors of clam CPUE, and all showed a negative correlation. When examining cellular-level stress indicators, we found a pattern consistent with increasing oxidative stress towards the distribution edge. While any of the three limnological variables could contribute to oxidative stress, we believe the evidence is strongest for DO.

Oxygen availability, particularly hypoxia, affects a wide variety of ecological processes in aquatic organisms (Baker and Mann, 1992; Diaz and Rosenberg, 1995; Rosenberger and Chapman, 2000). The ability of the sphaeriid clams in the Rwembaita Swamp to tolerate hypoxia is not surprising, given the well-documented ability of clams from this cosmopolitan family to tolerate DO fluctuations (Waite and Neufeld, 1977) and to aestivate during seasonal dry periods in ephemeral ponds (McKee and Mackie, 1983). However, the hypoxia tolerance of the sphaeriid clams in the Rwembaita Swamp appears to be greater than that demonstrated by congeners and closely related pisiid clams. For example, a European congener showed significantly decreased valve-movement behavior at DO levels comparable to that of the ecotone sites (Heinonen et al., 1997), and a North American sphaeriid and European pisiid had critical O_2 tensions comparable to the DO level at swamp sites (Waite and Neufeld, 1977; Hamburger et al., 2000).

While it is still unclear whether hypoxia causes elevated free radical production (e.g., Li and Jackson, 2002; de Oliveira et al., 2005), it is well established that hyperoxia (typically defined as O_2 concentrations higher than atmospheric levels) is linked to increased free radical production and oxidative damage (Boveris and Chance, 1973; Abele and Puntarulo, 2004). However, the concentration of O_2 necessary to cause oxidative damage may be much less than "hyperoxia" for isolated cells and small animals (Packer and Fuehr, 1977; Saito et al., 1995; Gray et al., 2004). Given that the sphaeriid clams in the Rwembaita Swamp system are adapted to hypoxia and appear to spend their entire life cycle in hypoxic conditions, we propose that clams living in the intermediate sites and distribution edge experience the equivalent of hyperoxic stress. The pattern of cellular-level indicators in our study is consistent with biomedical studies of hyperoxia (Wong et al., 1998; Freiberger et al., 2004; Roper et al., 2004; Olsvik et al., 2005). Therefore, "hyperoxia" stress may occur in any animal exposed to oxygen levels higher than those to which it is adapted, whether this is a normoxia-adapted animal exposed to hyperoxia, a hypoxia-adapted animal exposed to normoxia, or even, in the case of *Sphaerium* sp., an animal adapted to extreme hypoxia exposed to moderate hypoxia.

We cannot, however, rule out the possible influence of pH or transparency on clam distribution or cellular-level indicators of stress. In aquatic habitats, pH is influenced by CO_2 concentration, and the co-occurrence of hypoxia and acidic/hypercapnic conditions is widespread (Burnett, 1997). In the papyrus swamps of East Africa, both DO and pH are strongly influenced by organic matter decomposition, with 81% of the variance in DO explained by the concentration of free, dissolved CO_2 (Chapman et al., 2001). Interestingly, CO_2 may either promote or inhibit oxidative damage through a variety of reactions (Veselá and Wilhelm, 2002).

To our knowledge, there are no studies linking oxidative stress to environmental alterations of pH or CO_2.

Water transparency, which is influenced by concentrations of tannins (Labieniec et al., 2003) and suspended materials (Ward and Shumway, 2004), also was inversely correlated with clam abundance. Since tannin levels in the Rwembaita Swamp system are unknown, we are unable to evaluate their potential influence on clam physiology. Suspended materials, which may be inorganic or organic, also decrease water transparency, but the composition of suspended materials in this system is unknown. Since bivalve feeding efficiency decreases in water with high concentrations of inorganic suspended materials (Ward and Shumway, 2004), we would expect clam abundance to be lowest in the swamp, where water transparency is lowest, which is not consistent with our results or multi-year surveys (Chapman et al., 2004). However, when the suspended materials are primarily organic, clam abundance would be lowest where transparency is highest, as observed in this study. Nevertheless, the observed size/frequency distribution is inconsistent with nutrient limitation influencing the population distribution, and nutrient restriction does not cause increased oxidative stress (Gredilla and Barja, 2005).

Conclusions

In the Rwembaita Swamp system we found that the hypoxic, low-transparency, and acidic conditions of the swamp minimize oxidative damage, while the elevated DO, high transparency and neutral ph conditions of the stream increase oxidative damage. We propose that in this clam, DO above the extreme hypoxia of the swamp causes a physiological condition similar to that of other animals exposed to hyperoxia. A critical test of this hypothesis would entail long-term transplant experiments, coupled with direct manipulation of the physical environment. However, such experiments would be challenging at this site due to frequent disturbance by large animals and substantial fluctuations in water level during dry and rainy seasons. In addition, we found

that clams at the extreme edge showed decreased stress protein expression, despite decreased condition and elevated oxidative damage, which we propose is an indication of extreme stress. This demonstrates that analysis of stress protein expression alone may be a misleading index of stress and highlights the importance of sampling the extreme distribution edge. The integration of ecological and biochemical responses provides a useful approach to understanding the role of the physicochemical environment in structuring population distributions.

Table 4-1. Overview of stress protein functions.

Protein type	Protein	Function
Antioxidant	Manganese superoxide dismutase (MnSOD)	Catalyzes the dismutation of superoxide to the less reactive pro-oxidant H_2O_2, primarily mitochondrial (Halliwell and Gutteridge 1999).
	Copper/zinc superoxide dismutase (Cu/ZnSOD)	Catalyzes the dismutation of superoxide into H_2O_2, primarily cytoplasmic (Halliwell and Gutteridge 1999).
	Glutathione peroxidase (GPx)	Catalyzes the reduction of H_2O_2 to water with the concomitant oxidation of reduced glutathione (Halliwell and Gutteridge 1999).
Oxidative repair	Mitochondrial 8-oxoguanine DNA glycosylase (OGG1m)	Catalyzes the removal of the mutagenic 8-hydroxyguanine (8-oxodGuo) base lesion (Boiteux and Radicella 2000)
Chaperone	Heat shock protein 60 (Hsp60)	Aids in the folding of newly-formed proteins under normal physiological condition and refolds damaged proteins during stress (Hartl 1996; Kregel 2002).
	Heat shock protein 70 (Hsp70)	Has numerous roles involving chaperone functions, protein degradation and protein folding (Frydman 2001; Kregel 2002).
	Small heat shock proteins (sHsp)	Bind denatured proteins, preventing irreversible protein aggregation, and participate in the ubiquitin/proteasome system (Parcellier et al. 2005).

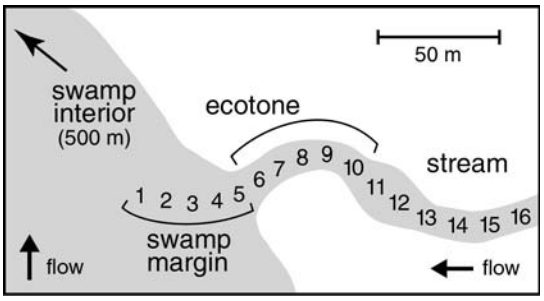


Figure 4-1. Schematic map of the study site in the Rwembaita Swamp system of Kibale National Park, Uganda showing direction of water flow from the stream and into the swamp. Numbered sites are at 10 m intervals. Sites 1-5 are considered swamp sites, 6-10 as ecotone sites, and 11-16 as stream sites.

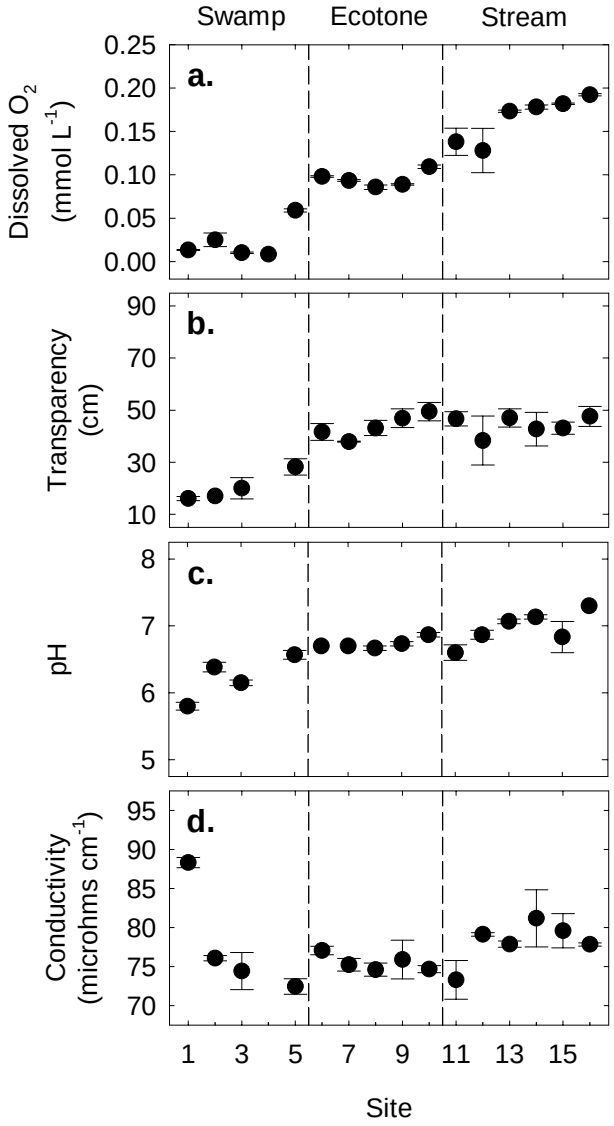


Figure 4-2. Limnological features of the stream-swamp transect. Mean (± 1 SE) of triplicate measures of **a** dissolved O_2 (mmol l^{-1}), **b** transparency (cm), **c** pH, and **d** conductivity (microhms cm^{-1}) . Sites correspond to the survey sites shown in Figure 4-1. We were unable to collect a sufficient volume of water from site 4 to measure conductivity, pH or transparency.

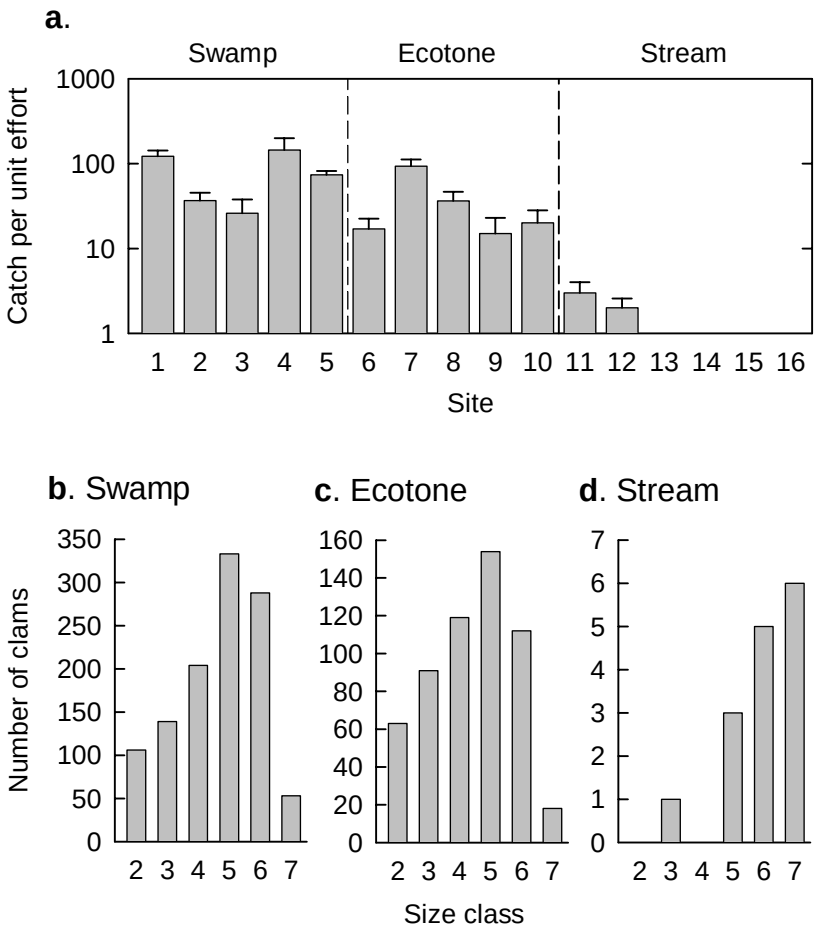


Figure 4-3. Catch per unit effort and size/frequency distribution of clams. Means (± 1 SE) of **a** triplicate measures of catch per unit effort of clams in each of the 16 sites on the transect and **b** size-frequency distribution of clams from pooled samples from the three characteristic site types (swamp, $n = 1209$ clams; ecotone, $n = 545$ clams; stream, $n = 15$ clams). Note the logarithmic scale used in the catch per unit effort graph. Shell length size classes of all collected clams ranged from 2 – 7 mm.

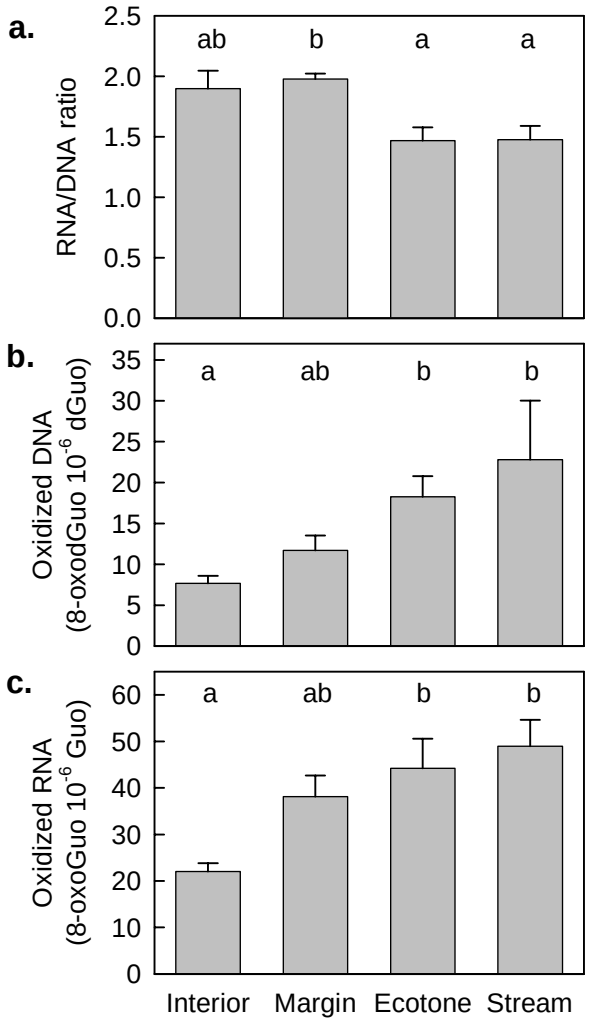
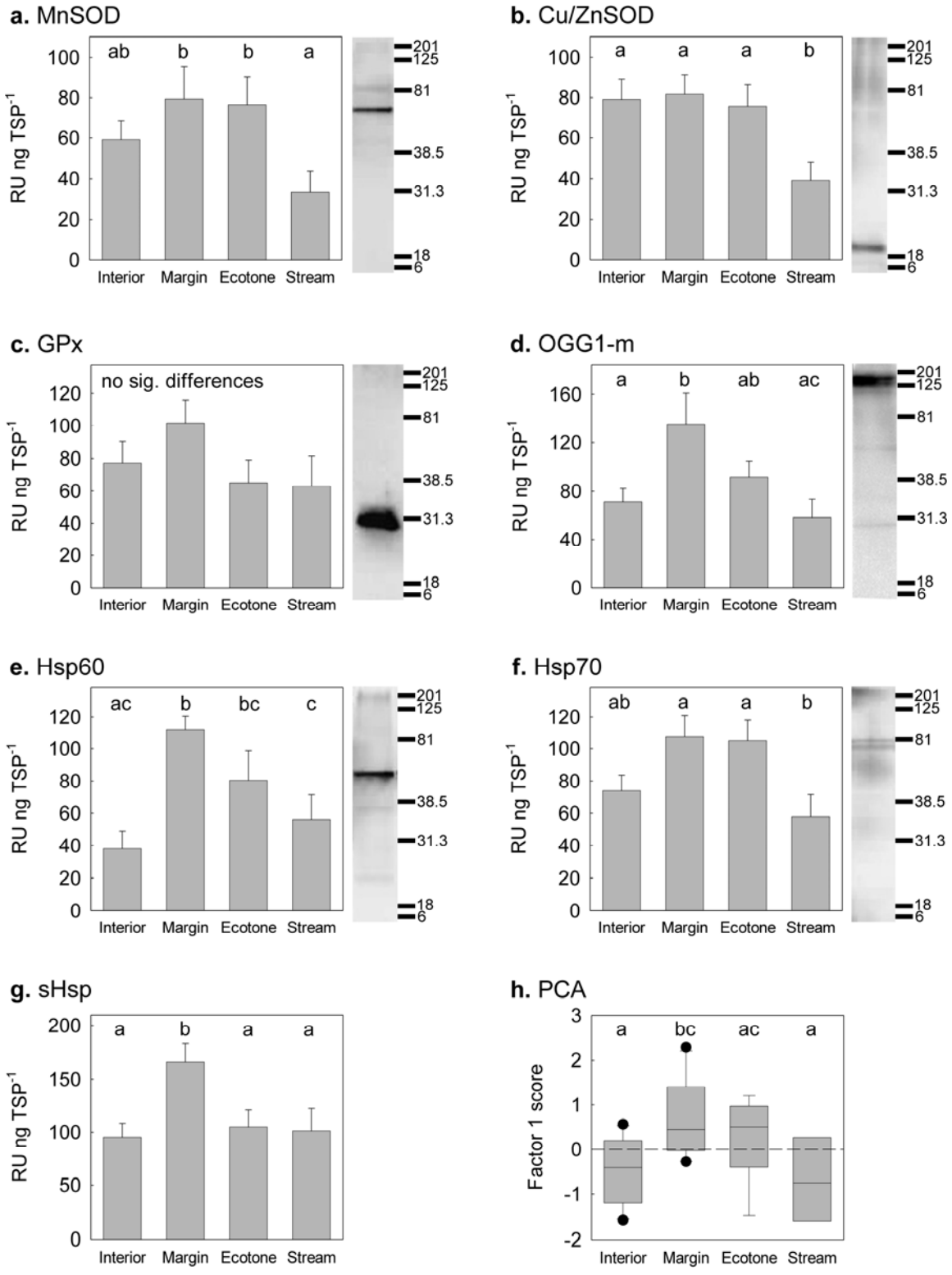


Figure 4-4. RNA:DNA ratio and oxidatively damaged nucleic acid in clams. Means (± 1 SD) of **a** RNA/DNA, **b** oxidatively damaged DNA nucleosides (8-oxo-dGuo, oxidized bases per 10^6 dGuo, black bars), and **c** oxidatively damaged RNA nucleosides (8-oxoGuo, oxidized bases per 10^6 Guo, grey bars) in 4-5 clams from each site. Nucleoside analyses were conducted in a subset of the clams used for stress protein analyses. Bars that share similar *letters* are not significantly different.

Figure 4-5. Antibody specificity tests and stress protein expression levels in clams. Levels are presented as relative units per nanogram of total soluble protein, (RU ng TSP^{-1}; *n* = 10 from each site). **a** manganese superoxide dismutase (MnSOD), **b** copper/zinc superoxide dismutase (Cu/ZnSOD), **c** glutathione peroxidase (GPx), **d** the DNA repair enzyme mitochondrial 8-oxoguanine DNA glycosylase (OGG1-m), **e** heat shock protein 60 (Hsp60), **f** heat shock protein 70 (Hsp70), and **g** small heat shock proteins (sHSP). Clams were collected from the swamp interior, swamp margin (site 2), ecotone (site 8), and stream (site 11). *Bars* depict means ± 1 SE. Similar *letters* indicate statistically indistinguishable samples according to Fisher's LSD post-hoc multiple comparison test. For antibody specificity tests, two random samples of *Sphaerium* sp. were pooled, subjected to SDS-PAGE, western blotted, and assayed with the appropriate antibodies. The positions of known molecular weight standards are indicated in kilodaltons. **h** Factor 1 loading scores from a principal components analysis of stress protein expression levels presented in box-and-whisker plots with the box defining data in the 25^{th} to 75^{th} percentiles, the line indicating the median, and the whiskers defining the 10^{th} and 90^{th} percentiles.


a. MnSOD
RU ng TSP-1
ab
b
b
a
Interior
Margin
Ecotone
Stream
201
125
81
38.5
31.3
18
6
b. Cu/ZnSOD
a
a
a
b
c. GPx
no sig. differences
d. OGG1-m
a
b
ab
ac
e. Hsp60
ac
b
bc
c
f. Hsp70
ab
a
a
b
g. sHsp
a
b
a
a
h. PCA
Factor 1 score
a
bc
ac
a

CHAPTER 5
SYNTHESIS

The research presented in this dissertation addresses three aspects of the overall question, "does the magnitude of the cellular stress response influence species distribution?" This research focused on the cellular stress responses of bivalves exposed to abiotic factors present in aquatic environments. The first part of this synthesis presents a discussion of what overall themes can be drawn from the three studies. The second part of this synthesis presents a summary of findings.

Sources of Variance

Students of physiology are taught that a well-designed laboratory experiment examining physiological processes should successfully minimize all potential sources of variance and that any detected variability in physiological response is an error that should be addressed with the appropriate statistics. In a recent book exploring patterns in physiological diversity and their impact on ecological processes, Spicer and Gaston (1999) emphasize that physiological variation pervades all hierarchical levels, from within an individual to within and between populations and species. They advocate embracing and studying this variation and its role in species distribution rather than attempting to minimize or ignore it. A number of sources of variation impact the ability of invertebrates to maintain an oxidative stress response and also impact the ability of an investigator to detect the oxidative stress response and properly interpret the results. Two of these sources are discussed below.

Season. As demonstrated in Chapters 2 and 3, seasonal differences in bivalve physiology affect both cellular-level and whole-organismal responses and susceptibilities to abiotic factors. These seasonal differences likely reflect variation in body composition (protein-rich or lipid-rich), reproductive versus growth cycle, degree of nourishment, age, and previous exposure to abiotic factors. All of these factors influence the oxidative stress response, as reviewed in the

previous chapters. Despite the categorization of *D. variabilis* (Chapter 2) as a species that is vulnerable to abiotic factors and that of *M. mercenaria* (Chapter 3) as a stress-tolerant species, a common seasonal pattern in susceptibility was detected, with the highest mortality occurring in the season for which the experimental conditions were most distinct from the environmental conditions (fall and winter). However, whether this pattern in susceptibility is related to cellular-level responses is unclear, since an oxidative stress response was detected in *D. variabilis* but not in *M. mercenaria*. In the case of *D. variabilis*, the abiotic factors, particularly hydrogen sulfide, instigated an oxidative stress response, but either this stress response was unrelated to survival or it was not sufficient to prevent clam death. *Mercenaria mercenaria* did not have a detectable cellular-level response to high temperature, the treatment that caused significant mortality in the winter experiments, but it is not known whether these clams died because they did not elevate stress protein expression or if they died from other causes. The seasonal pattern in mortality and functional responses like burial ability, particularly in the high temperature experiments, suggests that the cause of mortality was at a level of organization higher than the cellular level. These results highlight the importance of examining stress physiology over multiple seasons, regardless of whether the study subject is vulnerable to or tolerant of the abiotic factors.

Multiple levels of biological organization. The results from all three projects underscore the importance of examining responses at multiple levels of organization, a conclusion consistent with the views expressed by Spicer and Gaston (1999). The examination of stress protein responses as indicators of whole-animal tolerance and species distribution is a well-established technique. However, there is a large gap between the expression levels of a given stress protein and the documented distribution of a population, a gap that is not always bridged in ecological physiology studies and which therefore limits the power of inference. As demonstrated in both

Chapters 2 and 3, the upregulation or lack of upregulation of stress protein expression is not reliably predictive of whole-animal tolerances and therefore should not be used exclusively to draw conclusions about species distribution. As demonstrated in Chapter 4, elevated stress protein expression should not be interpreted strictly as evidence of conditions causing oxidative damage and the converse, decreased stress protein expression, expected to be found in individuals in benign conditions. These results confirm that stress protein expression data should not be interpreted in the absence of either cellular-level markers of damage or metabolic activity (as demonstrated in Chapter 4) or whole-organism markers (Chapters 2 and 3).

Spicer and Gaston (1999) emphasize that variation at all levels of biological organization are interrelated and that investigators should, when possible, look for correlation and causation at multiple levels. In some but not all of the experiments described in this dissertation, multiple assays were conducted on the same individual. Had this approach been made universal, even within an experiment, then it would have been possible to test for within-individual correlations using multivariate statistics. Such an analysis, if successful, would have facilitated an exploration into the causes underlying treatment-, season-, and species-dependent variation in stress tolerance. However, an informal analysis (not presented in this dissertation) of those instances in which multiple assays were conducted on the same individual reveals that high levels of variation, even within treatment and season, would make the detection of correlations unlikely.

Summary of Findings

In Chapter 2 I tested whether abiotic factors typical of aquatic habitats cause a cellular stress response consistent with free radical production. This study was conducted in the marine bivalve, *Donax variabilis*. The factors examined in this study, hypoxia, hyperoxia, and hydrogen sulfide, are not present in the high-energy sandy beaches inhabited by *D. variabilis*. This species was selected to test whether one could be more successful at detecting the consequences of the

abiotic factor in the absence of protective responses in an organism that presumably is not adapted to the stressors. The results show a strong seasonal pattern in susceptibility to and cellular-level responses to the stressors. The clams survived longer in the experiment conducted in the spring and, for the most part, had higher stress protein expression levels in the spring. The pattern of stress protein expression was consistent with elevated free radical production, particularly in the hydrogen sulfide treatment. These results demonstrated that exposure to hydrogen sulfide causes an oxidative stress response in a non-sulfide-adapted bivalve and highlighted the importance of examining seasonal variation in stress physiology.

In Chapter 3 I tested whether organisms can mitigate the cellular-level damage associated with exposure to single or multiple stressful abiotic factors. This study was conducted in the estuarine bivalve, *Mercenaria mercenaria.* The abiotic factors examined in this study, hypoxia, high temperature, and high temperature combined with hyposalinity, have been shown to cause elevated free radical production in invertebrates and vertebrates. Both cellular- and organismal-level responses to the abiotic factors were examined, and a seasonal pattern in susceptibility and magnitude of response was again detected. Although *M. mercenaria* did not have a detectable stress protein response to hypoxia or high temperature, the seasonal pattern in mortality and functional responses like burial ability, particularly in the high temperature experiments, suggests that the cause of mortality was at a level of organization higher than the cellular level. The additive effects of the dual factor treatments were evident in the functional responses of the clams, which underscore the importance of examining multiple factors when testing whether tolerance of abiotic factors affects distribution in complex environments. These clams have broad tolerances for extremes of temperature, dissolved O_2 level, salinity, and pH that reflect the species' widespread distribution in intertidal and subtidal coastal habitats. These results

demonstrate that physiological strategies at higher levels of organization buffer the need for a cellular-level oxidative stress response in a stress-tolerant organism.

In Chapter 4 I tested whether the capacity to produce an oxidative stress response affects species distribution. This combined field and laboratory study of the freshwater bivalve *Sphaerium* sp. demonstrated that individuals in a population that overlies an environmental gradient show a variation in the ability to maintain a cellular stress response that reflects position along the gradient. These results were consistent with the "abundant center distribution pattern," which dictates that abundance and individual condition decrease near the edge of the population's distribution if the distribution overlies a potentially stressful environmental gradient. In contrast to most previous studies linking stress protein expression to species distribution, these results showed that *decreases* in stress protein expression are more indicative of severe stress than are *increases*. Finally, since the clams were most abundant and experienced the least oxidative stress when living in conditions of extreme hypoxia, these results are the first whole-organism and field study to challenge long-held belief that normoxia is beneficial and hypoxia is stressful for aquatic organisms.

These three interrelated studies demonstrated that abiotic factors found in aquatic habitats do cause oxidative stress and that ability to respond to the abiotic factors correlates with distribution. The results highlight the importance of examining stress physiology over multiple seasons and underscore the importance of examining responses at multiple levels of organization. Since the upregulation or lack of change in stress protein expression was not reliably predictive of whole-animal tolerances, these data should not be interpreted in the absence of either cellular-level markers of damage or metabolic activity or whole-organism markers of physiological condition. Finally, these results demonstrate that future studies of

ecological physiology would benefit from new approaches, particularly the examination of multiple abiotic factors and the inclusion of ‘vulnerable’ species in the selection of model organisms.

APPENDIX
TRANSPLANT OF *SPHAERIUM* SP. ALONG A SWAMP/STREAM TRANSECT

Introduction and Methods

The study presented in this appendix is a continuation of Chapter 4. It was conducted in May – June 2004 at the Makerere University Biological Field Station in Kibale National Park, Uganda.

To examine whether *Sphaerium* sp. clams acclimatized to the hypoxic and acidic swamp conditions experience stress when exposed to the elevated dissolved O_2 (DO) and neutral pH conditions of the stream sites, we conducted a two-week transplant experiment. For a full description of the limnological characters of the transect sites, see Chapter 4 pages 119-120. Transect sites, numbered 1 – 16, were situated at 10 m intervals starting in the swamp (sites 1-5) and continuing through the ecotone (sites 6-10) and into Inlet Stream West (sites 11-16).

Clams (5-8 mm shell length) were collected from a swamp site (Site #1 in Figure 4-1, page 133) and kept in a bucket of swamp water until placed into cages. The cages were constructed of white PVC pipe (length approximately 25 cm, internal diameter 6 cm) with mesh placed over each end and secured with rubber bands. Holes were drilled at each end of the cage and fishing line was looped through the holes with enough slack to allow the cages to be suspended from overhanging vegetation. Five cages were placed at each of four sites (see site numbers in Figure 4-1, page 133): a) a swamp control site (Site 2), b) an ecotone site with a higher DO than the swamp and with clam abundance comparable to that of the swamp (Site 8), c) a stream site at the extreme edge of the clam population distribution that had the highest DO of any site with clams (Site 11), and d) a stream site with the highest DO of any site on the transect (Site 16). Thirty clams were placed into each cage, and cages were oriented to allow water flow through the cage. In most sites, the cages rested on top of the substrate.

Five clams per cage were collected at each of six time points (total 600 clams): 2 hours, 5 hours, 1 day, 2 days, 5 days, and 14 days. At each sampling, removed clams were replaced with newly collected clams (from Site 1) that were marked on both valves with pink nail polish. Collected clams were rinsed in water from the transect site, pierced with a dissecting needle to release water held in the mantle cavity, and flash-frozen in a liquid N_2 dry shipper (CX100, Taylor Wharton Cryogenics, Theodore, AL, USA). Clams were maintained in the dry shipper during transport back to the University of Florida, where they were stored at -80°C.

We analyzed stress protein expression levels in clams collected from all four sites at the six time points (3 per cage per site per sampling time) using Western Blotting and enzyme-linked-immunosorbent assay (ELISA). For details on the methods of stress protein analyses, see the descriptions in Chapters 2 and 4 (pages 31-34 and 117-119, respectively). For details about stress protein functions and the antibody binding patterns for Sphaeriid tissues, see pages 36-38 in Chapter 2 and Table 4-1 (page 132). Expression levels of the stress proteins were analyzed in triplicate by ELISA. The following proteins were examined: copper/zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx), mitochondrial 8-oxoguanine DNA glycosylase (OGG1-m), heat shock protein 60 (Hsp60), heat shock protein 70 (Hsp70), and small heat shock proteins (sHsp).

Statistical results for these data are not presented as all results were nonsignificant ($p > 0.05$). Each stress protein was analyzed independently. Since there were no clear trends across sampling times, the data were analyzed within sampling times by a nested ANOVA (Statistica), with cages nested within site and clams within cages treated as independent experimental units.

To examine whether the clams experienced oxidative damage during the transplant, we quantified the levels of oxidatively damaged RNA and DNA guanine bases in clams that had

been transplanted for 14 days. For details about the methods of sample preparation and analysis, see Chapters 4 (pages 117-118). We quantified oxidized RNA and DNA bases in five clams from each transplant site. Data were analyzed with one-way ANOVA.

Results and Discussion

The stress protein expression results are presented in Figure A-1. The data are presented as expression levels relative to the mean of the expression levels of clams collected at Time 0 to allow for temporal comparisons. Expression levels of all stress proteins except sHsp increased in clams transplanted to the ecotone, stream, and upstream sites in the 14-day period. Interestingly, the relative expression levels of all proteins (except sHSP) in clams from the swamp site tended to be close to or slightly below one.

There were no significant differences in stress protein expression levels following the 14-day transplant experiment. Expression levels of the two antioxidants (CuZnSOD and GPx) were constant across the four sites (Figure A-1A and B). Clams transplanted to ecotone and stream sites expressed slightly (but not significantly) higher levels of the DNA repair enzyme, OGG1m, than did clams maintained in the swamp site (Figure A-1C). There was some indication of elevated expression in heat shock proteins (Hsp60, Hsp70, and sHsp) in clams transplanted to the ecotone and stream, in relation to clams maintained at the swamp site (Figure A-1D, C, and E, respectively). This is consistent with the results of the survey of clams collected from the ecotone and stream sites (pages 121-123).

In summary, the general increase in stress protein expression in transplanted clams, particularly the elevated expression of the chaperone proteins Hsp70 and Hsp60, suggests that the clams experienced physiological stress during the transplant. This response was marginally greater in the clams transplanted to the ecotone and stream sites in comparison to the clams maintained in cages in the swamp site. However, since the highest overall expression levels and

the largest (but nonsignificant) differences in stress protein expression were detected in the chaperone proteins rather than in the antioxidants, these data do not support the hypothesis that the clams maintained an elevated oxidative stress response following transplantation to sites with elevated DO.

DNA and RNA oxidation levels in clams transplanted for 14 days are presented in Figure A-2A and B, respectively. There were no significant differences in the levels of oxidatively damaged DNA or RNA bases across transplant sites. Across sites, there was more RNA oxidation than DNA oxidation, which is consistent with the findings of the survey (page 121). Like the stress protein data, these data do not support the hypothesis that the clams experienced elevated oxidative stress following transplantation. An alternative interpretation is that they experienced oxidative damage to their DNA and/or RNA at earlier sampling times and repaired or removed the damaged nucleotides. However, nucleic acid oxidation was not assessed in clams from the earlier time points.

In summary, the stress protein expression and oxidative damage levels in clams transplanted for 14 days to sites with elevated DO and neutral pH do not support the hypothesis that the clams experienced elevated stress at the transplantation sites. The results do suggest that the clams experienced generalized stress (elevated chaperone expression), which could result from starvation or from being maintained in cages rather than buried in the substrate.

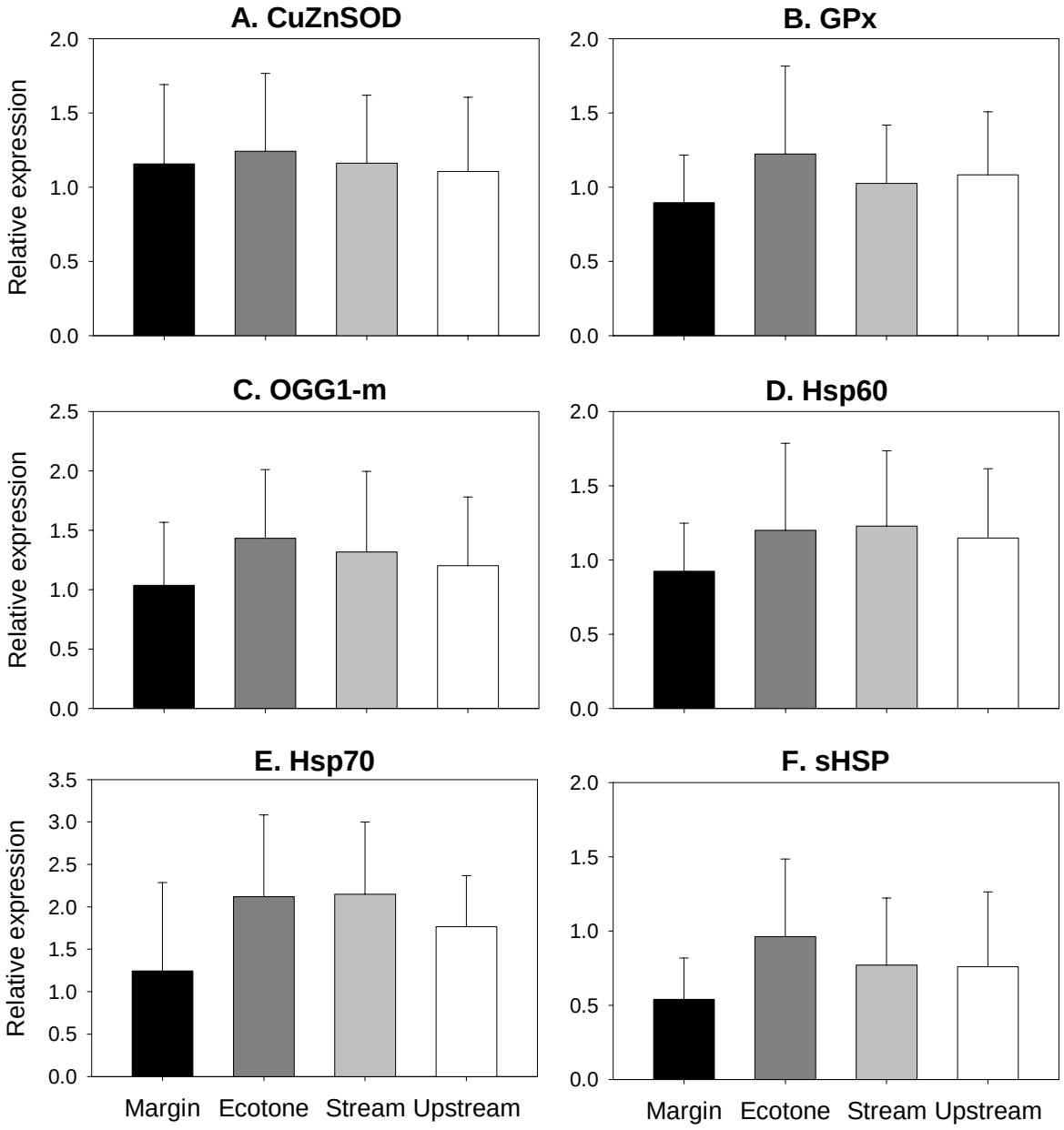


Figure A-1. Relative stress protein expression levels in *Sphaerium* sp. clams from the 14-day transplant experiment. Clams (15 per site) were collected at the swamp site and held in cages at each site for 14 days. Data are expressed as relative to the mean of the expression levels of the five clams collected at Time 0. Data are presented as mean ± standard deviation. Abbreviations: CuZnSOD, copper/zinc superoxide dismutase; GPx, glutathione peroxidase; OGG1m, mitochondrial 8-oxoguanine DNA glycosylase; Hsp60, heat shock protein 60; Hsp70, heat shock protein 70; sHsp, small heat shock protein.

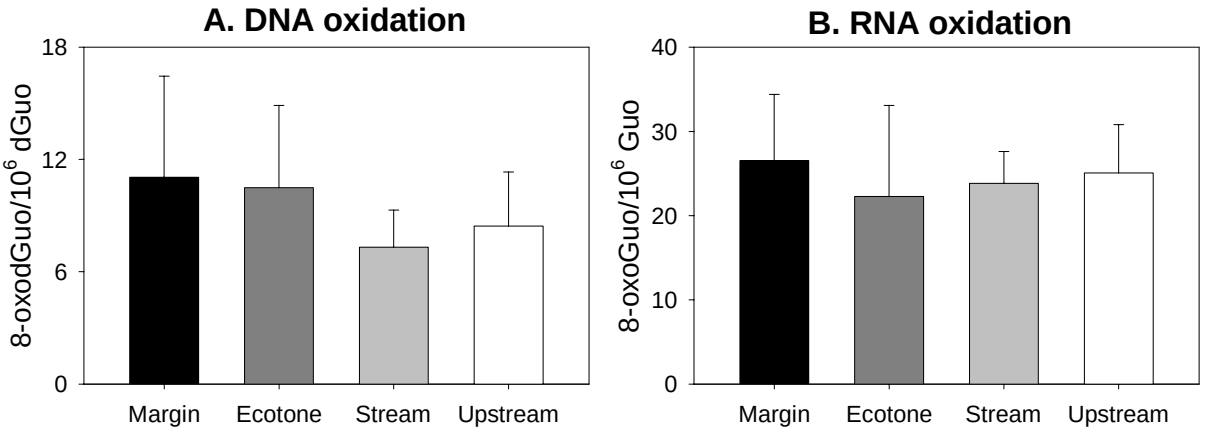


Figure A-2. Levels of oxidatively damaged DNA and RNA in *Sphaerium* sp. clams from the 14-day transplant experiment. Clams (5 per site) were collected at the swamp site and held in cages at each site for 14 days. Data are expressed as the number of oxidized bases (8-oxodGuo or 8-oxoGuo) per 10^6 non-oxidized bases (dGuo or Guo). Data are presented as means ± standard deviations. Abbreviations: 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine.

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BIOGRAPHICAL SKETCH

Joanna Lea Joyner was born in Guelph, Ontario, on November 2, 1977, to David and Pamela Joyner. Joanna has one older sister, Danielle. Joanna spent her first several years in Guelph, followed by three years in Carbondale, IL, and eighteen years in Salt Lake City, UT. She attended Skyline High School and graduated in 1995. She earned an Honors Bachelor of Science in Biology, with minors in chemistry and history, from the University of Utah (2000). Much of the minor in history was earned during a semester abroad at the University of Wales, Swansea (1997). In her Honors thesis she described the research she conducted in pediatric immunology under the supervision of Dr. Harry R. Hill at the University of Utah School of Medicine. Joanna served as a teaching assistant during her senior year, winning the "Teaching Assistant of the Year" award for her second semester of teaching.

In 2002 Joanna earned a Master of Science in Zoology from Washington State University (Pullman, WA). Dr. Raymond W. Lee was the chair of her thesis committee. Her research was supported by two student grants and was awarded an honorable mention for best student presentation at an international symposium. During these two years she was a teaching assistant for five different courses. She also met and married Luis F. Matos, a graduate student in the Department of Entomology.

In the fall of 2002 Joanna began her dissertation research in the laboratory of Dr. David Julian in the Department of Zoology, University of Florida (Gainesville, FL). During her graduate studies she earned three research grants, won the best student presentation competition at a national meeting, was a teaching assistant for two courses, and supervised the research experiences of five undergraduate students. She worked as an adjunct instructor at Santa Fe Community College (Gainesville, FL) for two semesters, team-teaching Introductory Biology for Non-majors with her husband, Luis F. Matos. During her final year, she celebrated the birth of

her son, Lucas David. After earning her Ph.D., Joanna plans to study the relationships among free radical metabolism, mutation accumulation, and evolution in the model organism *Caenorhabditis elegans*