Abstract

Title of dissertation: COMPARATIVE PHYSIOLOGICAL ECOLOGY OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA,* AND THE ASIAN OYSTER, *CRASSOSTREA ARIAKENSIS:* AN INVESTIGATION INTO AEROBIC METABOLISM AND HYPOXIC ADAPTATIONS

Sara Ann Lombardi, Doctor of Philosophy, 2012

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The Eastern oyster, *Crassostrea virginica*, has a remarkable ability to withstand low oxygen conditions. However, the taxonomically and morphologically similar Asian oyster, *Crassostrea ariakensis*, died earlier than *C. virginica* during hypoxic exposure in multiple studies. My dissertation research sought to understand the physiological basis for this difference in tolerance. The aerobic metabolic rate of these species was assessed and a theoretical investigation into the importance of correctly standardizing metabolic rate by mass was conducted by using multiple techniques to analyze the respiration data. *Crassostrea ariakensis* juveniles exhibited higher mass-specific respiration rates than *C. virginica*, while there was no difference between adults. Further, the approach used to standardize mass vastly affected the results. When adult oysters were exposed to hypoxic

water (oxygen concentration below 0.5mgL^{-1}) for 24 hours, *C. ariakensis* gaped more frequently and wider than *C. virginica* and gaping was associated with acidification of the ambient water. When gaping was restricted by clamping, the longer an oyster was clamped the more acidic the hemolymph became in both species and a more acidic shift was observed in *C. ariakensis*. This research also investigated whether hypoxic-induced gaping was a behavioral or physiological response by exposing oysters to hypoxic, hypercapnic, and both hypoxic and hypercapnic environments and assessing metrics of adductor muscle contraction strength and speed. No significant difference was observed in the contraction strength ($\log_{10}$ transformed grams) between species, gas type, or the length of time exposed to the gas treatment. While there was no significant species effect on the speed of contraction (square root transformed seconds until peak contraction strength), oysters within the combined hypoxic and hypercapnic environment contracted more slowly than those in other treatments. When oysters were exposed to gas treatments for eight hours they exhibited the slowest rate of contraction, but there was no significant linear relationship between time exposed and time (square root seconds) to peak contraction strength. This research indicates different biochemical responses to hypoxia between closely related species which may assist in identifying mechanisms responsible for hypoxia tolerance and may contribute to restoration decisions.

COMPARATIVE PHYSIOLOGICAL ECOLOGY OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA,* AND THE ASIAN OYSTER, *CRASSOSTREA ARIAKENSIS:* AN INVESTIGATION INTO AEROBIC METABOLISM AND HYPOXIC ADAPTATIONS

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Dedicated to

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Chapter 1

Introduction and Project Summary

Oysters can be found in diverse habitats throughout the world including both intertidal and subtidal zones of marine, estuarine, coastal, and mangrove environments (Carriker and Gaffney, 1996; Gosling, 2003). Oysters are vital to the ecosystems they inhabit as they filter the water column (Newell, 1988), promote biodiversity (Rodney and Paynter, 2006), act as benthic-pelagic couplers (Porter *et al*., 2004), and facilitate a commercially and nutritionally important fishing industry (Strachey and Major, 1849; Earle, 1979; Hargis and Haven, 2009; Earle, 2009; Landrum and Ache, 2010).

Two of the estuarine oyster species include the Eastern oyster, *Crassostrea virginica* (Gmelin, 1791) synonymous with *C. brasiliana*, *C. floridensis*, *C. guyenensis*, and *C. lacerata* and the Asian oyster, *Crassostrea ariakensis* (Fujita, 1913) synonymous with *Ostrea arborea*, *O. ariakensis*, *O. rivularis*, and *C. rivularis* (Carriker and Gaffney, 1996). These species belong to the family Ostreidae within the order Ostreoida and are therefore classified as "true oysters". Oysters within the *Crassostrea* genus are synchronous broadcast spawners and possess one adductor muscle responsible for valve closure (Refer to Figure 1.1 for internal anatomy of *C. virginica*). Despite different taxa, the morphology of *C. ariakensis* and *C. virginica* has been described as almost identical (Zhou and Allen, 2003; Newell *et al*., 2007). In these species, hemolymph (blood) circulates through the body by a three-chamber systemic heart which pumps hemolymph into arteries which lead to sinuses. A pair of accessory hearts then transports hemolymph

to the renal sinus and the mantle (Eble and Scro, 1996). In addition to taxonomic and morphologic similarities, these species also have exhibited similar size-selective filtration rates, temperature and salinity tolerances, and ability to form reefs (Foighil *et al*., 1995; Calvo *et al*., 2001; Zhou and Allen, 2003; NRC, 2004, Newell *et al*., 2007; Reece *et al*., 2008).

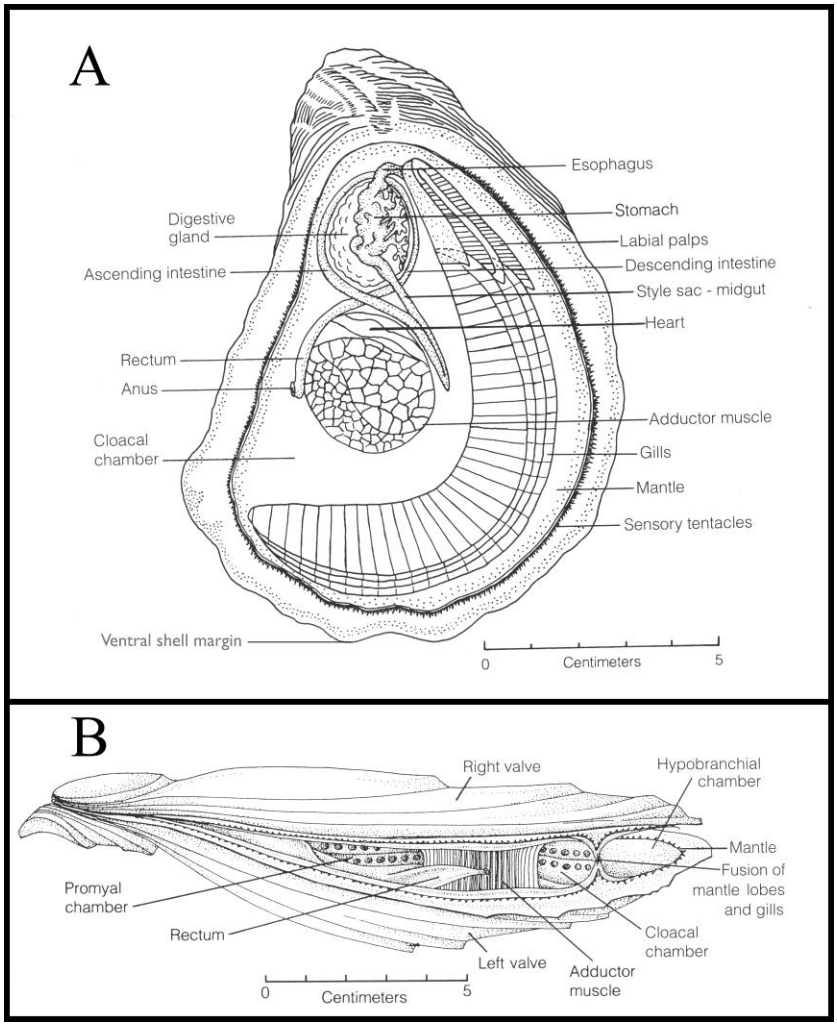

Figure 1.1 *Crassostrea virginica* anatomy. A) Sagittal view of the internal anatomy of a shucked oyster on the left valve. Modified from Eble and Scro (1996); Galtsoff (1964). B) Frontal view of *C. virginica* shell and internal anatomy. Modified from Eble and Scro (1996) as adapted from Galtsoff (1964).

Despite their many commonalities, these two species have unique ranges. *Crassostrea virginica* inhabits intertidal and subtidal estuarine and coastal regions from the Gulf of Saint Lawrence in Canada to the Gulf of Mexico along the eastern United States (Carriker and Gaffney, 1996). Some reports also indicate that *C. virginica* can be found along the South American coastline and in the Caribbean (Carriker and Gaffney, 1996; NRC, 2004). While the preferred habitat of *C. ariakensis* is also estuarine, the geographic range is distinct from that of *C. virginica*. *Crassostrea ariakensis* is found along the mouths of rivers in China and Japan (Reece *et al*., 2008) and potentially in India, Pakistan, Taiwan, the Philippines, Thailand, and Vietnam (Zhou and Allen, 2003; NRC, 2004) and is generally considered exclusively subtidal (Zhou and Allen, 2003; Kingsley-Smith and Luckenbach, 2008).

While their global distribution varies, estuarine environments are the preferred habitat of both *C. ariakensis* and *C. virginica* (NRC, 2004). As a result of both natural and anthropogenic processes, coastal estuaries are dynamic habitats and these varying conditions cause estuaries to be considered stressful environments for most species (Elliot and Quintino, 2007). Estuaries typically have lower salinities yet exhibit larger salinity ranges, larger temperature ranges, and are more prone to elevated levels of carbon dioxide than other marine systems (Hubertz and Cahoon, 1999; Ringwood and Keppler, 2002; Elliott and McLusky, 2002; McLusky and Elliott, 2007; Elliot and Quintino, 2007; Tomanek *et al*., 2011). As a result, estuarine species typically possess mechanisms to maintain internal homeostasis amid the external changes. One such approach exhibited by bivalves involves closing their valves, thereby isolating themselves from the external environment (Carriker, 1996). In addition to preventative

mechanisms, bivalves have exhibited adaptive responses such as reduced metabolic rate when exposed to stressful environments such as low oxygen (hypoxia) and high carbon dioxide (hypercapnia) concentrations (Shumway and Koehn, 1982; de Zwaan *et al*., 1983; Willson and Burnett, 2000).

Many estuarine systems routinely experience low oxygen concentrations particularly in summer months when considerable oxygen depletion occurs in the deeper waters (Taft *et al*., 1980; Breitburg, 1992; Murphy *et al*., 2011). When this depletion results in an oxygen concentration below 2 mgL^{-1} the environment is classified as hypoxic. While, low oxygen environments are natural in aquatic systems, they are becoming increasingly common and severe due to anthropogenic sources (Diaz, 2001; 2008; Hagy *et al*., 2004). Hypoxia has become a global issue particularly in marine and estuarine systems. It has been estimated that the prevalence of oxygen-depleted coastal marine environments has increased 1000% globally and 3000% in the United States over the past half a century (Diaz and Rosenberg, 2008; CENR, 2010). During this period hypoxia-induced dead zones have been reported in more than 245,000 square kilometers across more than 400 systems (Diaz and Rosenberg, 2008). Hypoxia can lead to habitat loss, reduction in fecundity and growth, reduction in species richness, and individual mortality (Diaz and Rosenberg, 1995; Rabalais and Turner, 2001; Breitburg, 2002; Diaz and Rosenberg, 2008; CENR, 2010). For instance, hypoxia in the Baltic Sea has been associated with a yearly reduction of 264,000 metric tons of carbon (MTC) (Karlson and Rosenberg, 2002). Therefore, hypoxia poses a threat to an array of habitats and species.

The ability to survive changes in environmental oxygen concentration varies among taxa. To examine the taxonomic differences in anoxia tolerance, Stickle *et al*.

(1989) compared the response of blue crabs, *Callinectes sapidus*, and other estuarine animals including *C. virginica* in a 10°C anoxic environment and found that *C. sapidus* exhibited a LT50 (the time associated with 50% mortality) of less than one day; whereas no mortality was observed in *C. virginica* for the entire 28-day study. Gray *et al*. (2002) noted a hierarchy to the anoxia tolerance, where molluscs appear to be the least sensitive to hypoxia, followed by annelids, then echinoderms and crustaceans, and noted that fish are particularly sensitive to oxygen deprivation. The hierarchy of anoxia tolerance proposed by Grey *et al.* (2002) also matches a hierarchy of mobility; anoxia tolerance likely negatively correlates with mobility, as sessile organisms cannot move to evade hypoxic conditions and therefore have likely evolved strategies to endure changing oxygen concentrations.

Bottom-dwelling, sessile, estuarine organisms are affixed furthest from atmospheric sources of oxygen and subject to oxygen stratification; thus, these species evolved in habitats vulnerable to low oxygen concentrations and as a result, some have developed means to survive in chronic low oxygen environments (Weisburg *et al*., 1997; Rabalais *et al*., 2002; Dauer *et al*., 2008). Specifically, bivalves have demonstrated substantial hypoxia/anoxia tolerance and metabolic adaptations to survive in these environments (Stokes and Awapara, 1968, de Zwaan and Zandee, 1972; Hochachka and Mustafa, 1972; de Zwaan and van Marrewijk, 1973; Malanga and Aiello, 1972; Collicutt and Hochachka 1977; Eberlee *et al*., 1983; Shumway and Koehn, 1982; de Zwaan, 1983; Stickle *et al*., 1989; Willson and Burnett, 2000; Matsche and Barker, 2006; Harlan, 2007). In particular, *C. virginica* has demonstrated a well-documented hypoxia tolerance (Eberlee *et al*., 1983; Stickle *et al*., 1989; Matsche and Barker, 2006; Harlan,

2007); however, the biochemical and physiological mechanisms by which that tolerance is accomplished remains only partially understood.

Although *C. virginica* has demonstrated adaptations enabling prolonged survival in oxygen-depleted environments, within the *Crassostrea* genus there are differences in anoxia and hypoxia tolerance. For instance, Harlan (2007) found that adult diploid and triploid *C. ariakensis* died significantly earlier when exposed to anoxia than *C. virginica*. Similarly, both *C. ariakensis* larvae (North *et al*., 2006) and juveniles (Matsche and Barker, 2006) died significantly earlier than *C. virginica* in laboratory studies. Thus, *C. ariakensis*, although closely related to *C. virginica* (Foighl *et al*., 1995; Reece *et al*., 2008), has not demonstrated a strong hypoxia tolerance (Matsche and Barker, 2006; North *et al*., 2006 Harlan, 2007). In addition to interspecific differences, anoxia and hypoxia tolerance can also vary intraspecifically based on environmental and physiological conditions. For instance, Dwyer and Burnett (1996) found that emersed *C. virginica* infected with the protozoan parasite *Perkinsus marinus* (the causal agent for the oyster disease dermo) exhibited more acidic hemolymph than that of uninfected oysters, suggesting that the extent of hypoxia tolerance may be affected by parasitic infections. However, the role of disease on the hypoxia tolerance of *C. ariakensi*s remains unknown.

In addition to differences in survival between *C. ariakensis* and *C. virginica* immersed in anoxic water, Harlan (2007) reported, but did not quantify, that when *C. ariakensis* was immersed in anoxic water it began gaping substantially earlier and to a greater extent than *C. virginica*. Potential differences in the gaping responses between *C. ariakensis* and *C. virginica* may indicate different physiological and/or behavioral

processes between these species. Additionally, gaping differences may reflect different selective pressures, and associated adaptive mechanisms, which enable some oyster species to survive in low oxygen environments.

Gaping, or abduction of the valves, results in an opening at the ventral shell margin and occurs when the adductor muscle relaxes, allowing for a separation of the two oyster valves. This opening facilitates an exchange between the internal and external environment, thus enabling spawning, phytoplankton consumption, and respiratory gas exchange. Conversely, when the adductor muscle contracts it causes valve closure which isolates the internal tissues from the external environment. Valve closure is the primary predatory defense of oysters as the hard calcium carbonate valves act as barriers between the internal tissues and predators. However, the closed valves inhibit the ability to exchange respiratory gases and can quickly induce hypoxia (Crenshaw and Neff, 1969; Moon and Pritchard, 1970). The longer an oyster can endure hypoxia, the longer it can evade a potential predator by staying closed. Therefore, there has likely been strong selective pressure for metabolic adaptations in order to survive predator-induced organismal hypoxia, even before environmental hypoxia became more frequent and severe. Thus, metabolic adaptations to survive hypoxic conditions may not only have evolved to survive environmental hypoxia frequent in estuaries, but possibly as secondary mechanism to avoid predation.

Different life histories may contribute to different mechanisms to survive hypoxic episodes. One such reason for these differences may be due to differing environmental selective pressures to survive hypoxia. In its native range in China, *C. ariakensis*,

originally classified with *Crassostrea hongkongensis* as *Crassostrea rivularis* (Wang *et al*., 2004), is referred to as *Jinjiang-muli* meaning "oyster close to river" (Zhou and Allen, 2003), as it is predominantly found at the mouths of rivers. Due to river flow and tidal actions, the mouths of rivers exhibit high turbulence and mixing, and therefore these areas may not be prone to long hypoxic episodes. In its natural habitat there may not have been strong selective pressures to develop tolerance to hypoxia in *C. ariakensis*. *Crassostrea virginica* populations, on the other hand, have not only evolved in regions characterized by significant hypoxia, but in some southern regions, many *C. virginica* reefs are intertidal, and therefore, the oysters routinely undergo regular tidal emersion. In contrast, while Zhang *et al*. (1960) found *C. rivularis* at the high water mark, *C. ariakensis* has been found in the sublittoral zone up to 10 meters deep and has been reported to be unable to tolerate aerial emersion (review by Zhou and Allen, 2003; Kingsley-Smith and Luckenbach, 2008). These differences in habitat and distribution may help explain why *C. ariakensis* may not be as well-adapted to hypoxia as *C. virginica*.

Through a series of studies investigating the metabolic rate and the effects of hypoxia and metabolic byproducts on physiological function and behavioral responses of *C. virginica* and *C. ariakensis*, my dissertation research aims to better understand behavioral and physiological adaptations responsible for hypoxia tolerance. These are ideal species to compare, as *C. virginica* has demonstrated an ability to withstand weeks of exposure to low oxygen (Stickle *et al*., 1989; Harlan, 2007; Chapter 3), while *C. ariakensis* died earlier than *C. virginica* during anoxic/hypoxic exposure (Matsche and Barker, 2006; North *et al*., 2006; Harlan, 2007). Thus, these oysters share similar

taxonomy (refer to genetic analysis by Foighil *et al*., 1995; Reece *et al*., 2008) and morphology (Zhou and Allen, 2003); yet have exhibited a striking difference in hypoxia tolerance. By comparing the physiological convergences and divergences in *C. virginica* and *C. ariakensis* during exposure to low oxygen environments, research may yield insight into the physiological adaptations that enable organismal and tissue survival during low oxygen conditions. These findings may have implications for oyster restoration decisions and may contribute to biomedical research by understanding how tissues survive low oxygen conditions. Hypoxia research may be relevant to, and have implications for, biomedical research and innovation as tissue hypoxia is a major cause of human mortality and is considered the primary cause of sudden infant death syndrome (SIDS) (Rognum *et al*., 1988; Rognum and Saugstad, 1991). Further, tumor hypoxia is a major area of oncology research (Vaupel *et al*., 1991; Zhong *et al*., 1999; Semenza *et al*., 2002). Therefore, studying adaptations enabling organismal survival (during environmental hypoxia) and tissue survival (during organismal hypoxia) may be relevant for physiological and ecological studies but may also provide insight to biomedical fields.

Understanding the basal metabolic rate of *C. ariakensis* and *C. virginica* can provide valuable information into the physiological ecology, including physiological responses to environmental hypoxia, of these species. Previous studies documented a faster growth rate in *C. ariakensis* than *C. virginica* (Calvo *et al*., 2001; Paynter *et al*., 2008); this difference in growth rate could be due to a higher basal metabolic rate, providing more energy for growth or due to differences in the allocation of resources. Further, studying metabolic rate yields insight into acid-base homeostasis by providing information on the rate of accumulation of acidic metabolic products. As an organism

metabolizes energy, acidic intermediate and end products are formed which can result in a reduced tissue pH. During aerobic respiration, carbon dioxide is produced which can react with water to lower pH (Equation 1.1), while during anaerobic respiration oysters produce alanine and succinate as compared to the typical mammalian anaerobic end product of lactate (Collicutt and Hochachka, 1977; Hochachka 1980; Eberlee *et al*., 1983; Storey, 1993; Figure 1.2). Thus, studying metabolic rate yields information about the bioenergetics and acid-base balance of these species.

Equation 1.1 Acid-base balance. In this reversible equation, carbon dioxide and water react to form the intermediate compound carbonic acid, which then dissociates into hydrogen and bicarbonate ions.

$$\mathbf{H_2O_{(l)} + CO_{2\,(g)} \leftrightarrow H_2CO_{3\,(aq)} \leftrightarrow H^+_{(aq)} + HCO_3^-{}_{(aq)}}$$

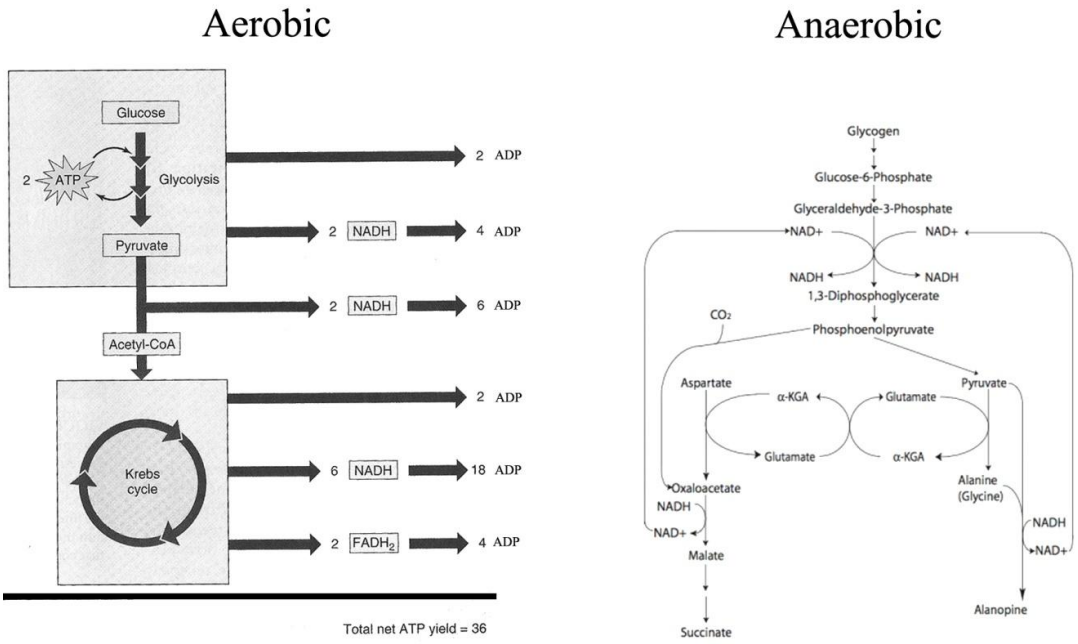

Figure 1.2 Oyster metabolic pathways. The figures above show the metabolic pathways exhibited by oysters in the presence (left) and absence (right) of oxygen. Modified from Raven and Johnson (2002) and Hochachka (1980).

Respiration is one of the most basic physiological functions. In animals, aerobic respiration involves the utilization of oxygen and production of carbon dioxide. Oxygen is utilized in aerobic metabolism where catabolic reactions yield energy in the form of ATP. As external respiration provides the oxygen used in aerobic metabolism, respiration is often used to gauge metabolic rate in animals, which can contribute to understanding their energy budget. Energy is needed for an organism to perform vital functions such as breathing and cell transport, as well as to grow, move, hunt, and reproduce (Blaxter, 1989); energy for these activities is made available by metabolic breakdown, catabolism, of an organism's energy reserves. Additionally, changes in

metabolic rate can be used to assess toxicity and disease (Singhal *et al*., 1993; Heath, 1995). Therefore, studying metabolic rate by means of oxygen consumption analysis may yield substantial information about the life history and physiology of an organism as well as the ecosystem in which it resides.

Metabolism is not only linked to acidosis and respiration, but also growth rate. Since oxygen is the final electron acceptor in the electron transport chain, the metabolic production of ATP is maximized in the presence of oxygen; the more ATP that is produced translates to more energy available to allocate to growth. Therefore, analyzing oxygen consumption may contribute to understanding differences in growth rate and energy allocation. As per the first law of thermodynamics, energy is finite, meaning it cannot be created or destroyed but rather transferred between systems or transformed within a system. For animals, energy is gained through the input of food resources, consumption (C) (Equation 1.2). Energy from an organism is "lost" via excretion (E) which includes fecal loss (F) and urinary loss (U). The net energy acquired can then be used to sustain life (R_m), move and hunt (R_a), breakdown food (R_f), reproduce (P_r), repair and replace tissues (P_e), and grow (P_g) (Equation 1.2). Energy can be allocated to reflect the life history and needs of an organism. The ability of an organism to grow depends on the energy which can be allocated towards P_g; this is termed "scope for growth" (Naylor *et al*., 1989). When the scope for growth is positive there is energy which can be allocated for growth. If the value is zero, no energy can to be allocated towards growth, and when the value is negative there is an energy deficit which requires the reabsorption of tissues (Calow and Sibley, 1990; Widdow *et al*., 1997). Therefore, differences in growth rate between organisms could reflect differences in metabolic rate, conversion

efficiency, differences in consumptions, or differences in the allocation of resources. An elevated aerobic metabolic rate could yield more energy which could be allocated towards growth. In particular, if *C. ariakensis* exhibits a higher metabolic rate than *C. virginica* it could explain the observed difference in growth between these species. Overall, metabolic analyses may provide an opportunity to identify both a mechanism for the observed differences in growth rate as well as to better understand the rate of metabolic acid accumulation. Understanding the differences in the scope for growth and rate of acid accumulation of *C. ariakensis* and *C. virginica* may provide valuable insight into the physiology of these two species, which may be used as a stepping-stone to answer other physiological and bioenergetic questions as well as help inform restoration decisions.

Equation 1.2 Energy budget. Energy consumed (C) is allocated to one of three major paths--respiration (R), production (P), or excretion (E). These are then further broken down into respiration for maintenance/survival (R_m) , respiration for activity (R_a), respiration to support feeding (R_f), growth (P_g), reproduction--shed gametes (P_r) replacement of loss tissues (P_e), urinary loss (U), feces (F_1) , and pseudo feces (F_2).

$$\mathbf{C= R_m +R_a + R_f + P_g+ P_r+ P_e + U + F_1+ F_2}$$

The introduction of *C. ariakensis* has been proposed in multiple areas where there has been a collapse of the *C. virginica* population, including the Chesapeake Bay (NRC, 2004). The introduction of *C. ariakensis* has been considered because of its fast growth rate and resilience to the effects of the protozoan parasite *P. marinus* which causes dermo (Calvo *el al*., 2001). Understanding differences in the oxygen requirements (Chapter 2)

of the two species may be important in order to inform restoration efforts in areas prone to low oxygen. If *C. ariakensis* has a higher oxygen demand, it is possible that with seasonal bouts of hypoxia in the Chesapeake Bay (Hagy *et al*., 2004; Murphy *et al*., 2011) the species might not survive following introduction. Additionally, if one of the species has high oxygen consumption, then congregations of oysters may induce areas of localized oxygen depletion within benthic habitats. Therefore, comparative metabolic research has direct restoration and management implications, and may further explain fundamental differences between two closely related species. These data may also be used as ground work to further analyze physiological differences between *C. virginica* and *C. ariakensis*. For example, understanding the basal metabolic rate of these two organisms (Chapter 2) is fundamental to understanding metabolic adaptations, like the demonstrated ability of *C. virginica* to reduce its basal metabolic rate and oxygen consumption rate up to 90% when exposed to hypoxic and anoxic environments (Shumway and Koehn, 1982; Stickle, 1989; de Zwaan *et al*., 1991). Further, metabolic rate information may be useful when comparing the acidification of hemolymph (Chapter 3) by providing insight into acidic metabolic byproduct production and when assessing the associated effects of these byproducts on muscle function (Chapter 4). Therefore, comparative metabolic experiments may provide the foundation for more comparative physiology and comparative physiological ecology studies in these two species.

While measuring oxygen consumption can be a tool to understand metabolic differences between species, comparing metabolic rate requires oxygen consumption values to be standardized by mass. However, the relationship between mass and metabolic rate has been a source of scientific debate for almost a century (Agutter and

Wheatley, 2004; Isaac and Carbone, 2010). While both small and large organisms respire, the amount of oxygen consumed per gram is not constant across all body sizes. Instead, there is an allometric relationship between oxygen consumption and mass (Rubner, 1883; Kleiber, 1932; Hemmingsen, 1960) This relationship applies to size differences throughout development (Riisgård, 1998) as well as larger scale size differences between taxa (Brody and Proctor, 1932; Hemmingsen, 1960) This difference in metabolic rate per gram is shown through the scaling exponent also known as the metabolic or power scaling exponent, "b" in the equation $R = aM^b$, where "a" is a normalizing constant, "M" is the mass, and "R" is the respiration rate, which because of cellular respiration corresponds to metabolic rate (hence in this dissertation respiration rate will be used synonymously with aerobic metabolism). Historically, it was believed that endotherm oxygen consumption scaled as $mass^{2/3}$, because body surface to volume ratios scales to $mass^{2/3}$; therefore, to regulate body temperature organisms should metabolize energy, creating heat, to the same scale as that heat could be released (Rubner, 1883). However, Kleiber (1932) found that multiple endothermic species scale to $mass^{3/4}$ rather than $mass^{2/3}$. By the 1960s studies had shown that the metabolic scaling exponent for unicellular, endothermic, and ectothermic organisms conformed to ¾, making the case for the universality of the ¾ power scaling law (Brody and Proctor, 1932; Benedict 1938; Hemmingsen 1960; Figure 1.3).

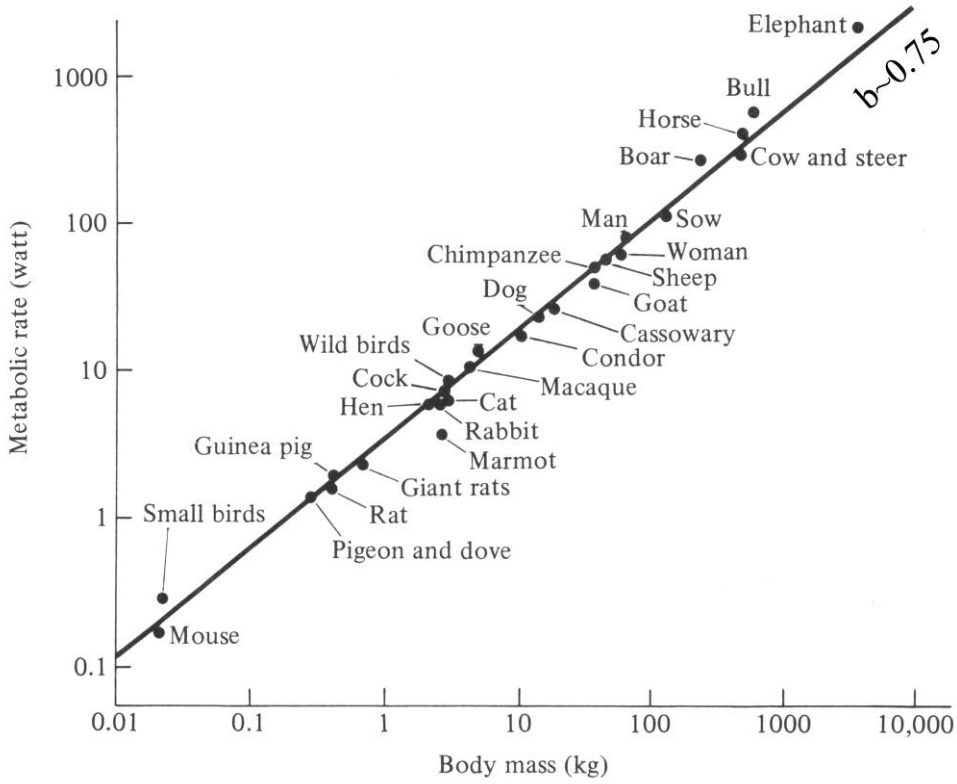

Figure 1.3 "3/4" metabolic scaling law. This figure depicts the widespread convergent relationship between body mass and metabolic rate. The slope of this line (b) is approximately 0.75. Modified from Schmidt-Nielson (1984) as adapted from Benedict (1938).

The foundation for the ¾ power law was grounded in empirical evidence; however until the 1990's no mechanism was proposed to explain this convergence. In 1997, West *et al*. proposed that the relationship was due to physiological fractal symmetry within transport systems. However, this explanation has not been universally accepted. Kozlowski and Konarzewski (2004) argued that multiple taxa have vascular and pulmonary systems that do not adhere to the assumptions of the West *et al.* model, and that due to size-specific variations in blood vessels and blood volume this model cannot apply to large animals. However, the West *et al*. (1997) model is not the only

available explanation. Banavar *et al.* (1999) have also put forth a distribution network driven explanations for scaling, while Glazier (2005; 2010) and Glazier *et al.* (2011) have focused on a more top-down approach suggesting that activity and ecology can alter metabolic variables and scaling.

Another reason the reductionist transport models (e.g. West *et al*., 1997; Banavar *et al*., 1999) have not been universally accepted is because not all organisms exhibit a metabolic scaling exponent of ¾ (Dodds *et al*., 2001; Glazier, 2005; Glazier, 2006; Riisgård, 1998). Glazier (2005) showed that intraspecific metabolic scaling exponents varied from 0.1 to 1.6 and interspecifically from 0.5 to 1.1. Riisgård (1998) found that intraspecific power scaling exponents varied based on age, where older organisms experienced a lower metabolic scaling exponent. Additionally, it is possible that growth mechanisms can affect the scaling exponent. It has been proposed that when an organism grows by increasing its number of cells, the metabolic scaling exponent is 1; however, when growth is solely achieved through increasing cell size the exponent is 0.6 (Davison, 1955; 1956, Chown *et al*., 2007). Furthermore, Glazier (2006) found that habitat type can lead to differences in scaling exponents; specifically, that benthic invertebrates exhibited significantly lower scaling exponent than pelagic invertebrates. Even within pelagic environments, epipelagic cephalopods had different metabolic scaling exponents than mesopelagic cephalopods (Seibel, 2007). Similarly, captive-reared birds exhibited a lower metabolic scaling exponent than wild birds (McKechnie *et al*., 2006). These studies indicate that ecology and habitat type can influence the metabolic scaling exponent. This theory is further validated by Glazier *et al*. (2011) who found predation pressure altered the metabolic scaling exponent of freshwater amphipods (*Gammarus minus*). These

studies contradict the reductionist theories and present evidence that scaling may not merely be a function of organismal morphology but may be influenced by ecological factors. Consequently, given that many organisms experience a scaling exponent significantly different from 0.75, many believe it may be an over-generalization to declare that all organisms exhibit a metabolic scaling exponent of 0.75 (Riisgård, 1998; Kozowski and Konarzewski, 2004; Glazier, 2005).

As there is variation in the allometric relationship, in order to assess metabolic rate across a size range, an appropriate means to standardize metabolic rate by mass must first be determined. This means a scientist must deduce or calculate a metabolic scaling exponent that is biologically relevant to their system or use an analytical technique which does not require designating a scaling exponent. This may include different scaling exponents for different age groups, seasons, body designs, or habitats since scaling exponents have been shown to change across these factors (Riisgård, 1998, de Souza *et al*., 2004; Glazier, 2006; McKechnie *et al*., 2006, Seibel, 2007; Glazier, 2010). Historically, mass-specific metabolic rate was assessed via the ratio method which involved dividing metabolic rate by mass. In this dissertation, I am also including dividing metabolic rate by mass scaled to a fixed scaling exponent ("b") as part of the ratio method, as it still creates a ratio between metabolic rate and mass (refer to Equation 1.3). The ratio method has been criticized as it assumes a fixed relationship between mass and metabolic rate and can give misleading conclusions (Packard and Boardman, 1984; Poehlman and Toth, 1995). A more currently accepted approach is to use mass as a covariate for metabolic data and perform an Analysis of Covariance (ANCOVA), however even using ANCOVA use is not without criticism (Tracy and Sugar, 1989).

However, both using ANCOVA (Mueller and Diamond 2001; Geiser 2004; Piiroinen *et al*., 2010) and the ratio method (Pellymounter *et al*., 1995; Erickson *et al*., 1996; Harlan, 2007; Dewey *et al*., 2008; Chantler, 2009, Kaiyala *et al*., 2010) for calculating mass-specific metabolic rate are prevalent in recent literature.

As there are multiple approaches currently being employed to account for metabolic rate by mass, the proposed research (Chapter 2) not only aims to understanding the mass-specific respiration rate of *C. virginica* and *C. ariakensis* in an attempt to understand growth rates and metabolic acid-base balance, but also investigates the importance of correctly standardizing metabolic rate by mass through a series of theoretical, empirical, and statistical manipulations.

Equation 1.3 Standardizing metabolic rate by mass. The variable "b" is the metabolic scaling exponent, time refers to the length of time the organism was in sealed in the chamber in which the oxygen consumption was measured.

$$\textbf{Standardized metabolic rate} = \textbf{Oxygen consumed}/(\textbf{Time} * \textbf{Weight}^{b})$$

Respiration and the circulatory system are intrinsically linked since oxygen obtained through respiration and respiratory byproducts, like carbon dioxide, are transported to and from the tissues via hemolymph. In most animals, oxygen and carbon dioxide are carried by respiratory pigments such as hemoglobin or hemocyanin. However, oyster blood, hemolymph, does not have respiratory pigments; rather, oxygen and carbon dioxide are dissolved within the hemolymph (Galtsoff, 1964).

During emersion, predation attempts, or exposure to hypoxia or pollution, *C. virginica* and other bivalves often close their valves (cease gaping) which can induce hypoxia and acidosis within the tissues, including hemolymph (Moon and Pritchard, 1970; Widdows *et al*., 1979; Akberali and Black, 1980; Akberali and Trueman, 1985; Truchot, 1990; Paynter 1996). Hypercapnia and acidosis can occur since both oxygen and carbon dioxide exchange with the external environment are halted during valve closure. Therefore, during hypoxic and anoxic stress, the accumulation of carbon dioxide and other acidic metabolic byproducts can induce metabolic acidosis. Acidosis can occur when carbon dioxide accumulates and reacts with water in the hemolymph, resulting in the formation of a hydrogen ion, thereby reducing pH (Equation 1.1). Other acidic metabolic products such as aspartic acid, succinate, and alanine are formed through anaerobic respiration, and can accumulate in the tissues as well (Collicutt and Hochachka, 1977; Hochachka 1980; Eberlee *et al*., 1983; Storey, 1993; Stickle *et al*., 1989; Figure 1.2). As metabolic products can be carried in the blood, acidic metabolic byproducts can accumulate in the hemolymph and lead to a reduced pH which may affect the other tissues in contact with the hemolymph. The acidic environment created as a result of these compounds can affect a variety of physiological functions and organ systems (Downing *et al*., 1965; Mongin, 1968; Sutton *et al*., 1979; May *et al*., 1986; Machado *et al*., 1988; Orchard and Kentish, 1990; Mitch *et al*., 1994). As a result mechanisms have evolved in some species to maintain internal acid-base balance. Some bivalve species mitigate acidosis through buffering via carbonate mobilization from the shell (Dugal, 1939; Akberali and Trueman, 1985; Byrne and McMahon, 1994; Dwyer and Burnett, 1996; Michaelidis *et al*., 2005a) or prevent acidosis by reducing metabolic

rate (Shumway and Koehn, 1982; de Zwaan, 1983; Willson and Burnett, 2000). Overall, metabolic rate, acidosis, and acid-base homeostasis are fundamentally linked to respiration. Understanding what factors contribute to these processes and the interactions between them may provide insight into adaptations to prevent and tolerate hypoxia and hypoxia-induced acidosis.

While there are many organs which can be affected by changes in pH, any effect on the adductor muscle can severely impact oyster survival. The adductor muscle controls the gape of an oyster and thus plays a large role in predatory defense and osmotic control (Carriker, 1996). Harlan (2007) noted, but did not experimentally test, that when exposed to anoxia, *C. ariakensis* began gaping almost immediately, whereas *C. virginica* remained closed for weeks up until 24-48 hours before its death. Additionally, Harlan (2007) estimated that *C. ariakensis* had a gape between 5-20mm, whereas, in *C. virginica* only dead oysters gaped more than 5mm. These drastic differences in the observed gape during hypoxia could be due to compromised adductor muscle function, possibly due to altered internal chemistry due to hypoxia. However, before mechanisms for these differences can be identified, studies should be conducted testing the observations of Harlan (2007) (Chapter 3).

The primary function of muscles, either voluntary (skeletal muscles) or involuntary (smooth or cardiac), is movement. However, a variety of conditions including hypercapnia (Hammer *et al*., 2011); hypoxia (review by Taggart and Wray, 1998); low pH (Bing *et al*., 1973; Metzger and Fitts, 1987; Hui, 2006); and combined low pH and hypoxia (the effects of which may be additive (Bing *et al*., 1973)) can affect the

strength of muscle contraction. Hypoxia, hypercapnia, and acidosis can occur within bivalve tissues when gaping is inhibited (Crenshaw and Neff, 1969; Moon and Pritchard, 1970; Widdows *et al*., 1979; Akberali and Black, 1980; Booth *et al*., 1984; Akberali and Trueman, 1985; Truchot, 1990). However, the effect of these conditions, especially their interactive effects is largely unknown (Burnett 1997), especially in *C. ariakensis* and *C. virginica*. While muscle contraction typically requires continuous energy input (Equation 1.4), oysters have evolved catch muscles which allow the adductor muscle to remain contracted with minimal energetic expense (Millman, 1964**;** Morrison, 1996; Schmidt-Neilson, 1997). It is possible that gaping may be a result of muscle fatigue due to environmental and internal stress or energy limitations. Alternatively, gaping may be a behavioral response to prevent tissue damage and maintain acid-base balance by removing acidic metabolic byproducts and permitting oxygen and carbon dioxide exchange. However, the mechanism inducing gaping response during hypoxia needs to be investigated (Chapter 4).

Equation 1.4 Muscle contraction. Actin (A), myosin (M), adenosine triphosphate (ATP), adenosine diphosphate (ADP), inorganic phosphate (Pi).

$$\mathbf{A + M + ATP \rightarrow A + M + ADP + Pi + Force}$$

In order to better understand the basic physiology of *C. virginica* and *C. ariakensis*, I examined the oxygen consumption rates (Chapter 2), assessed the gaping response (width, frequency, and role of gaping on acidification) during low oxygen environments (Chapter 3), measured changes in hemolymph pH when oysters were clamped and emersed (Chapter 3), analyzed the role of *P. marinus* infection on acid-base

balance (Chapter 3), and measured the function of the adductor muscle (contraction strength and speed) during environmental stress (low oxygen, high carbon dioxide; low oxygen and high carbon dioxide; Chapter 4). My hypothesis is that these species exhibit different metabolic strategies to facilitate survival during low oxygen, as well as demonstrate different responses to mitigate metabolic byproducts. Specifically, I propose that gaping may be a response employed to release acids from the tissues to the external environment, thus preventing metabolic acidosis, and that *C. virginica* may have evolved different mechanisms to prevent acidosis and its associated effect on tissues. Differences in acid-base regulation could manifest themselves in the hemolymph, and since the adductor muscle is in contact with hemolymph, changes in the blood chemistry could adversely affect adductor function and therefore influence gaping responses and survival.

The components of this research are intrinsically linked. Metabolic rate is fundamentally linked to the hemolymph pH, since the higher the metabolic rate, the more metabolic byproducts are likely to be present in the hemolymph. If the oysters do not gape, then both gas exchange with the external environment and the release of acidic metabolic byproducts from their system may be inhibited; this could lead to an acidic shift within the hemolymph. Since hemolymph flows through the adductor muscle via the adductor muscle sinus, the acidic hemolymph could impact adductor muscle function. A compromise in adductor muscle function may reduce survival, since relaxation in the adductor muscle results in gaping, which substantially increases an oyster's risk of predation (Tomkins, 1947; Loosanoff, 1956; Menzel and Niche, 1958; Carriker, 1996). Therefore, understanding the role of hypoxia and hypercapnia (and associated acidosis) on adductor muscle function not only yields insight into the effects of metabolic

physiology but also provides valuable information for restoration. The findings of these research projects may help explain the mechanisms for the difference in gaping response of the two species as well as the physiological mechanisms by which *C. virginica* can tolerate hypoxic and anoxic stress.

Chapter 2

Importance of correctly standardizing metabolic rate by mass shown through interspecific analysis of the Eastern oyster, *Crassostrea virginica*, and the Asian oyster, *Crassostrea ariakensis*

2.1 Introduction

While both small and large organisms respire, the amount of oxygen consumed per gram tissue weight is not constant across body sizes; rather, there is typically a linear allometric relationship between oxygen consumption and mass. Thus, larger organisms have a greater gross oxygen consumption rate than smaller organisms, however smaller organisms have a greater oxygen consumption rate per gram body mass than larger organisms. Based on the seminal work of Brody and Proctor (1932), Kleiber (1932), Benedict (1938); Hemmingsen (1960) and the more recent explanations for this pattern proposed by West *et al.* (1997), the relationship between mass and metabolic rate is generally considered to scale as mass$^{3/4}$ (Figure 1.3). However, multiple studies (Davison, 1955; 1956; Riisgård 1998, Dodds *et al*., 2001; Bokma, 2004; de Souza *et al*. 2004; Glazier, 2005; Glazier 2006; McKechnie *et al.* 2006, Hawkins *et al*., 2007; Seibel 2007; Glazier, 2010; Chown *et al*., 2007; Glazier *et al*., 2011) have demonstrated scaling exponents which deviate from ¾. Overall, metabolic scaling exponents have been shown to vary across different age groups, seasons, body designs, habitats, and predation pressure (Riisgård, 1998, de Souza *et al*., 2004; Glazier, 2006; McKechnie *et al*., 2006,

Seibel, 2007; Glazier, 2010; Glazier *et al*., 2011). Therefore, the assertion that metabolic rate scales to mass$^{3/4}$ may be both an overgeneralization and an oversimplification.

Given the variation in scaling exponents, assessing metabolic rate across a size range requires an appropriate means to standardize metabolic rate by mass. Therefore, a scientist must deduce or calculate a metabolic scaling exponent that is biologically relevant to their system or use statistical techniques which incorporate body size into the analysis. However, multiple methods for this standardization exist in the literature. Two such approaches recently employed are Analysis of Covariance (ANCOVA) (Mueller and Diamond 2001; Geiser 2004; Piiroinen *et al*., 2010) and the ratio method (Pellymounter *et al*., 1995; Erickson *et al*., 1996; Harlan, 2007; Dewey *et al*., 2008; Chantler, 2009, Kaiyala *et al*., 2010). With ANCOVA, mass can be used as a covariate and, therefore, the metabolism is assessed while taking mass into account. With the ratio method, mass can be standardized through Equation 1.3, which accounts for the allometric relationship between body mass and oxygen consumption, and then Analysis of Variance (ANOVA) can be performed. However, as deviations from the ¾ are prevalent in nature, there is ambiguity as to what value to use for the scaling exponent when using the ratio method. Therefore, I performed an academic investigation to assess the effect of using different scaling exponents and analytical techniques on metabolic rate. My hypothesis is that when the scaling exponent used in Equation 1.3 changes, or if ANCOVA is used rather than the ratio method, there will be differences in the results and subsequently different conclusions reached. This research may highlight the importance of using a biologically appropriate mass standardization approach by illustrating that the variation in results can have strong implications on the inference made from the data.

The importance of appropriately standardizing metabolic rate by mass will be demonstrated through a case study of the metabolic rates of the Eastern oyster, *Crassostrea virginica*, and the Asian oyster, *Crassostrea ariakensis*. Understanding the mass-specific metabolic rate of these species may be important as it may contribute to understanding mechanisms for differing traits and may impact restoration decisions. These two species share similar morphology and taxonomy; however, there are notable differences in growth rate, where *C. ariakensis* has been shown to grow significantly faster than *C. virginica* (Calvo *et al.*, 2001; Paynter *et al*., 2008). Since growth is contingent upon metabolic energy which is maximized through aerobic respiration, my hypothesis is that *C. ariakensis* will exhibit a higher mass-specific oxygen consumption rate ($\mu l 0_2 h^{-1} g^{-1}$) than *C. virginica*. A previous study by Harlan (2007) found that metabolic rates of adult oysters of the two species did not significantly vary. However, Harlan (2007) only investigated adults, and the oxygen concentrations within the experimental chambers were allowed to reduce to hypoxic levels, which may alter metabolic rate, particularly in oysters which can be oxygen conformers (Shumway and Koehn, 1982; de Zwaan *et al*., 1983; Willson and Burnett, 2000). Thus, this study aims to investigate the inter and intraspecific oxygen consumption ($\mu l 0_2 h^{-1} g^{-1}$) of juvenile oysters (spat) and adults, while simultaneously using these data to test the effects of using different techniques to standardize by mass.

When using the ratio method, a scientist must assess or choose an appropriate value to use as the scaling exponent ("b"). However, there are multiple potential scaling values which could be applied to the oyster samples to standardize oxygen consumption by mass. For instance, Hamburger *et al*. (1983) found that in another bivalve, the

common muscle, *Mytilus edulis*, adults exhibited a scaling exponent of 0.7, however there is also a strong case to use 0.75, as per the power scaling law. Another possibility is to empirically calculate the scaling value for each species. However, some studies show that within molluscs different age classes had different scaling exponents (Riisgård *et al*., 1980, Riisgård *et al*., 1981, Hamburger *et al*., 1983; Riisgård, 1998); this suggests that scaling exponents should be calculated for each life stage for each species. Additionally, rather than analyzing the data using Equation 1.3 coupled with ANOVA, ANCOVA can be used to assess mass-specific oxygen consumption. Changing the metabolic exponent and the statistical analysis is likely to affect the results. Thus, I conducted a series of analyses, applying different statistical techniques and varying metabolic scaling exponents (literature and empirically derived), and demonstrated the ensuing different results and implications in order to highlight the importance of choosing a biologically and statistically valid approach to standardize metabolic rate by mass.

The implications of this study can extend beyond oyster physiology and standardizing oxygen consumption data by mass. In particular, it can be applied to other physiological fields, since most anatomical and physiological processes (ranging from brain size, egg incubation time, circulation time, drug absorption rates, and the ease of moving up inclines) scale allometrically (Schmidt-Nielson, 1984; Packard and Boardman, 1987; Pokras *et al*., 1993). Therefore, any analysis comparing these or other allometric processes need an appropriate means of standardizing by mass. Thus, this research provides information about differences in the respiration rate of *C. ariakensis* and *C. virginica* while illustrating the larger issue of the implications of incorrect

standardization by mass in allometric relationships, which may be applied to an array of topics within physiology, ecology, organismal biology, medicine, and other related fields.

2.2 Materials and Methods

2.1 Specimen collection and oxygen consumption measurements

Crassostrea virginica and *C. ariakensis* were obtained from the University of Maryland's Center for Environmental Science Horn Point Lab Oyster Hatchery in Cambridge, MD and transported to the University of Maryland College Park campus. Cultchless juvenile oysters (spat) were fasted in aerated 25°C water with a salinity of 15 for 24 hours, while adults were starved for a week. After the fasting period, oysters were transported to Juniata College and were cleaned in 9:1 water-chlorine bleach (HOCl) solution, rinsed in a freshwater bath, and then scrubbed to remove encrusting and burrowing organisms, bacteria, and other foreign debris. Spat were individually placed into glass syringes sealed with wax and each adult was put into an airtight chamber. The apparatuses were filled with 25°C water with a salinity of 15 and placed in a 25°C environmentally controlled room. After two hours the oxygen tension in each chamber was assessed using a Strathkelvin respirometry system. These values were compared against controls, water-filled respiration chambers without an oyster, to determine the amount of oxygen consumed by each organism in the two hour timeframe. Each oyster was shucked and the flesh was removed from the shells and wet tissue was dried in a 60°C oven. Using a microbalance, a dry weight was determined for each sample. Using

these values and Equation 2.1 (as referenced by Glazier, 1991), the oxygen consumption rate, µl 0_2 h^{-1}, for each individual oyster was calculated. A total of 91 oysters (spat and adults of *C. virginica* and *C. ariakensis*) across four orders of magnitude of body mass were used in this study.

Equation 2.1 Respiration calculation. Where "R" equals respiration, "P" equals oxygen reading (mmHg), "e" represents experimental samples and "c" represents control samples, "S" equals the solubility coefficient of oxygen (µmol $liter^{-1}$ $mmHg^1$ at 25°C and salinity of 15), "V" equals the volume of water (liters), "A" equals the volume of 1 mole of oxygen at standard temperature and pressure (liters mol^{-1} and "t" is the time in the respiration chamber (hours)

$$\mathbf{R= [(P_e-P_c) * S * V * A]/t}$$

2.3 Standardizing by mass

To account for the allometric relationship between body mass and oxygen consumption, the respiration rates were standardized by mass by either using the ratio method, Equation 1.3, with ANOVA, or by using ANCOVA with mass as a covariate. When using the ratio method, different scaling exponents were applied (Trials 1-8); some values were derived from the literature and some empirically calculated. The intra and interspecific null and alternate hypotheses tested in this study are shown in Table 2.1.

In trials where the metabolic scaling exponent was empirically calculated, the scaling value was determined by assessing the slope using Least Squares Regression (LSR) with $\log_{10}$ transformation of mass (g) as the independent variable and $\log_{10}$ transformation of respiration ($\mu l 0_2 h^{-1}$) as the dependent variable. Least Squares Regression was used rather than Reduced Major Axis (RMA) Regression since the results

tend to be similar, but LSR is likely more accurate and the assumptions of RMA may not be met (Calder, 1987; Glazier, 2005; White, 2011). Additionally, data was transformed to convert an exponential relationship to a linear relationship, thereby allowing linear ANCOVA analysis. Additionally log transformed data may more accurately represent metabolic rate relationships than non-linear, untransformed data (Kerkhoff and Enquist, 2009; White, 2011).

Table 2.1. Respiration hypothesis tested with multiple scaling exponents. Null hypotheses are represented by "H_o", and each alternate hypothesis is shown by "H_a".

Intraspecific analyses	**H_o1**	*Crassostrea virginica* spat have the same mass-specific oxygen consumption rate as *Crassostrea virginica* adults.
	H_a1_a	*Crassostrea virginica* spat have a lower mass-specific oxygen consumption rate than *C. virginica* spat.
	H_a1_b	*Crassostrea virginica* spat have higher mass-specific oxygen consumption rate than *C. virginica* adults.
	H_o2	*Crassostrea ariakensis* spat have the same mass-specific oxygen consumption rate as *C. ariakensis* adults.
	H_a2_a	*Crassostrea ariakensis* spat have a lower mass-specific oxygen consumption rate than *C. ariakensis* adults.
	H_a2_b	*Crassostrea ariakensis* spat have a higher mass-specific oxygen consumption rate than *C. ariakensis* adults.
Interspecific analyses	**H_o3**	*Crassostrea ariakensis* spat have the same mass-specific oxygen consumption rate as *C. virginica* spat.
	H_a3_a	*Crassostrea ariakensis* spat have lower mass-specific oxygen consumption rate than *C. virginica* spat.
	H_a3_b	*Crassostrea ariakensis* spat have a higher mass-specific oxygen consumption rate than *C. virginica* spat.
	H_o4	*Crassostrea ariakensis* adults have the same mass-specific oxygen consumption rate as *C. virginica* adults.
	H_a4_a	*Crassostrea ariakensis* adults have a lower mass-specific oxygen consumption rate than *C. virginica* adults.
	H_a4_b	*Crassostrea ariakensis* adults have higher mass-specific oxygen consumption rate than *C. virginica* adults.

2.3a Different approaches to standardizing mass

Trial 1: Isometric scaling: b=1

In this trial the mass-specific respiration rate was calculated by functionally removing "b" from Equation 1.3 by setting it equal to 1. This assumes an isometric, and not allometric, relationship between oxygen consumption and body mass.

Trial 2: Literature derived. The power-scaling law

In this trial, the value of 0.75 was used for the metabolic scaling exponent as per the power-scaling law. This value is considered by some to be a universal scaling exponent applying to mitochondria, mice, elephants and most everything in between (Hemmingsen, 1960; Calder 1984; Schmidt-Neilson, 1984) and should therefore apply to both species of oysters across age classes.

Trial 3: Literature derived. Kleiber's empirically calculated exponent

One of the central foundations of the ¾ power law was Kleiber's (1932) empirical evidence suggesting that mass scales to ¾ and not 2/3. However Kleiber's actual experimental value was not 0.75, but in fact 0.73 (Kleiber, 1932; Schmidt-Neilson, 1984). The value 0.75 was chosen instead of 0.73 to simplify mathematical calculations (Schmidt-Neilson, 1984). Therefore, in this trial I applied the original scaling exponent calculated by Kleiber (1932).

Trial 4: Literature derived. The common mussel, *Mytilus edulis*, scaling exponent values

The scaling exponents used in this trial were derived from a taxonomically, morphological, and functionally similar species, *Mytilus edulis*.

Thus, a scaling exponent value of 0.9 was applied to spat of both *C. ariakensis* and *C. virginica*, and a scaling exponent of 0.7 was applied to adults because these values were associated with the physiologically similar benthic bivalve, *Mytilus edulis* (Riisgård *et al*., 1980; Riisgård *et al*., 1981; Hamburger *et al*., 1983).

Trial 5: Empirically calculated. Regression analysis for each species

In this trial the scaling exponent was calculated as 0.7091 for *C. ariakensis* and 0.8462 for *C. virginica*. The values for the scaling exponent in this trial were calculated using the data collected on spat and adults with almost four orders of magnitude size range. Scaling exponents were empirically derived because studies have shown interspecific differences in scaling exponents (Riisgård, 1998; Glazier, 2005). Additionally, studies have suggested that ecology, specifically habitat type, can cause deviations in the expected value of 0.75 (Glazier, 2006; McKechnie *et al*., 2006; Seibel, 2007). This is particularly relevant in this study since the native range of *C. ariakensis* and *C. virginica* do not overlap and the species have demonstrated different growth rates, so these species may have different energetic requirements resulting in different scaling exponents.

Trial 6: Empirically derived. Regression analysis on adults of each species

The scaling exponents used in this trial were calculated for each species using adult oysters only, but the scaling exponent was applied to all sizes. This approach yielded a scaling exponent of 0.0014 for *C. ariakensis* and 0.1418 for *C. virginica*. These scaling exponents were then applied to all samples (spat and adults) of each species. This approach was employed because energy

requirements and allocation may change with different body designs and life stages as Riisgård *et al*. (1980; 1981) and Hamburger *et al.* (1983) observed intraspecific differences by age in bivalves. This approach may yield valuable insight into the effect of using a sample of a limited size range and generally applying it to the entire size range of a species.

Trial 7: Empirically derived. Regression analysis on spat for each species.

Similar to the adult study (Trial 6), the scaling exponents calculated in this trial were empirically derived from only the spat samples but applied to both spat and adult samples. Using this approach, the scaling exponent for *C. ariakensis* was calculated as 0.7672 and 1.0313 for *C. virginica*. An analysis based on spat was included because there can be intraspecific differences in energy usage based on age which can cause intraspecific difference in the scaling exponent. This analysis was applied to adults to show the effect of generalizing the exponent value to different size classes.

Trial 8: Exponent empirically derived. Regression analysis on each age class and species.

The value for the scaling exponent was calculated for each age class (spat and adults) for each species. Using this approach, the scaling exponent for *C. ariakensis* spat was 0.7672, for *C. ariakensis* adults was 0.0014, for *C. virginica* spat was 1.0313, and for *C. virginica* adults was 0.1418. This approach was employed to account for differences between species as well as age classes, both of which have been shown to cause deviations in the metabolic scaling exponent.

Therefore, this approach allows for the analysis of the respiration data while accounting for both inter and intra specific differences in respiration.

Trial 9: Analysis of Covariance

Using mass as the covariate eliminated the need to use Equation 1.3, and therefore there was no need to assign a scaling exponent. Intraspecific age relationships cannot be compared using traditional ANCOVA since mass, the covariate, and age are fundamentally linked with oysters. Since ANCOVA analysis revealed that *C. ariakensis* and *C. virginica* exhibited different slopes ($p=0.035$), indicating different scaling exponents, the Johnson-Neyman Technique modified by White (2003) was employed because it allow analysis of the height of the lines (metabolic rate) despite heterogeneous slopes (different scaling exponents).

2.3. Results

In seven of the nine trials there was a significant ($p<0.05$) interaction between species and age on the mass-specific aerobic respiration rate. In the other two cases there was marginal significance ($p=0.060$, $p=0.090$) and unprotected pair-wise comparisons were performed. Results of Trials 1-9 can be found in Table 2.2; the results of Trial 9 can also be seen in Figure 2.1.

Trial 1: Isometric

In this trial the value of 1 was used for the metabolic scaling exponent, thus standardizing the data by mass but not accounting for a negative allometric

relationship. In this trial, *C. virginica* spat possessed a higher mass-specific oxygen consumption rate than adults (p_{Ho1}=0.001). Similarly, *C. ariakensis* spat exhibited a higher mass-specific oxygen consumption rate than adults (p_{Ho2}<0.001). *Crassostrea ariakensis* spat also exhibited a higher rate than *C. virginica* (p_{Ho3}<0.001), whereas the oxygen consumption rate of adults were not significantly different (p_{Ho4}=0.270; Table 2.2 Column 3)

Trial 2: Power-scaling law

When the value 0.75 for the metabolic scaling exponent was used, there was no intraspecific shifts in mass-specific oxygen consumption in *C. virginica* (p_{Ho1}=0.066) or *C. ariakensis* (p_{Ho2}=0.317). Additionally, there was not a significant difference in the rate between adults of *C. virginica* and *C. ariakensis* (p_{Ho4}=0.270), but *C. ariakensis* spat exhibited a higher mass-specific oxygen consumption rate than *C. virginica* spat (p_{Ho3}=0.014; Table 2.2 Column 4).

Trial 3: Kleiber's empirically calculated exponent

When the value 0.73 was applied to the data set, *C. virginica* adults exhibited a higher mass-specific oxygen consumption rate than spat (p_{Ho1}=0.025) whereas *C. ariakensis* adults and spat demonstrated the same rate (p_{Ho2}=0.636). Interspecifically, *C. ariakensis* adults exhibited a higher mass-specific oxygen consumption rate than *C. virginica* adults (p_{Ho4}=0.014), while spat of each species exhibited the same rate (p_{Ho3}=0.696; Table 2.2 Column 5).

Trial 4: The common mussel, *Mytilus edulis*, scaling exponent values

When a scaling exponent of 0.7 was applied to spat of both species, and a value of 0.9 to adults of both species, spat exhibited a higher mass-specific

oxygen consumption rate than adults in *C. ariakensis* (p_{Ho2}<0.001), while in *C. virginica* there was no intraspecific difference (p_{Ho1}=0.344). In this trial, *C. ariakensis* spat demonstrated higher mass-specific oxygen consumption values than *C. virginica* spat (p_{Ho3}=0.002), while the rates of adults were not significantly different (p_{Ho4}=0.806; Table 2.2 Column 6).

Trial 5: Empirically calculated for each species

When scaling exponents calculated for each species through regression analysis were applied, there was no intraspecific difference in the mass-specific oxygen consumption rate for *C. virginica* (p_{Ho1}=0.859) or *C. ariakensis* (p_{Ho2}=0.936), nor was there any interspecific difference based for spat (p_{Ho3}=0.763) or adults (p_{Ho4}=0.972; Table 2.2 Column 7)

Trial 6: Empirically calculated for adults of each species

When scaling exponents calculated for adult oysters of each species were applied to all samples within a species, adults exhibited a higher mass-specific oxygen consumption rate than spat in *C. virginica* (p_{Ho1}<0.001; Table 2.2 Column 8) and in *C. ariakensis* (p_{Ho2} <0.001). *Crassostrea virginica* spat exhibited a higher oxygen consumption rate than *C. ariakensis* (p_{Ho3}=0.002), but there was no significant difference in adult mass-specific oxygen consumption rates (p_{Ho4}=0.054; Table 2.2 Column 8).

Trial 7: Empirically calculated for spat for each species

When the scaling exponent calculated for spat of each species was applied to all samples, spat exhibited a higher mass-specific oxygen consumption rate than that of adults in *C. virginica* (p_{Ho1}<0.001), but there was no significant

intraspecific difference based on age in *C. ariakensis* (p_{Ho2}=0.148). Additionally, *C. virginica* spat had a higher mass-specific oxygen consumption rate than adults (p_{Ho3}=0.014), but there was not an interspecific difference in adults (p_{Ho4} =0.990; Table 2.2 Column 9).

Trial 8: Empirically calculated for each age class and species

When a scaling exponent was empirically calculated for each age class of each species and applied to the dataset, *C. virginica* spat had a higher mass-specific oxygen consumption rate than adults (p_{Ho1}=0.014), whereas in *C. ariakensis* spat had a lower mass-specific oxygen consumption rate than adults (p_{Ho2}=0.034). Also, *C. virginica* spat had a higher mass-specific oxygen consumption rate than that of *C. ariakensis* spat (p_{Ho3}=0.012), but *C. ariakensis* adults had a higher rate than *C. virginica* adults (p_{Ho4}=0.039; Table 2.2 Column 10).

Trial 9: Analysis of Covariance

In this trial, Analysis of Covariance with mass as a covariate was used, rather than the ratio method used in the previous trials (Trial 1-8). The Johnson-Neyman approach (modified by White, 2003) was used employed since there was a significant interaction between species and mass (p=0.035), indicating the species exhibited different metabolic scaling exponents. Here, *C. ariakensis* exhibited a higher mass-specific oxygen consumption rate than *C. virginica* for oysters with a dry mass less than 0.04g, (p_{Ho3} <0.05; Table 2.2 Column 11; Figure 2.1). All adults tested had a dry mass greater than 0.04g, while all spat had a dry mass less than 0.04g, and thus this analysis suggests that *C. ariakensis* spat

exhibited a higher mass-specific oxygen consumption rate than *C. virginica*, but that there was no interspecific difference between adults (p_{Ho4}>0.05). The Johnson-Neyman Technique extrapolated that a difference may occur for oysters greater than 4.6x10^13g, which is an unrealistic size for an oyster, and thus for oysters above 0.04g there is likely no significant difference in the mass-specific oxygen consumption of *C. virginica* and *C. ariakensis*.

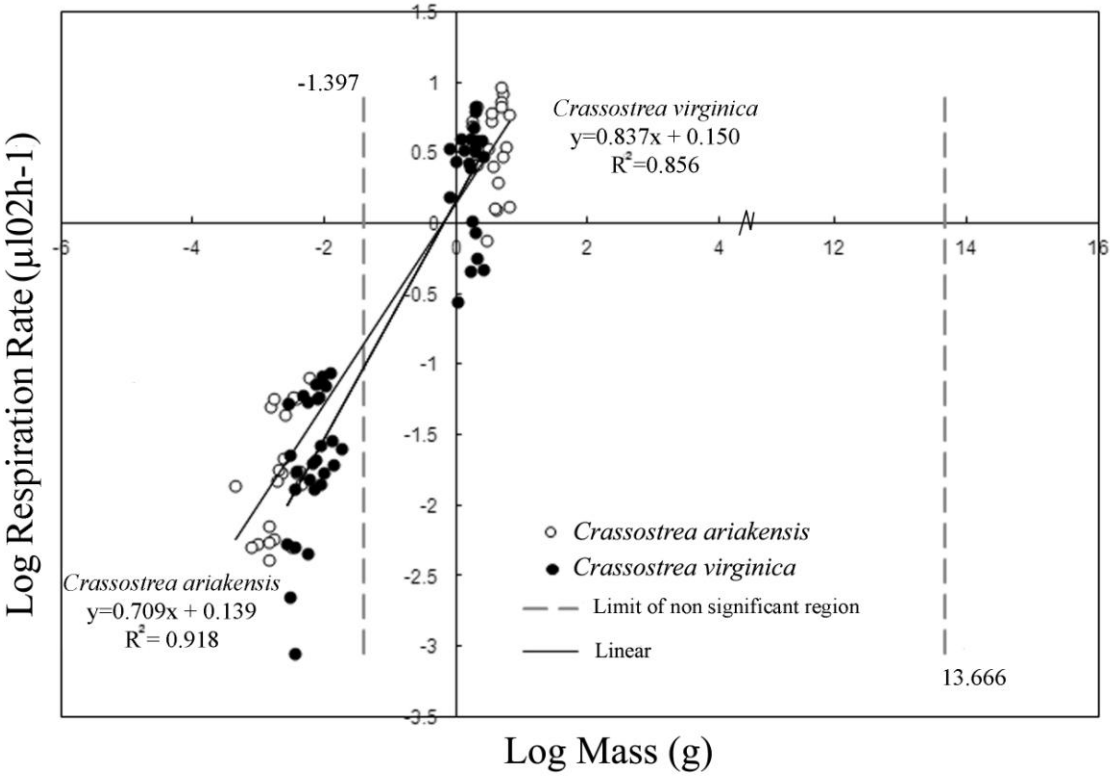

Figure 2.1 *Crassostrea virginica* and *Crassostrea ariakensis* Analysis of Covariance with Johnson-Neyman Technique. The covariate is $\log_{10}$ mass in grams, while the treatment is species (open circles *C. ariakensis*; closed *C. virginica*) and the response variable is the $\log_{10}$respiration ($\mu l 0_2 h^{-1}$). Area between the dashed vertical lines indicate the size range in which the species exhibited the same mass-specific respiration rate (p>0.05).

Trials 1-9 summary

Overall, every null hypothesis and all but one of the eight alternate hypotheses can be either rejected (based on an alpha of 0.05) or accepted, depending on which technique was used (Table 2.2). For instance, three out of the eight trials which studied intraspecific differences in *C. virginica* found that there was not a significant difference in the mass-specific oxygen consumption rate between age classes, yet five out of eight of the approaches indicated a difference between ages (H_o1). Of those five, three trials found that spat demonstrated a higher rate, while two found that adults exhibited a higher mass-specific oxygen consumption rate. For intraspecific analysis of *C. ariakensis* (H_o2), the results are more split, with half of the tests suggesting there was no difference and the remainder suggesting there was a difference. Of the four that suggested there was a difference, two suggested that adults exhibited higher values, and the other half suggested that spat had higher values. For hypothesis three, assessing the metabolic rate of both species of spat, eight out of nine approaches found that there was a difference between species (Table 2.2; Figure 2.1), but five approaches found that *C. ariakensis* exhibited higher values, while three found that *C. virginica* exhibited higher values. Lastly, eight out of nine (Table 2.2; Figure 2.1) of the approaches found that there was not a significant difference between adults of each species with probability (p) values as high as 0.972 (Trial 5) and 0.990 (Trial 7), yet when empirically calculated values were applied for each species and each age class *C. ariakensis* adults had a higher mass-specific oxygen consumption rate than *C. virginica* adults (p=0.039).

Table 2.2 Summary of the respiration results based on different mass standardization techniques for *Crassostrea virginica* and *Crassostrea ariakensis*. Columns 3-10 represent the ratio method, while Column 11 represents Analysis of Covariance. Hypotheses (refer to Table 2.1) are presented in rows two through fourteen, and each method of standardization has its own column. The last column summarizes the number of test which suggested to not reject each of the null hypotheses compared to the number which suggested to reject the hypothesis (based on an alpha of 0.05).

Null hypothesis	**Alternate Hypothesis**	**Trial 1** Isometric	**Trial 2** Power-scaling law	**Trial 3** Kleiber's value	**Trial 4** *Mytilus*	**Trial 5** Empirical for each species	**Trial 6** Empirical adult	**Trial 7** Empirical spat	**Trial 8** Empirical age and species	**Trial 9 ANCOVA**	**Accept -reject Ratio**
$H_0$1- Intraspecific *C. virginica*		**Reject p=0.0011**	Do not reject p=0.0662	**Reject p=0.0247**	Do not reject p=0.3435	Do not reject p=0.8588	**Reject p<0.001**	**Reject p<0.0001**	**Reject p=0.0139**	**n/a**	**3:5**
	Ha1a: spat<adult			Accept			Accept				
	Ha1b: spat>adult	Accept						Accept	Accept		
$H_0$2 Intraspecific *C.ariakensis*		**Reject p<0.0001**	Do not reject p=0.3165	Do not reject p=0.6355	**Reject p<0.0001**	Do not reject p=0.9361	**Reject p<0.001**	Do not reject p=0.1481	**Reject p=0.0341**	**n/a**	**4:4**
	Ha2a: spat<adult						Accept		Accept		
	Ha2b: spat>adult	Accept			Accept						
$H_0$3 Interspecific spat		**Reject p=0.0007**	**Reject p=0.0135**	**Reject p=0.0158**	**Reject p=0.0021**	Do not reject p=0.7625	Reject p=0.0018	**Reject p=0.0142**	**Reject p=0.0119**	**Reject p<.05**	**1:8**
	Ha3a: C.a.<C.v.						Accept	Accept	Accept		
	Ha3b: C.a.>C.v.	Accept	Accept	Accept	Accept					Accept	
$H_0$4 Interspecific adult		Do not reject p=0.2702	Do not reject p=0.6963	Do not reject p=0.7400	Do not reject p=0.8063	Do not reject p=0.9718	Do not reject p=0.0541	Do not reject p=0.9901	**Reject p=0.0389**	Do not reject p<.05	**8:1**
	Ha4a: C.a.<C.v.										
	Ha4b: C.a.>C.v.								Accept		

2.4 Discussion

Changing the scaling exponent or the statistical analysis used resulted in vastly different conclusions being reached in this study, which highlights the importance of using the most appropriate scaling exponent. Contradicting results and conclusions were reached when the ontogenetic-intraspecifc and interspecific oxygen consumption rates of *C. virginica* and *C. ariakensis* were compared using the nine approaches (Analysis of Covariance and the ratio method with different scaling exponent values; Table 2.2). All of the four null, and all but one (of eight) of the alternate hypotheses could either be accepted or rejected based on which standardization approach was employed. If a scientist haphazardly only chose one of these approaches, there is a strong likelihood that their findings would not reflect that of the population studied. Of these approaches, I believe the most valid for this dataset was the ANCOVA analysis (see rational below), and therefore *C. ariakensis* spat exhibited higher respiration rate, translating to a higher aerobic metabolic rate, than *C. virginica*, but adults experienced similar metabolic rates. This analysis identified differences in the ontogenetic metabolic rate of two oyster species, while highlighting the effects of using different statistical approaches and scaling exponents to standardize metabolic rate by mass.

Even small, non-statistically significant changes in the scaling exponent resulted in different conclusions. For instance, shifting the scaling exponent 0.02 units, from 0.75 to 0.73 resulted in the conclusion that *C. virginica* adults had a higher mass-specific metabolic rate than *C. virginica* spat, whereas when using 0.75, there was no significant intraspecific difference based on age. This is similar to the findings of Hayssen and Lacy

(1985) which showed that changing the scaling exponent between these two values could change the metabolic rate of a 1kg mammal by 15% and by 26% for a 100kg mammal. Even slight changes in the scaling exponent can lead to dramatic differences in the calculation of metabolic rate, thus emphasizing the importance of assessing and utilizing an appropriate scaling exponent.

For this reason, it is important to determine the appropriateness of the various approaches used in each trial. Specifically, some of the prescribed trials were more biologically and statistically appropriate than others. For instance, Trial 6 (adult values applied to all sizes) and Trial 7 (spat values applied to all sizes), were not ideal approaches as the scaling exponents were calculated using a small data sets with a limited size range but the scaling exponents were applied to a larger size range. Further, Trial 6 found extremely low and unrealistic scaling exponents (0.0014 & 0.1418), though interestingly, these scaling exponents were not significantly different from those calculated on adult *C. ariakensis* and *C. virginica* oysters by Harlan (2007 and Harlan unpublished data). These low scaling exponents can likely be explained by the limited size range of adults studied (less than an order of magnitude), as smaller size ranges can inhibit the calculation of meaningful scaling exponents (Calder, 1987) and can lead to the calculation of lower scaling exponents (Brown *et al*., 1997). Datasets with limited size ranges, less than two orders of magnitude (Schmidt-Neilson, 1984), can affect the results, since growth trajectories and metabolic scaling interpretations can be incorrect (Schmidt-Neilson, 1984; Refinetti, 1989). Further, using small datasets and over-generalizing the patterns (as in Trials 6 and 7) can lead to erroneous conclusions (compare Galvão *et al.*, 1965 and Dmi'el, 1972). Therefore, Trials 6 and 7 act as a warning of the variation in

results which can occur when scaling exponents are calculated on a specific or narrow size range but are generally applied; they also highlight the general danger of using limited size ranges.

The results of Trial 1 indicate that there was likely an allometric, and not isometric, relationship in metabolic scaling of these species. A negative allometric relationship would manifest as spat consuming more oxygen per gram dry tissue than adults. Trial 1 showed that both *C. ariakensis* and *C. virginica* spat exhibited higher respiration rates per gram than adults. This is not surprising as it is generally agreed that physiological variables scale allometrically with mass (Packard and Boardman, 1987). Therefore, Trial 1 may not be an appropriate technique to use for mass standardization.

Here, I provide a ranking (from worst to best) of the approaches used in this study. I argue the least valid approaches are those where a scaling exponent for one size class was calculated and applied to the whole dataset (Trials 6 and 7; rationale above). As there has been extensive work showing inter and intraspecific variation (Riisgård, 1998, Glazier, 2005; 2006; Seibel, 2007; McKechnie *et al.*, 2006; Glazier *et al*., 2011), I propose the next worst are assuming both species scale isometrically (Trial 1), to ¾ (Trial 2), or to 0.73 (Trial 3). Since scaling relationships have been shown to vary based on age in bivalves, empirically calculating a single value for each species (Trial 5) is not ideal. Similarly, as there is much interspecific variation, it may not be valid to assume that similar taxa, such a *Mytilus* and *Crassostrea* exhibit the same scaling value (Trial 4). I believe the second best approach is the ratio method with empirically derived scaling

values for each age class and species assuming each age class has sufficient sample size and size range (Trial 8) and the best approach was ANCOVA (Trial 9; rationale below).

While there is still controversy over the usefulness of the ratio method (Packard and Boardman, 1984; Poehlman and Toth, 1995) and how to appropriately use ANCOVA (Tracy and Sugar, 1989) for calculating mass-specific metabolic rate, ANCOVA has less mass bias than the ratio method. Some have found the ratio method to give "misleading conclusions" (Poehlman and Toth, 1995); similarly, this study found the ratio method (Trials 1 through 8) gave contradicting results and, therefore, in some cases yielded entirely wrong conclusions. Therefore, I believe ANCOVA, which accounts for mass across all sizes and limits subjectivity (by removing the election of a "b") to be a better approach. Thus, I propose that, of the approaches tested, ANCOVA was the most biologically valid approach. However, scientists must determine the best method for their data, by taking into account the life history and ontogeny of their taxa.

The implications of this study extend beyond theoretical discussions of metabolic scaling, its universality, and methods of standardization. In the case of the Eastern and Asian oysters, using different scaling exponents could result in different findings about the metabolic rate of these oysters. Since, in this situation, ANCOVA was the most biologically sound analysis, it can be inferred that *C. ariakensis* spat exhibited a higher oxygen consumption rate than *C. virginica* spat, but adults of both species had the same mass-specific respiration rate. Additionally, *C ariakensis* and *C. virginica* had significantly different metabolic scaling exponents. The observed elevated metabolic rate in *C. ariakensis* spat compared to *C. virginica* spat could explain the faster growth

rate of *C. ariakensis* as juveniles. If *C. ariakensis* had a higher metabolic rate, then when food is bountiful this may explain the elevated growth rate exhibited by *C. ariakensis*. An elevated metabolic rate would release energy obtained from food at a faster rate, which could be allocated to facilitate growth. However, since there was no difference in the mass-specific oxygen consumption between adults (based on ANCOVA), the elevated growth rate in these species as adults cannot be explained. Therefore, other bioenergetic differences likely exist between these species. *Crassostrea ariakensis* may have a greater anaerobic metabolic rate, facilitating a greater scope for growth for *C. ariakensis* than *C. virginica*. Another possibility is that energy production is the same for each species but energy is allocated differently between these species as adults. Specifically, there could be differing costs for reproduction or different energetic requirements for growth. To better understand the mechanisms associated with the observed difference in growth rates between these species (Calvo *et al*., 2001; Paynter *et al*., 2008), further studies should be performed analyzing reproductive energetic contributions in both species, energetic contributions for somatic and shell growth, and anaerobic metabolic rate using calorimetry. Understanding oxygen requirements and energy budgets of these species could aid in understanding scope for growth, reproductive success, carbon dioxide accumulation, acid-base balance, and mechanisms associated with hypoxia tolerance (Chapter 3 and 4) which may have applications in comparative physiology, restoration, and oyster management. Thus, as the results varied based on technique applied, using a mass standardization technique that is not the most appropriate for the data could lead to incorrect physiological interpretations and detrimental restoration decisions.

Beyond understanding the differences in *C. ariakensis* and *C. virginica,* correctly assessing mass-specific metabolic rates may be applicable to an array of fields. Veterinary and human medical fields depend on understanding variables that scale allometrically including drug absorption rates, drug response times, and hypothermic risk (Pokras *et al*., 1993). Further, Guiot *et al.* (2003) found the rate of malignant tumor growth also scaled allometrically to tumor mass. Thus, understanding power scaling relationships and appropriate means to standardize by mass can aid in the understanding and predicting of disease proliferation, which is instrumental to implementing successful treatment strategies (Guiot *et al*., 2003). The biomedical applications further highlight the importance of correctly standardizing metabolic rate.

Metabolic scaling and metabolic rate are fundamentally linked to an array of physiological mechanisms. Thus, incorrectly standardizing allometric relationships by mass may have a profound impact on a variety of fields ranging from theoretical biology to oncology. The diverse array of interpretations of oyster metabolic rate based on which scaling exponent was used acts as a reminder of the importance of choosing an appropriate analysis and scaling exponent to assess traits that scale allometrically. Therefore, this study both identified different aerobic metabolic rates in *C. ariakensis* and *C. virginica* spat while simultaneously highlighting the importance of appropriately standardizing allometric physiological processes, specifically metabolic rate, by mass through the analysis of *Crassostrea virginica* and *Crassostrea ariakensis.*

Chapter 3

Hypoxic gaping responses and hemolymph pH during clamped emersion in the Eastern oyster, *Crassostrea virginica*, and the Asian oyster, *Crassostrea ariakensis*

3.1 Introduction

Low dissolved oxygen levels are common in estuarine systems such as the Chesapeake Bay, particularly in summer months, when considerable oxygen depletion occurs in the deeper stratified waters (Taft *et al*., 1980; Breitburg, 1992; Murphy *et al*., 2011). Since near-bottom waters are more prone to hypoxia due to decomposition of organic matter and stratification, benthic communities are disproportionately affected by hypoxia (Weisburg *et al*., 1997; Rabalais *et al*., 2002; Dauer *et al*., 2008). Sessile benthic organisms, such as oysters, are particularly vulnerable to low oxygen because they cannot move to evade hypoxic zones.

Many sessile members of the benthic community, including the Eastern oyster, *Crassostrea virginica*, have evolved metabolic adaptations that facilitate survival in low oxygen environments. One such adaptation in *C. virginica* is its ability to decrease its oxygen consumption and reduce its metabolic rate by up to 90% when exposed to hypoxia and anoxia (Shumway and Koehn, 1982; de Zwaan *et al*., 1983; Willson and Burnett, 2000). Hypoxic environments can lead to the accumulation of carbon dioxide and other acidic metabolic byproducts (Figure 1.2) in the tissues of some organisms

resulting in metabolic acidosis (Cochran and Burnett, 1996; Burnett, 1997; Michaelidis *et al*., 2005a). When *C. virginica* closes its valves, it prevents the uptake of oxygen or release of carbon dioxide from its tissues into the surrounding water. Thus, as the shell remains closed, acidic metabolic byproducts accumulate within and around the tissues inducing an acidic environment (de Zwaan and Wijsmas, 1976; Byrne *et al*., 1991). Reducing the metabolic rate can decrease the accumulation of metabolic byproducts thus limiting acidosis, and some bivalves including oysters, are able to mobilize carbonate from their calcium carbonate shells increasing the buffering capacity of the hemolymph and other tissues (Dugal, 1939; Crenshaw and Neff, 1969; Byrne *et al*., 1989; Dwyer and Burnett, 1996; Michaelidis *et al*., 2005b).

Although *C. virginica* has evolved mechanisms to survive in oxygen-depleted environments, the closely related Asian oyster, *Crassostrea ariakensis,* has not demonstrated the same survival rates. In a comparative study, Harlan (2007) found that adult diploid and triploid *C. ariakensis* died significantly earlier when exposed to hypoxia than *C. virginica*, and reported, but did not experimentally test, that when immersed in hypoxic water *C. ariakensis* began gaping earlier and to a greater extent than *C. virginica*. Similarly, during anoxic exposure *C. ariakensis* spat (Matsche and Barker, 2006) and larvae (North *et al.*, 2006) reportedly died earlier than those of *C. virginica*.

While the hypoxia tolerance of *C. virginica* has been well documented, the adaptations that give rise to this tolerance are not completely understood. The differences in mortality (Matsche and Barker, 2006; North *et al.*, 2006; Harlan, 2007) and the potential differences in gaping (Harlan, 2007) between *C. ariakensis* and *C. virginica*

may indicate different physiological responses to emersion and low oxygen that may manifest in the hemolymph of the oysters. As a means to better understand gaping responses, acid-base balance, and interactions between the two in *C. ariakensis* and *C. virginica*, the gaping response (width, frequency, and correlation between acidification and gaping) during hypoxia and the hemolymph pH and the change in hemolymph pH from normal conditions of *C. ariakensis* and *C. virginica* during clamped emersion were assessed. Additionally, Dwyer and Burnett (1996) suggested that *Perkinsus marinus* (dermo) infection impaired the ability of *C. virginica* to maintain acid-base homeostasis. While previous studies (Calvo *et al*., 2001; NRC, 2004) found *C. ariakensis* demonstrated reduced physiological effects of dermo compared to *C. virginica*, there are no data on the influence of *P. marinus* on *C. ariakensis* homeostasis. Therefore, the impact of *Perkinsus* infection on the acid-base balance of *C. ariakensis* was also assessed. Findings from this research may shed light on the biochemical effects of hypoxia and thereby aid in understanding the physiological responses to hypoxia between these two closely-related species.

3.2 Materials and Methods

3.2.1 Oyster collection

Diploid *C. ariakensis* and *C. virginica*, approximately 100 mm in shell height, were obtained from University of Maryland's Center for Environmental Science at Horn Point Lab Oyster Hatchery in Cambridge, MD. Oysters were transported to the

University of Maryland College Park campus and acclimated without food for a week in aerated seawater of salinity 15 at 25°C.

3.2.2 Gaping response to hypoxic immersion

After the acclimation period, oysters were cleaned in a 9:1 water-chlorine bleach (HOCl) solution, rinsed in freshwater, and scrubbed to remove epifaunal and burrowing organisms. Individual oysters were then placed into ½" plexiglass respiration chambers (approximately 1790 ml) filled with artificial seawater of salinity 15 at 25°C. The seawater within the chamber was monitored with a YSI dissolved oxygen probe and sparged with nitrogen gas until the oxygen concentration was below $0.5 mgL^{-1}$, and the chamber was then immediately sealed. Gaping frequency and gape width was assessed by visual inspection at the ventral shell margin at 5, 10, and 30 minutes, 1 hour, 2 hours, and then every other hour for the first 12 hours, and every 24 hours after hypoxic immersion, until the oysters did not respond to the external stimulus of knocking a blunt metal object (large bolt) on the exterior of the chamber, and were then classified as dead. Additionally, at each sample period between 8-72 hours after hypoxic immersion, the pH of the seawater surrounding each live oyster was analyzed using a Cole Parmer combination micro pH electrode and Orion Two Star pH meter. Controls included chambers with sparged seawater without oysters, with sparged seawater with either *C. ariakensis* or *C. virginica* valves (to account for any bacteria or cryptogenic species not removed during bleaching and scrubbing), and with live oysters immersed in normoxic chambers.

3.2.3 Hemolymph extraction and analysis

The oysters were clamped closed using two-inch binder clips while underwater and placed on the lab bench for 0, 2, 4, 6, 8, 10, 12, or 24 hours, presumably inducing a hypoxic environment by preventing normal ventilation and gaping (Moon and Pritchard, 1970; Truchot, 1990). At each sampling time, the dorsal and ventral edges of the oyster were quickly notched using a grinding wheel (approximately 5-15 seconds) and pallial fluid was drained. A 5ml glass syringe with 19 gauge needle was immediately inserted into the adductor muscle sinus to collect hemolymph. As the extracted hemolymph was decanted into a 1.5 ml eppendorf tube, hemolymph pH was measured using a Cole Parmer combination micro pH electrode/Orion Two Star pH meter. Each oyster was sampled only once to limit stress which has been shown to affect hemolymph pH (Jones *et al*., 1993).

3.2.4 Role of *Perkinsus marinus* infection on acid-base balance

To test if *C. ariakensis* exhibited impaired acid-base balance when infected with *P. marinus*, the hemolymph pH of oysters with low-moderate and moderate dermo (infection intensity of 3, on a scale from 0-5) was compared to that of oysters with no to light infection rates (infection intensity of 1 or less). *Crassostrea virginica* was included in the analysis to compare to the finding of Dwyer and Burnett (1996).

3.2.5 *Perkinsus marinus* diagnosis for hemolymph studies

Since the ability of *C. virginica* to maintain acid-base balance has been shown to be influenced by infections of *P. marinus* (Dwyer and Burnett, 1996), all samples were

diagnosed for *P. marinus* infection. Gill, mantle, and rectal tissue samples were excised and incubated in *Ray's Fluid Thioglycolate Media* and analyzed 5-7 days later in accordance to the procedures of Ray (1952; 1966) as modified by Burreson (2009). Disease intensity was assessed on a scale of 0-5 based on the microscopic abundance of *P. marinus* cells (Burreson, 2009), and any oyster with a *P. marinus* score greater than one was excluded from pH analysis. *Perkinsus marinus* diagnosis is a lethal technique and hence was performed after pH analysis except when testing for any effect of disease. As *C. virginica* had higher *P. marinus* scores than that of *C. ariakensis*, more samples of *C. virginica* were excluded from analysis than *C. ariakensis*, resulting in an unequal number of replicates.

3.2.6 Statistical analysis

For the gaping study, Repeated Measures Analysis of Variance was performed to determine if *C. ariakensis* and *C. virginica* gape width differed in the first 72 hours of hypoxic exposure and if the pH of the ambient water surrounding gaping oysters was lower than that of oysters that were not gaping. A one-way Analysis of Variance (ANOVA) was used to test if gape frequency, defined as the percent of time an oyster was gaping across all time levels, differed between *C. ariakensis* and *C. virginica*.

For the hemolymph study, a two-way fixed-factor ANOVA was performed to determine if species or time emersed affected hemolymph pH, or if there was an interaction between time and species. A separate ANOVA was performed to test if these variables affected the ΔpH of the hemolymph. The ΔpH response variable was obtained by subtracting the average pH after zero hours of clamped treatment for each species,

from the pH of each subsequent sample. A ΔpH of zero indicated there was no change in the hemolymph pH from the average time zero unclamped value. Whereas, an acidic shift over time is indicated by a negative ΔpH, and an alkaline shift over time is represented by a positive ΔpH. With both hemolymph pH and ΔpH two-sided contrasts between each species at each time level were performed to determine at what times these species hemolymph deviated from one another. To test the influence of *P. marinus* infection, a three-way ANOVA was performed with species, time clamped and emersed, and dermo infection level (high or low) as treatments, and hemolymph pH as the response variable. All statistical analyses were performed using R (http://www.r-project.org/)

3.3 Results

3.3.1 Gaping during hypoxia

During hypoxic immersion, a significantly higher percent of *C. ariakensis*, 55.6±7.1%(SEM), gaped than *C. virginica*, 17.1±5.6% ($p<0.001$ $n_{C.ariakensis}=9$; $n_{C.virginica}=9$; see Figure 3.1A for percent frequency at each time level). When the oysters gaped, *C. ariakensis* on average gaped 4.1±0.4 mm which was significantly wider than the 2.0±0.7 mm gape of *C. virginica* ($p<0.006$, Figure 3.1B) during the first 72 hours. However, once the oysters did not respond to external stimuli and were assumed to be dead, *C. ariakensis* and *C. virginica* did not exhibit statistically different gape widths ($p=0.493$). Additionally, the seawater surrounding live gaping oysters between 8-72

hours, without regard to species, was significantly more acidic (pH 7.06±0.08) than the seawater surrounding oysters that were not gaping (pH 7.35±0.06; p=0.012; Figure 3.1C), but the pH of the seawater surrounding gaping *C. virginica* was not significantly different than that of gaping *C. ariakensis* (p=0.205).

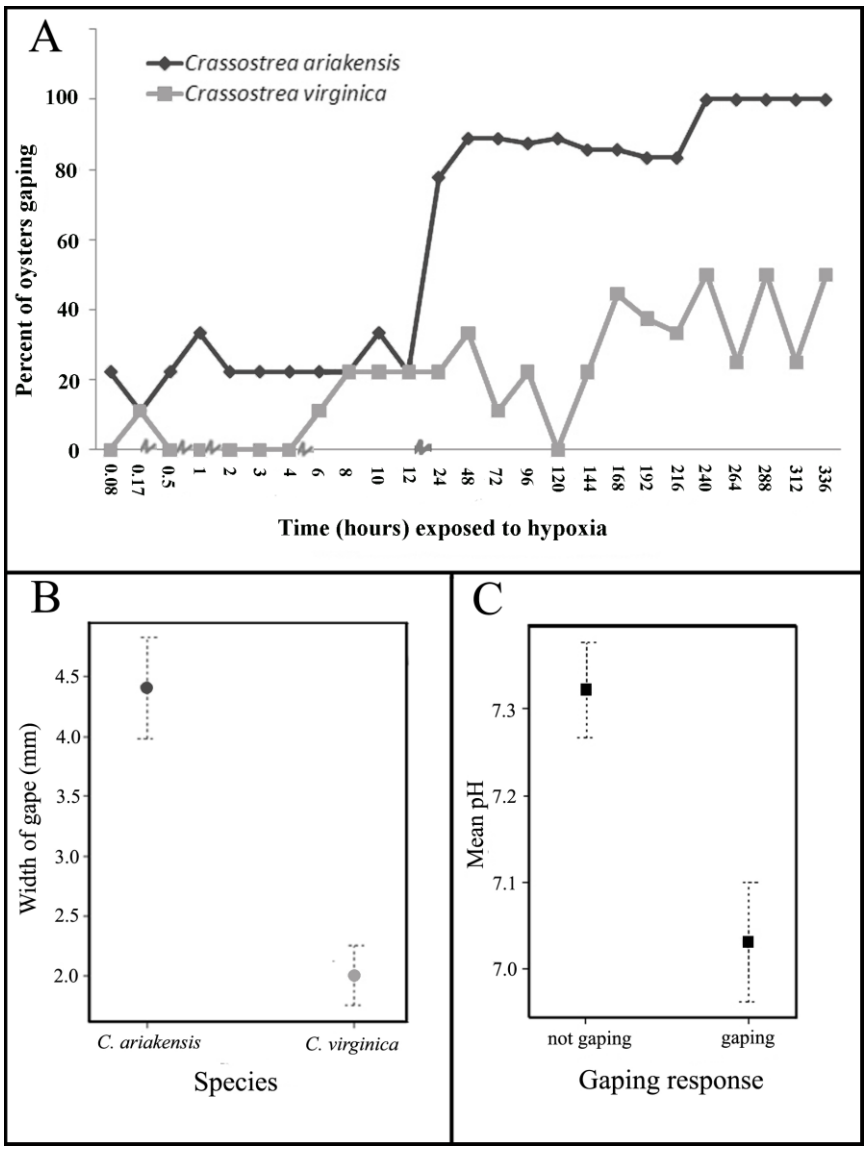

Figure 3.1 Gaping responses in *Crassostrea ariakensis* and *Crassostrea virginica* after hypoxic immersion. Graph A represents the percent of oysters (n=9) of each species gaping at each time level after hypoxic immersion within the first 14 days of hypoxic immersion. The X axis represents the duration, in hours, that oysters were exposed to hypoxic water. Graph B represents the average gape width (mm) for each species in the first 72 hours of hypoxic exposure, when they were exhibiting live gaping responses. Graph C depicts the mean pH of the water surrounding live gaping and non-gaping oysters of both species after 8-72 hours in hypoxic water. In graphs B and C error bars indicate standard error of the mean.

3.3.2 Hemolymph pH after emersion

There was no significant interaction between time emersed and species on hemolymph pH ($p=0.293$). The longer an oyster was emersed and clamped, the more acidic the hemolymph pH became for both species ($p<0.001$; Figure 3.2A). *Crassostrea ariakensis* hemolymph pH became significantly more acidic than that of *C. virginica* ($p<0.001$; Figure 3.2A). Despite no significant interaction between time emersed and species, two-sided contrast revealed that the difference in hemolymph pH between *C. ariakensis* and *C virginica* manifested itself at hours 8 ($p=0.002$), 10 ($p<0.001$), and 12 ($p=0.035$) after being clamped (Figure 3.2A). Therefore, for both species, time clamped and emersed led to an acidic shift in hemolymph pH, with *C. ariakensis* exhibiting a hemolymph pH more than 0.6 units lower than *C. virginica* at 10 hr ($\mu_{C,ariakensis}=6.15\pm0.11$; $\mu_{C,virginica}=6.78\pm0.16$).

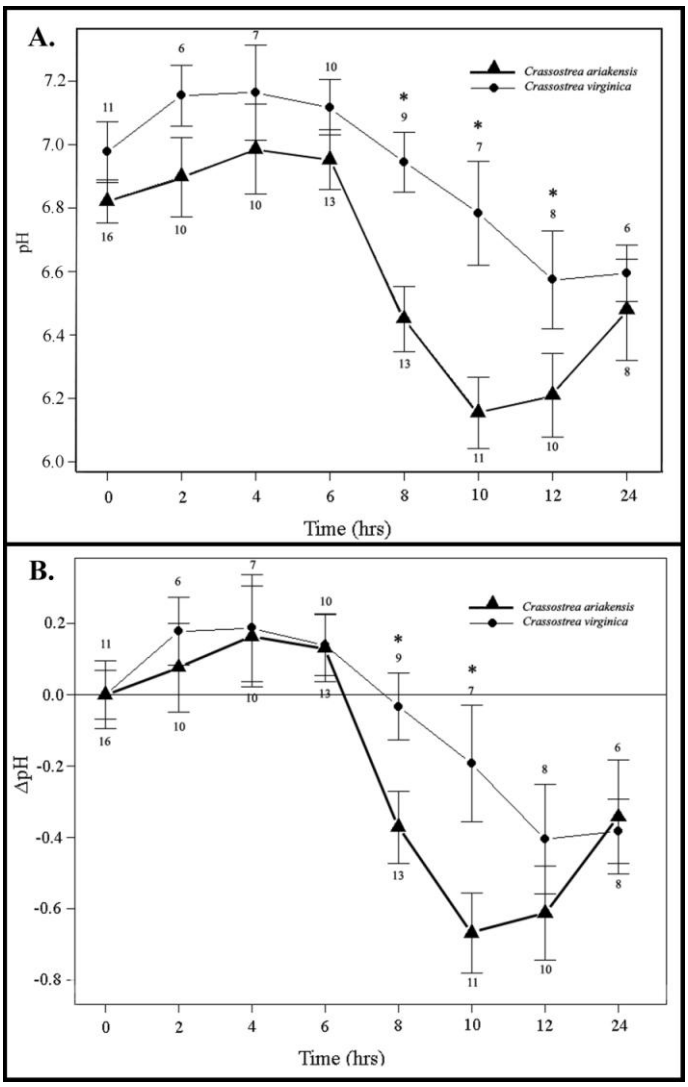

Figure 3.2 Hemolymph (A) and change in hemolymph (B) pH of *Crassostrea virginica* and *Crassostrea ariakensis* after clamped emersion. The X axis represents the time, in hours, that the valves of each oyster were clamped closed and placed on a lab bench preventing normal gaping. In panel B, the response variable, change in pH from normal conditions (ΔpH), was calculated by subtracting the average time zero value each species from each subsequent sample. The black line shows a 0 ΔpH indicating no change from the normoxic hemolymph pH. In both panels, error bars indicate standard error. Numbers above (*C. virginica*) and below (*C. ariakensis*) represent sample size at each time level. Asterisks represent statistical significance at an alpha of 0.05 using two-sided contrasts between species at the indicated time levels.

3.3.3 Change in hemolymph pH after emersion

There was no significant interaction between time emersed and species on the change of hemolymph pH (ΔpH) from normal unclamped conditions (p=0.293). Time clamped and emersed had a significant effect on pH change in *C. virginica* and *C. ariakensis* hemolymph (p=0.021). As time clamped and emersed increased, the |ΔpH| increased (p=0.021) as a result of an acidic shift in the hemolymph in both species. Additionally, there was a significantly different ΔpH between species (p=0.021; Figure 3.2B). The hemolymph pH of *C. ariakensis* declined significantly more from time zero than that of *C. virginica* (p=0.021). Similar to the observations above, two-sided contrast revealed that hemolymph ΔpH differences manifested themselves at hour 8 (p=0.031) and hour 10 (p=0.007), where *C. ariakensis* had a significantly greater hemolymph ΔpH than *C. virginica* (Figure 3.2B).

3.3.4 Role of *Perkinsus marinus* infection on acid-base balance

Moderate dermo (score 3) was the highest intensity observed in any of samples. There was no significant interaction between clamped emersion time level, species, and dermo infection level on hemolymph pH (p=0.140); additionally, there were no significant two-way interactions (p>0.05). Further, disease had no influence on hemolymph pH in either species (p=0.579); oysters with low infection had an average hemolymph pH of 6.75 ± 0.04, and oysters with higher infection levels exhibited a pH of 6.81 ± 0.05.

3.4 Discussion

This study found differences in the physiological responses of taxonomically similar oysters with varying degrees of hypoxia and anoxia tolerance. When gaping responses were not inhibited, *C. ariakensis* gaped more often and wider than *C. virginica* during hypoxic immersion, and in both species gaping was associated with the acidification of seawater surrounding the oyster in the chamber. When gaping was prevented by clamping the oysters, *C. ariakensis* hemolymph pH declined to nearly 6.1 within 10 hours, which was significantly more acidic than that of *C. virginica* during any period of the study (minimum at hour 12; 6.57±0.15). The findings indicate these two species responded differently to hypoxia and demonstrated potentially different metabolic responses. These results may help account for the lower anoxia tolerance in *C. ariakensis* compared to *C. virginica* (Matsche and Barker, 2006; North *et al*., 2006; Harlan, 2007), by demonstrating different macroscopic (gaping) and metabolic (hemolymph pH) responses to low oxygen environments. Changes in these factors may influence an organism's ability to maintain acid-balance, organ function, and survive natural hypoxic and anoxic episodes. However, a hemolymph pH of 6.1 is probably not lethal in and of itself. Additionally, the average hemolymph of dermo infected oysters was approximately 0.06 pH units lower than that of uninfected oysters ($p=0.579$). At the level of disease observed in the samples, which is comparable to level observed at most reefs in the Maryland portion of the Chesapeake Bay (Paynter *et al*., 2010; Lombardi personal observations), *P. marinus* infection intensity did not influence the ability to maintain hemolymph acid-base balance in either species. However, the findings would be more

conclusive with a larger sample size and the ability to compare oysters with moderate (or higher) dermo infection to no infection.

One caveat to these findings is that the control pH values were slightly lower than that of previous *Crassostrea* hemolymph studies in the reported literature (Dwyer and Burnett, 1996; Michaelidis *et al*., 2005a; Lannig *et al*., 2010). This may be due to the temperature chosen (25°C) or the inclusion of oysters with low *P. marinus* infections.

Bivalves typically exhibit one of two responses when emersed. First, the organism may close its valves, reduce oxygen consumption and shift to anaerobic metabolism as tissue and pallial fluid oxygen declines; or second, permit gas exchange between the tissues and the external environment through periodic gaping (Widdows *et al*., 1979; Byrne *et al*., 1990). Based on the results, it appears that the latter mechanisms may be employed by *C. ariakensis*, where as the former may be employed more by *C. virginica*; since in this study, when gaping was permitted, *C. ariakensis* gaped more often and wider than *C. virginica* during hypoxic immersion. This difference in gaping width is not likely the result of different ligament physiology and elasticity, as the gaping width of both species was larger and not significantly different between species for dead and dying oysters. This suggests that both species are mechanically capable of gaping to the same extent. Additionally, gaping was correlated with the acidification of the ambient seawater in the chamber. Thus, gaping may reduce metabolic acidosis by allowing acidic products to escape from the tissues into the external environment.

During the hemolymph pH study, *C. ariakensis* and *C. virginica* were clamped closed which inhibited normal gaping responses. Since gaping may facilitate an release

of acidic end-products to the external environment, clamping shells inhibited this exchange and likely resulted in a hypoxic pallial environment (Moon and Pritchard, 1970; Truchot, 1990). The lower hemolymph pH exhibited by clamped *C. ariakensis* in comparison with *C. virginica* is likely the result of one of three potential mechanisms: 1) different metabolic byproduct accumulation rates 2) different levels of carbonate buffering 3) combination of byproduct accumulation and buffering differences.

First, metabolic byproducts, including carbon dioxide, could be accumulating in the hemolymph and other tissues to a greater extent in *C. ariakensis.* Studies by Harlan (2007) and presented in Chapter 2 found that *C. ariakensis* and *C. virginica* adults exhibited similar aerobic basal metabolic rates. However, differences may exist in the alternate metabolic pathways used during hypoxic stress. Burnett and Stickle (2001) found that the majority of *C. virginica* energy was obtained through anaerobic respiration during hypoxic stress; however the dependence on anaerobic metabolism in *C. ariakensis* is not well documented. Further, studies have found when exposed to hypoxic environments *C. virginica* reduced its aerobic metabolic rate to only 10% of its normoxic metabolic rate (Willson and Burnett, 2000) and that other bivalves reduced their ATP requirements during anaerobiosis to 5% of aerobic levels (de Zwaan, 1983). Therefore, while *C. virginica* has exhibited metabolic changes when exposed to low oxygen, it is possible that *C. ariakensis* does not engage in as substantial hypoxic-induced metabolic depression, or the hypoxic metabolism is less efficient, creating more acidic metabolic byproducts resulting in reduced hemolymph acid-base homeostasis. This is supported by Harlan (2007) who proposed, based on findings that of different anaerobic intermediate substrates and end-products, that *C. ariakensis* and *C. virginica* species may engage in

different anaerobic pathways. This potential mechanism of hemolymph acidification due to metabolic byproducts production is supported by the findings that when oysters are permitted to gape during hypoxic stress, that *C. ariakensis* gaped more often than *C. virginica* and that the ambient seawater of gaping oysters was more acidic than that of non-gaping oysters, suggesting the release of acids during gaping.

A second means to explain the different hemolymph pH, may be differences in the buffering capacity between the species, as the oyster shells have demonstrated different properties including density and load compression strengths (Newell *et al*., 2007; Lombardi *et al*., unpublished data). Some molluscs, including *C. virginica*, react to metabolite-induced acidosis by mobilizing calcium carbonate from their valves to buffer their tissues (Dugal, 1939; Akberali and Trueman, 1985; Byrne and McMahon, 1994; Dwyer and Burnett, 1996; Michaelidis *et al*., 2005a). While tidally emersed *C. virginica* typically gape and likely exchanges gases with the air, metabolites can accumulate whenever gaping is prevented or reduced such as during emersion, predator attack, or environmental stresses such as hypoxia or pollution, inducing acidosis within tissues (Widdows *et al*., 1979; Akberali and Black, 1980; Akberali and Trueman, 1985). Therefore, it is possible that the difference in pH between species can be explained not only by different rates of accumulating carbon dioxide and other acidic products, but also different rates of calcium carbonate mobilization into the hemolymph during clamped emersion. In addition to buffering through the mobilization of carbonate from the shell, it is possible that oysters can buffer their tissues using non-bicarbonate buffers including phosphates and imidazole compounds which have been documented in other taxa (Burton, 1978; Castellini and Somero, 1981; Eberlee and Storey, 1984) Thus, the

difference in hemolymph pH may reflect different buffering capabilities in *C. virginica* and *C. ariakensis* as well.

Finally, the difference in hemolymph pH between species may be a combination of differences in carbon dioxide and calcium concentrations in the blood. For instance, Crenshaw and Neff (1969) reported increased calcium, carbon dioxide, and hydrogen ion concentrations when valves were closed in the hard clam *Mercenaria mercenaria.* Further studies quantifying hemolymph biochemistry and calorimetric anaerobic respiration studies during hypoxic exposure are needed to understand the mechanisms for the observed differences in hemolymph pH when clamped and emersed.

Crassostrea ariakensis and *C. virginica* have different life histories and preferred habitats, which may have contributed to the evolution of different mechanisms to respond to and to survive hypoxia. *Crassostrea virginica* populations have not only evolved in regions characterized by natural episodes of hypoxia, but in some southern regions, most *C. virginica* reefs are intertidal and therefore the oysters routinely undergo regular tidal emersion. In contrast, *C. ariakensis* has been found in the sublittoral zone up to 10 meters deep and has been reported to be unable to tolerate aerial emersion (see review by Zhou and Allen, 2003; Kingsley-Smith and Luckenbach, 2008). These physiological differences observed during clamped emersion and hypoxia, coupled with differing hypoxic exposure due to habitat and distribution may help explain why *C. ariakensis* is not as well-adapted to hypoxia as *C. virginica*.

Comparative research into *C. ariakensis* and *C. virginica* physiology and biochemistry to identify convergences or divergences in responses may yield insight into

adaptations responsible for the remarkable hypoxia tolerance of *C. virginica.* Overall, the results suggest that *C. ariakensis* may depend on gaping for acid-base homeostasis more than *C. virginica* since gaping was associated with the acidification of the external environment, and *C. ariakensis* gaped both more often and wider, and its hemolymph pH was more affected by clamping than *C. virginica*. These findings indicate differences in the physiological response of these oysters to low oxygen, which in turn may help illuminate adaptations enabling *C. virginica* to survive weeks without oxygen.

Chapter 4

Strength of muscle contraction and speed of contraction of the Eastern oyster, *Crassostrea virginica*, and the Asian oyster, *Crassostrea ariakensis*, when exposed to high carbon dioxide and low oxygen environments

4.1 Introduction

Oysters have two calcium carbonate valves which provide space for internal organs and act as a shield providing protection from environmental factors including predators, salinity changes, desiccation, changes in oxygen tension, or contaminated water (Carriker 1996). The two valves are attached to one another via the adductor muscle located in the posterior region of the body and a hinge ligament at the dorsal shell margin (See Figure 1.1 for anatomy of *Crassostrea virginica*). When the adductor muscle relaxes the ligament forces the valves apart which creates a gap at the ventral shell margin of the oyster; this process is known as gaping (Carriker 1996).

Gaping allows an oyster to spawn, feed, and exchange respiratory gases. However, gaping has also been shown to substantially increase an oyster's vulnerability to predation since the soft flesh is not fully enclosed within the protective valves (Tomkins, 1947; Loosanoff, 1956; Menzel and Nichy, 1958). In order for the oyster to be closed, the adductor muscle must be contracted. Maintaining closed valves typically requires energy input in the form of ATP to sustain adductor muscle contraction

(Equation 1.4). However, oysters have evolved catch muscles within the adductor muscle which allows for the muscle to remain contracted for long periods of time with little to no additional energy input (Millman, 1964**;** Morrison, 1996; Schmidt-Neilson, 1997).

The adductor muscle's ability to keep the valves shut is an oyster's main predatory defense. However, many animals have evolved strategies to prey upon oysters despite their hard shell. For instance, sea stars employ two methods to prey upon oysters. Sea stars use their tube feet to attach to the valves and apply a force that overpowers the adductor muscle; additionally, sea stars can secrete an anesthetic chemical which causes the adductor muscle to relax, thereby inducing the oyster to gape (Galtsoff, 1964; White and Wilson, 1996). In both cases, once the oyster gapes the sea star can easily feed upon the oyster tissue. Some gastropod species also feed on oysters; species such as *Urosalpinx cinerea*, *Eupleura caudata*, *Thais haemastoma,* and *Murex pomum* consume oysters by drilling through the shell, whereas others, such as *Melongena corona,* insert their proboscis into a gaped oyster (Menzel and Nichy, 1958; White and Wilson, 1996). Since oysters are also preyed upon by polychaetes, flatworms, crabs, birds, and fish having a functioning adductor muscle and maintaining the ability to close are vital for the survival of an oyster (White and Wilson, 1996). Gaping is so intrinsically linked to mortality that many studies have considered unresponsive gaping oysters as dead (EPA, 1996; Harlan, 2007; Thessen *et al*., 2010). Despite different native ranges, the Eastern oyster, *Crassostrea virginica*, and the Asian oyster, *Crassostrea ariakensis*, likely share similar predation pressures as there are crustacean, echinoderm, and gastropod predators

in both habitats (Carriker, 1996, Zhou and Allen, 2003), however there is ambiguity about the predation pressures exerted by native fish on *C. ariakensis*.

Crassostrea virginica and *C. ariakensis* engaged in different gaping responses when exposed to low oxygen environments (Chapter 3). *Crassostrea ariakensis* gaped on average 2.1mm wider than *C. virginica* during the first 72 hours of hypoxic exposure (Chapter 3), and over the course of the study, gaped on average more than three-times as often as *C. virginica* (Chapter 3). While gaping results from the relaxation of the adductor muscle, the specific mechanisms behind the observed difference in hypoxic gaping response are not clear. The differences in gaping could be due to differing behavioral responses, or it may be a result of adductor muscle fatigue or failure leading to involuntary gaping.

Muscles can be affected by a variety of factors including salinity (Wickes and Morgan, 1976), vitamin deficiency (Janssen *et al*., 2002), disease (Lopes *et al*., 1982), temperature (Rome, 1990; Bennett 1985), and pH (Orchard and Kentish, 1990; Ueno *et al*., 2002). The effect of decreasing pH on a muscle varies depending on the level of the acidification. If an environment becomes very acidic, this can lead to the denaturing of the proteins within the muscle or if the shift is less severe, lowered pH can be linked to muscle fatigue (Metzger and Fitts, 1987; Hui, 2006). Muscle tissue may be exposed to pH levels lower than their natural range due to metabolic byproducts or acidic contaminants (Mitch *et al.,* 1994; Rayne and Forest, 2009). The results from Chapter 3 demonstrated that *C. ariakensis* hemolymph was significantly more acidic, presumably due to increase acidic products, than that of *C. virginica* across the 24 hour study and that

C. ariakensis hemolymph was approximately 0.6 pH units more acidic than that of *C. virginica* after 8 hours of clamped emersion (Chapter 3). Since pH can alter the function of muscles (Bing *et al*., 1973; Metzger and Fitts, 1987; Hui, 2006), the reduced pH observed in Chapter 3 could compromise adductor muscle function leading to gaping. Further, hypercapnic (Hammer *et al*., 2011), and hypoxic (Bing *et al*., 1973; see review by Taggart and Wray, 1998) environments been shown to affect muscle physiology and reduce function. Hypoxic environments are typically accompanied by elevated carbon dioxide concentrations and associated pH reduction (Burnett, 1997). Thus, hypercapnic, acidic, and hypoxic environments are linked, and any of these environments may influence adductor muscle function.

A series of studies were designed which assessed the strength of valve closure following gaping in *C. ariakensis* and *C. virginica* after acute and chronic exposure to hypercapnic/acidic, low oxygen, both hypercapnic and low oxygen, and standard oxygen and standard pH/carbon dioxide conditions (control) environments. As hypercapnic and acidic environments are intrinsically linked (refer to Equation 1.1 for the relationship between carbon dioxide and pH) rather than describing environments as hypercapnic/acidic, I will simply refer to the environment as hypercapnic in all future references, leaving acidic to be implied. These studies also assessed the contraction speed following gaping in these different environments. The aim of this research was to determine which environmental condition, if any, affected muscle physiology, and to identify a mechanism for the observed difference in gaping response between *C. ariakensis* and *C. virginica* (Chapter 3). Additionally, these findings may help explain

the survival of each species in hypoxic environments since in the wild a gaping oyster is at enhanced risk of predation (Tomkins, 1947; Loosanoff, 1956; Menzel and Nichy, 1958). Therefore, understanding the underlying mechanisms behind hypoxic-induced gaping, including the possible role of adductor muscle function, may provide valuable physiological and ecological information.

4.2 Materials and Methods

4.2.1 Collections and Acclimation

Crassostrea ariakensis was obtained from Horn Point Laboratory's Oyster Hatchery Program; *Crassostrea virginica* was obtained from restored oyster reefs in the Chesapeake Bay and conditioned at the hatchery for approximately one year. Oysters were transported to the University of Maryland where they were acclimated and fasted for one week in aerated artificial seawater at a salinity of 15 and approximately 25°C.

4.2.2 Contraction strength following acute exposure

Waterproof adhesive was used to attach a string loop to the convex shell (left valve; Figure 1.1) superficial to the adductor muscle. Respiration chambers were filled with artificial seawater at a salinity of 15 and approximately 25°C and sparged with either nitrogen, 5% carbon dioxide balance air, 5% carbon dioxide balance nitrogen, or ambient air (control) gas treatment. After 10 minutes of sparging, the gas flow rate was decreased, but sparging continued throughout the 12 hour study. Oysters were then

placed into respiration chambers which were sealed with parafilm with a small incision to accommodate surgical string which connected the oyster to a force transducer. Force transducers were calibrated at the start of each trial and connected to data loggers which recorded measurements on computers with iworx and Labscribe2 software (http://www.iworx.com/content/?id=14). This allowed continuous data collection as well as basic data analysis.

The strength (g) associated with valve closure was assessed at 5 minutes, 15 minutes, 30 minutes, and 1, 2, 4, 6, 8, 10 and 12 hours after immersion if the oyster was gaping. Valve closure was induced by using a blunt metal object (shucking knife) to tap the side of the chambers of gaping oysters. The strength of the closure was assessed using iworx and Labscribe2 software with the "analysis" tool. Repeated Measures Analysis of Variance was used to test if the valve closure strength differed across time levels, treatments, or species and if any significant interactions existed between treatments. Square root transformations were performed on the data to meet assumptions of normality and homogeneity of variance.

4.2.3 Speed of Contraction

Using the data obtained from the acute exposure study (methods section 4.2.2), time until peak (maximum) closure strength for each time level, species, and gas treatment was calculated. This was performed to test if the rate of contraction slowed over time, if the species exhibited different contraction rates, or if the gas treatments altered contraction speed. Additionally, this was performed to better understand if oysters could become habitualized to external stimuli. Time ($\log_{10}$ transformed seconds)

to peak closure strength was used as a metric for valve closure speed, because the force transducer and Labsribe2 output did not identify the time in which the valve fully closed (See example output in Figure 4.1). Repeated Measures Analysis of Variance was used to test if the closure time differed between time levels, species, or gas treatment and to test for any interaction between these treatments. To better understand if oysters became acclimated to external stimuli, regression analysis was performed to assess if there was a significant linear relationship between time exposed to treatments (correlated with number of times the oyster was disturbed) and closure time. Log_{10} transformations were performed on the data to meet the assumptions of normality and homogeneity of variance.

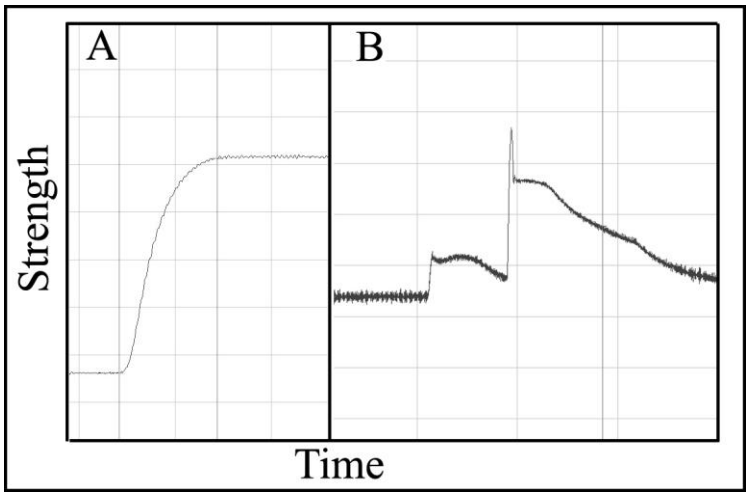

Figure 4.1 Iworx and Labscribe output. This figure illustrates the graphical output of software program Labscribe2 when the contraction strength of *Crassostrea ariakensis* and *Crassostrea virginica* were exposed to different gas treatments and gape closure was induced. Specially, Figure A illustrates a smooth valve closure, where the oyster closed in one continuous motion, whereas panel B shows stepwise contractions to induce valve closures. Panel B illustrates the issues calculating valve closure force using with the dataset.

4.2.4 Contraction strength following chronic exposure

Additionally, the effect of chronic exposure to hypoxia on muscle contraction strength in these two species was tested by exposing oysters to either normoxic or hypoxic treatments for five days and then assessing the strength of contraction. This was achieved by housing oysters in individual chambers with water sparged with nitrogen gas for five days. Then, the methods outlined in the methods section 4.2.2 were followed to attach oysters to the force transducers. Once oysters gaped naturally, the side of the chamber was tapped with a shucking knife or the internal tissues were prodded with a syringe tip to initiate valve closure and the strength of contraction was captured using Labscribe2 software and iworx. If oysters did not gape within five hours of being monitored by the force transducer, or were unresponsive gapers (could not induce valve closure), then they were discarded from analysis. A two-way Analysis of Variance was used to test if the adductor closing strength of normoxic immersed *C. ariakensis* and *C. virginica* differed from that of oysters exposed to hypoxia and if there was a difference between species.

4.3 Results

4.3.1 Strength following acute exposure

Repeated Measures Analysis of Variance revealed no significant interaction was present between treatments (three-way or two-way interaction; $p>0.4$). The valve closure strength (square root (sqrt) transformed to meet assumptions) of *C. ariakensis* (2.17±0.11

sqrt grams) was not significantly different from that of *C. virginica* (2.17±0.15 sqrt grams; p=0.993). Transformed closure strength was not significantly affected by time exposed (p=0.615), nor gas treatment (carbon dioxide: 2.18±0.11 sqrt grams; nitrogen 2.30±0.22 sqrt grams; carbon dioxide and nitrogen: 2.19±0.34 sqrt grams; aerated: 2.08±0.15 sqrt grams; p=0.569). In summary, there was no difference (p>0.05) in the transformed strength of valve closure between species (*C. ariakensis* or *C. virginica*), gas treatment (nitrogen, carbon dioxide, combined nitrogen and carbon dioxide, or air), or time (up to 12 hours).

4.3.3 Speed of Contraction

Repeated Measures Analysis of Variance with species, gas, and time exposed to gas environments as treatments revealed there was no significant interaction between time exposed, species, and gas treatment (p=0.142), gas and species (p=0.132), time and species (p=0.162), time and gas (p=0.437) on log transformed time to peak contraction strength. Further, *C. ariakensis* reached maximum valve closure strength at approximately 0.19±0.04 $\log_{10}$ seconds which was not significantly different from the closure speed of *C. virginica* (0.23±0.06 $\log_{10}$ seconds; p=0.067). Time ($\log_{10}$ transformed seconds) to peak closure strength did not vary significantly between oysters immersed in aerated (0.21±0.06 $\log_{10}$ seconds), nitrogen (0.17±0.08 $\log_{10}$ seconds) and carbon dioxide (0.19±0.59 $\log_{10}$ seconds) sparged seawater. Time (log transformed seconds) to peak contraction strength was significantly greater for oysters immersed in the seawater sparged with combined nitrogen and carbon dioxide (0.42±0.11 $\log_{10}$ seconds) than all other treatments (p=0.012; Figure 4.2) Further, there was a significant

effect of treatment exposure time (p=0.039; Figure 4.3), where at hour 8, oysters of both species took the longest longer to reach peak closure strength. However, there was no significant linear relationship between valve closure (sqrt seconds) and time (hrs) exposed when a linear regression analysis was performed (R^2=0.212; y=0.016x +0.116); p=0.181; Figure 4.3).

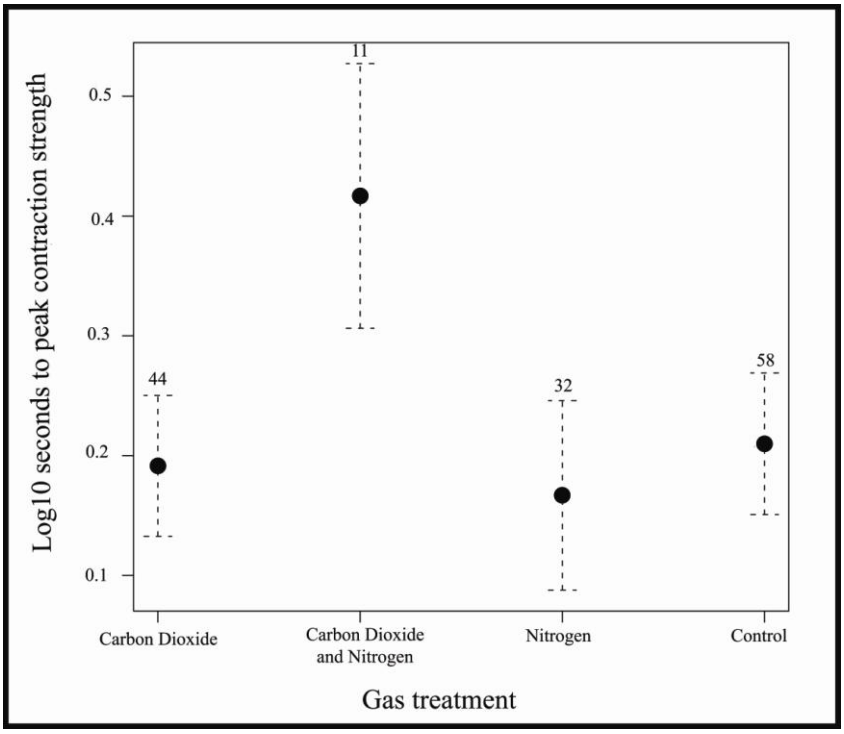

Figure 4.2 Time (log_{10} seconds) until peak valve closure strength after immersion in seawater sparged with different gases. *Crassostrea virginica* and *Crassostrea ariakensis* were immersed into seawater sparged with either 5% carbon dioxide balance air, 5% carbon dioxide balance nitrogen, nitrogen, or a control (sparged with air) for 12 hours. At specific intervals during the 12 hours, the time until oysters reached peak contraction strength following gaping (a measure of the speed of valve closure) was assessed. Error bars represent standard error of the mean and samples size is given above each error bar. To meet assumptions of normality seconds to peak contraction strength was log_{10} transformed.

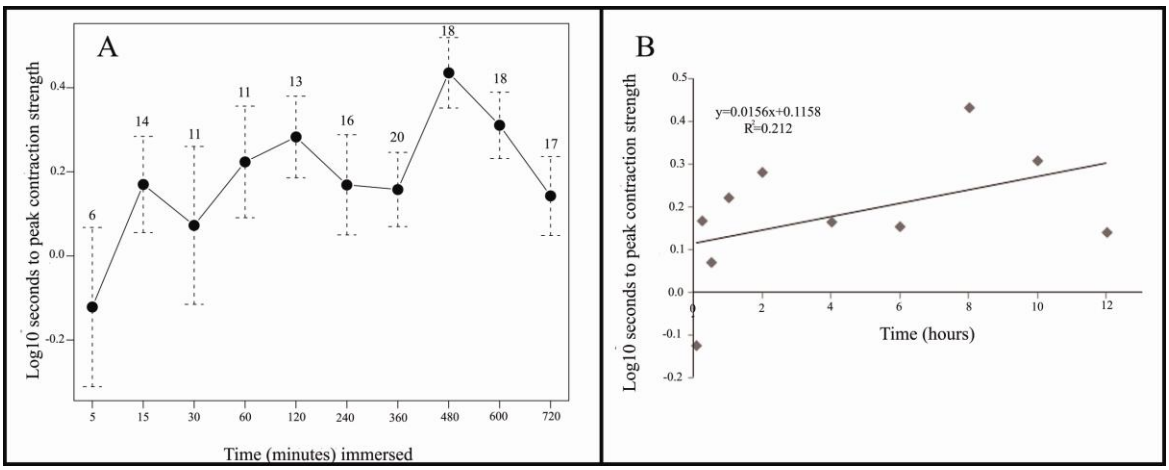

Figure 4.3 Time (log_{10} seconds) until peak valve closure strength as a function of time immersed. *Crassostrea virginica* and *Crassostrea ariakensis* were immersed into seawater sparged with either carbon dioxide, carbon dioxide and nitrogen, nitrogen, or a control environment for 12 hours. At specific intervals during the 12 hours, the time until oysters reached peak contraction strength following gaping (a measure of the speed of valve closure) was assessed. Panel A shows means and standard error of the mean at each time level with sample size given above the error bar. Panel B shows regression analysis with time in hours as the dependent variable and time (log_{10}seconds) to peak contraction strength as the independent variable. Each point represents the average time to peak contraction for all oysters during a time level. Analysis of variance revealed that time had a significant effect (Panel A; p=0.039) indicating that at least one time level was significantly different than another, however there was no pattern for the effect of time (Panel B; p=0.181). To meet assumptions of normality seconds to peak contraction strength was log_{10} transformed for all analyses.

4.3.4 Contraction strength following chronic exposure

Contraction strength following chronic exposure could not be assessed as few (eight) oysters both gaped and responded to external stimuli by closing their valves. These eight oysters represent control oysters (aerated water) as well as experimental oysters (nitrogen exposure) in *C. ariakensis* and *C. virginica* and there was not enough

replication to perform statistical analysis. Descriptive statistics did not yield reliable information for comparisons as only one nitrogen immersed *C. ariakensis* gaped and engaged in valve closure.

4.4 Discussion

The contraction strength of the adductor muscle of *C. ariakensis* and *C. virginica* was not significantly affected by up to 12 hours of exposure to low oxygen (nitrogen sparged), hypercapnic (carbon dioxide sparged), low oxygen and hypercapnic (nitrogen and carbon dioxide sparged), or normal conditions (sparged with air). This suggests that the hypoxic induced gaping observed in Chapter 3 was not the result of adductor muscle failure due to low oxygen concentration or associated acidosis. Further, it was not likely an indirect physiological response, such as reduced nerve impulse transmission, affecting adductor muscle contraction as the study used intact oysters, since any indirect physiological effect would have still induced changes. While there was no difference in the strength of contraction in these environments, oysters in combined hypoxic and hypercapnic environments contracted more slowly ($\log_{10}$ transformation seconds until peak contraction strength) than oysters in any other treatment. The speed of contraction data suggest that combined low oxygen and hypercapnic environments are more stressful than each of these conditions individually. This is important since studies have shown that hypoxia and acidosis can have additive effects (Bing *et al*., 1973), but the combined effects of hypoxia and hypercapnia are largely understudied (Burnett, 1997). Additionally, these finding may suggest that the combined hypoxic and hypercapnic

environment may lead to a reduction in force, as the strength of contraction remains constant but the speed decreases. However, different times to peak strength do not automatically indicate differences in force, as in some cases oysters closed in a stepwise fashion with bursts of closure with intermittent pauses (Figure 4.1b) rather than a constant rate of closure (Figure 4.1A). Therefore, these findings cannot conclude if the force was lower in hypoxic and hypercapnic environments. However, since the oysters were capable of value closure and strength was not compromised in any environment, early gaping in hypoxic environments is likely not due to muscle failure or fatigue but is rather likely a behavioral response to hypercapnic and hypoxic environments. Experiments in Chapter 3 found that the external environment became more acidic when oysters were gaping, thus gaping may be a behavioral response to release acidic products from the tissues into the external environment as a means to mitigate and prevent acidosis during early hypoxic exposure.

It is possible that reduced speed of valve closure is the first manifestation of environmental stress in the adductor muscle, and that if chronically exposed to nitrogen and carbon dioxide sparged environments that the strength of contraction may vary. However, the effects of chronic exposure were unable to be assessed due to the limited number of oysters which were both gaping and responsive when chronically exposed to hypoxic. Therefore, using excised muscles (see Pérez *et al*., 2009 for force comparisons between excised and intact bivalve adductor muscles) may be a better method for assessing the impact of long-term hypoxic and/or hypercapnic environments on adductor muscle strength. Additionally, future studies should be conducted isolating the

physiological effects of carbon dioxide from pH, possibly through adding buffers to carbon dioxide sparged water and lowering pH through means other than carbon dioxide.

This research also provides insight into the potential effects of ocean acidification on adductor muscle function and ultimately oyster survival. Currently, atmospheric carbon dioxide concentration is at the highest levels observed over the past 740,000 years (approximately 390 ppm) and expected to more than double this century (Hoegh-Guldberg *et al*., 2007; IPCC, 2010; Reaka and Lombardi, 2011). Increased atmospheric carbon dioxide diffuses into the ocean waters and can lead to increased ocean acidity. Haugan and Drange (1996) estimate that during the 21st century the oceans will become 0.4 pH units more acidic. This could translate to stark differences in environmental conditions as when an oyster gapes these external environments may affect the internal homeostasis of an oyster. This research found that neither hypercapnia nor acidic environments lead to reduced muscle contraction strength, which suggests that ocean acidification may not inhibit adductor muscle contraction. However, chronic exposure studies would be more indicative of adductor performance following acidification.

This dataset may also provide insight into potential habitualization of oysters to external stimuli by comparing the contraction time of oysters across time. Previous observations suggest that oysters may be able to become habitualized or desensitized to external stimuli, as oysters may close their valves less often in response to light changes, sounds, and vibrations after repeatedly exposed to these stimuli (Paynter unpublished observations; Lombardi unpublished observations) At minute 5 oysters had only been disturbed through tapping the external chamber with a shucking knife once, while oysters

at hour 12 had experienced chamber tapping a up to ten times. Comparing the contraction speeds across time levels could provide insight into the ability of oysters to be acclimated to stimuli. While there was a general positive trend (increasing 0.02 $\log_{10}$ transformed seconds, approximately 1 second an hour), this trend was not significant and there was then no significant effect of exposure time on contraction speed (transformed; p=0.181; Figure 4.3b). The nonsignificant relationship persisted even when the one treatment environment (hypercapnic and hypoxic) that affected contraction speed was removed ($y=0.021x+0.061$; $R^2=0.277$; p=0.118). Thus, this study does not demonstrate acclimation or habitualization to stimuli over time, though this hypothesis could be better tested through longer exposure times, on the order of days rather than hours, as well as through more frequent stimuli exposure.

Overall, early gaping in hypoxic environments in both *C. ariakensis* and *C. virginica* was not likely the result of hypoxia-induced adductor muscle failure. The strength of the adductor muscle was not compromised by reduced oxygen concentration and or increased carbon dioxide concentration of the external water. However, the speed of contraction was affected by the combined hypoxic and hypercapnic environment, suggesting that this environment may have evoked a change in typical muscle response and possibly in force. While chronic exposure studies were inconclusive, these results along with the gaping results presenting in Chapter 3, suggest that early gaping is likely a behavioral response to hypoxia that differs between *C. ariakensis* and *C. virginica*.

Chapter 5

Discussion and Significance

The Eastern oyster, *Crassostrea virginica*, is well known for its ability to withstand low oxygen conditions. However, the taxonomically and morphologically similar Asian oyster, *Crassostrea ariakensis*, has died significantly earlier than *C. virginica* during hypoxic exposure (Matsche and Barker, 2006; North *et al.*, 2006; Harlan, 2007). My dissertation aims at understanding the physiological basis for this difference in tolerance, while contributing to a greater body of knowledge of *C. ariakensis*, *C. virginica*, and comparative physiology.

Crassostrea virginica and *C. ariakensis* adults exhibited the same mass-specific respiration rate, but *C. ariakensis* spat exhibited a higher mass-specific respiration rate than that of *C. virginica* spat (Figure 2.1; Chapter 2). Further, by altering the approach used to standardize by mass, different respiration and metabolic rates were generated (Table 2.2), illustrating the importance of choosing a biologically relevant and statistically appropriate means to account for mass (Chapter 2).

When both species of oysters were exposed to water with oxygen concentration below 0.5mgL^{-1} for 24 hours, *C. ariakensis* gaped wider (4.1±0.4mm) in the first 72 hours and more frequently (55.6 ±7.1%) than *C. virginica* (width: 2.0±0.7 mm, $p<0.006$; frequency*:* 17.1±5.6%; $p<0.001$; Figure 3.1). Gaping was also associated with acidification of the surrounding water during hypoxic exposure; water that surrounded live gaping oysters was significantly more acidic (pH 7.06±0.08) than water surrounding

closed oysters (pH 7.35±0.06**;** p=0.012; Figure 3.1; Chapter 3). When gaping was inhibited by clamping, the time an oyster was clamped had a significant effect on the hemolymph pH for each species and the hemolymph exhibited a more acidic shift in *C. ariakensis* than *C. virginica* (pH values varied over time, see Figure 3.2; Chapter 3). Additionally, the hemolymph pH of oysters with low to moderate *Perkinsus marinus* (the causal agent for the oyster disease dermo) infections was 6.81 ± 0.05 pH units which was not significantly different than that the hemolymph pH of 6.75 ± 0.04 associated with oysters with lower infection levels (p=0.579). Thus, at the level of *P. marinus* infection observed in our samples, disease did not influence hemolymph acid-base homeostasis, contrary to Dwyer and Burnett (1996) who found differences in *C. virginica*.

Gaping responses were tested to determine if gaping was a behavioral or a physiological, involuntary response triggered by low oxygen, high carbon dioxide (and associated acidic shifts; see Equation 1.1 and Chapter 4 introduction) or a combination of the two environments. This was accomplished by assessing the strength of muscle contraction of both *C. virginica* and *C. ariakensis* during short-term exposure to hypercapnic, low oxygen, low oxygen and hypercapnic, or aerated seawater, as well as during long-term hypoxic exposure. Acute exposure (up to 12 hours) to these environments did not alter the strength of contraction (square root (sqrt) transformed) during 12 hours of exposure (carbon dioxide: 2.18±0.11 sqrt grams; nitrogen 2.30±0.22 sqrt grams; carbon dioxide and nitrogen: 2.19±0.34 sqrt grams; aerated: 2.08±0.15 sqrt grams; p=0.5689). Nor was the 2.17±0.11 sqrt grams strength exerted by *C. ariakensis* significantly difference from the 2.17±0.15 sqrt grams strength exhibited by *C. virginica* (p<0.993; Chapter 4). Similarly, *C. ariakensis* reached peak contraction strength in

0.19±0.04 $\log_{10}$ seconds which was not significantly different than the 0.23±0.06 $\log_{10}$ seconds of *C. virginica* (p=0.067). However, oysters in the combined low oxygen, acidic environment did take a longer time, 0.42±0.11 $\log_{10}$ seconds, to reach peak contraction strength than any other treatment (carbon dioxide: 0.19±0.06 $\log_{10}$ seconds; nitrogen 0.17±0.08 $\log_{10}$ seconds; aerated: 0.21±0.06 $\log_{10}$ seconds; p=0.012**;** Chapter 4). Further, time exposed had a significant effect (p=0.039; Figure 4.3); hour 8 had the longest time to peak contraction, however regression analysis demonstrated there was no significant trend with regards to time exposed to the environment (R^2=0.212; y=0.016x +0.116); p=0.181). No conclusions can be reached on the strength of contraction or contraction speed after chronic hypoxic exposure as not enough chronically exposed oysters both gaped and responded to stimuli to perform statistical analysis.

In summary, this research found *C. ariakensis* spat exhibited higher mass-specific aerobic respiration rate than *C. virginica*. *Crassostrea ariakensis* gaped wider and more frequently than *C. virginica* and gaping was associated with the acidification of the external environment. When gaping was prevented, clamped and emersed *C. ariakensis* hemolymph was more acidic than that of *C. virginica*, but disease intensity had no role on the hemolymph pH. The strength of the valve closure (square root transformed strength in grams) was not significantly affected by low oxygen, hypercapnic, or combined low oxygen and hypercapnic environment. However, a metric of contraction time (log transformed seconds until peak contraction strength) indicated that oysters closed more slowly in combined hypercapnic and hypoxic environments

The cumulative findings of this research may assist in identifying mechanistic differences in the hypoxic responses of *C. ariakensis* and *C. virginica*. Specifically, I hypothesize that interspecific differences in hypoxic metabolic rate could be responsible for many of the observed differences between the species including hemolymph pH when clamped (Chapter 3), hypoxic gaping width and frequency (Chapter 3), adult survival (Harlan, 2007), growth (Calvo *et al.*, 2001; Paynter *et al*., 2008), and intertidal survival abilities (Zhou and Allen, 2003; Kingsley-Smith and Luckenbach, 2008). The ability of *C. virginica* to survive hypoxia is likely due to its demonstrated ability to reduce its aerobic metabolic rate (Shumway and Koehn, 1982; Stickle, 1989; de Zwaan *et al*., 1991) coupled with its ability to catabolize glucose into alanine and succinate as well as the metabolism of aspartate (Collicutt and Hochachka, 1977; Hochachka 1980; Eberlee *et al*., 1983; Storey, 1993; Figure 1.2) during exposure to low oxygen environments; however these metabolic adjustments and anaerobic pathways may not be present, or as efficient, in *C. ariakensis*. If *C. ariakensis* does not exhibit as strong of an anaerobic capacity as *C. virginica*, it may then be more dependent upon aerobic pathways to yield ATP. This increased dependence upon aerobic pathways could require *C. ariakensis* to maintain a higher aerobic metabolic rate than *C. virginica* in order to sustain its energetic requirements during hypoxia. Different interspecific hypoxic-induced metabolic rates may explain some of the observed behavioral and physiological differences between these species.

For instance, *C. ariakensis* not reducing it metabolic rate as substantially as *C. virginica* during hypoxia could result in a greater accumulation of metabolic acids, thereby explaining the observed differences in clamped acid-base balance between species (Chapter 3). Further, if during hypoxia there is limited metabolic depression in *C. ariakensis*, then in order to meet the oxygen demand it may attempt to extract additional oxygen from the environment. As Tran *et al.* (2000) found increased ventilation was the main metabolic adaptation during early hypoxic exposure in the freshwater clam, *Corbicula fluminea,* an increase in gape width and frequency (as observed in *C. ariakensis* in Chapter 3) may enable additional oxygen extraction by filtering more water, thereby facilitating more substrate-level ATP production via aerobic pathways. Further, if gaping is an approach to obtain more oxygen, then it could also explain the difference in intertidal distribution (Zhou and Allen, 2003; Kingsley-Smith and Luckenbach, 2008). If when emersed *C. ariakensis* gapes, it could inhibit survival as emersed gaping may exacerbate osmotic stress (Carriker, 1996), could lead to desiccation (McMahon, 1963; Marsden and Weatherhead, 1998; Nicastro et al., 2010) and may lead to elevated predation risks (Tomkins, 1947; Loosanoff, 1956; Menzel and Nichy, 1958; Carriker, 1996;). Additionally, this hypothesis may explain the observed difference in growth rates (Calvo *et al.*, 2001; Paynter *et al*., 2008) when hypoxic stress is not lethal. If at sublethal hypoxic levels *C. virginica* substantially decreases its metabolic rate while *C. ariakensis* maintains a higher rate, then during acute hypoxia *C. ariakensis* may have more energy to allocate towards growth (Equation 1.2). Further, muscle contraction (Chapter 4) results are congruent with this hypothesis, as the data suggest that early gaping may have been a behavioral response induced by physiological conditions since

there was no difference in muscle contraction strength following hypoxic exposure. Therefore, I propose that early gaping in *C. ariakensis* may be a behavioral approach to increase exchange with the external environment by both removing acids as well as obtaining additional oxygen to maintain a higher hypoxic-induced metabolic rate than *C. virginica*. However, gaping after long-term exposure may be due to a lack of energy, driven by limited oxygen concentrations, leading to tissue and system failure, which could explain the difference in adult mortality observed by Harlan (2007). The findings of my dissertation research are congruent with the hypothesis that *C. virginica* reduces it metabolic rate more than *C. ariakensis* during hypoxia, however further research is needed to test this hypothesis. This potential mechanism can be tested by exposing *C. ariakensis* and *C. virginica* to both normoxic and hypoxic environments and comparing the aerobic metabolic rate in both environments.

The hypothesis that *C. ariakensis* exhibits a higher metabolic rate than *C. virginica* during hypoxia is one possible mechanism to explain the observed interspecific differences with *C. virginica*; however, others mechanisms could be working in conjunction with or instead of this hypothesis. The similar adult mass-specific respiration rates, implying similar aerobic metabolic rates, between *C. virginica* and *C. ariakensis* (Chapter 2) can help test mechanisms responsible for both the interspecific differences in growth rate (Calvo *et al*., 2001; Paynter *et al*., 2008) and hemolymph pH during clamped emersion (Chapter 3). A higher metabolic rate could be associated with the catabolism of more resources, thereby freeing up more energy which could be allocated towards growth. While spat of *C. ariakensis* exhibited a higher metabolic rate compared to *C. virginica*, there was no significant difference in the metabolic rate of adults, despite a

difference in growth rate. Therefore, these metabolic data do not explain differences in the growth rate of these species. Other mechanisms for this difference could be differences in assimilation efficiencies, consumption rate (though this is unlikely as NRC (2004) found filtration rates to be similar), energy allocation, or investment into growth. Further, Chapter 2 respiration data helps eliminate a possible explanation (see explanation 1 below) for the more acidic shift (ΔpH) and the overall more acidic hemolymph observed in *C. ariakensis* compared to *C. virginica* during clamped emersion (Chapter 3). Possible explanations include: 1) Higher aerobic metabolic rate in *C. ariakensis* than *C. virginica*. 2) Higher hypoxic-induced metabolic rate in *C. ariakensis* than *C. virginica* 3) Higher anaerobic metabolic rate in *C. ariakensis* than *C. virginica* 4) Different, more acidic, metabolic byproducts in *C. ariakensis* than *C. virginica* or 5) Different buffering and acid-base balance mechanisms in *C. ariakensis* than in *C. virginica*. Overall, this research may help identify possible mechanisms for the interspecific difference in hypoxia tolerance, however additional studies may be needed to conclusively isolate the adaptation(s) responsible for survival.

Potential mechanisms #1-3 assume that higher metabolic rates (anaerobic or aerobic) result in more acidic metabolic products and thereby could result in more severe metabolic acidosis. As in mammals, during aerobic respiration oysters undergo glycolysis, pyruvate oxidation, Krebs cycles, and the electron transport chain with oxygen being the final electron acceptor (Figure 1.2). The aerobic catabolism of glucose and the associated processes yields 30-36 ATP per glucose molecule (Raven and Johnson, 2002). However, during low oxygen conditions different metabolic pathways can occur; for instance *C. virginica* can engage in glycogen and aspartate fermentation

resulting in alanine and succinate (Eberlee *et al*., 1983; Storey, 1993) where alanine is produced by the metabolism of glucose, while succinate by aspartate (Collicutt and Hochachka, 1977; Figure 1.4) Additionally, carbon dioxide is a product of both aerobic and anaerobic respiration, which can react with water to lower tissue pH (Equation 1.1). Therefore, a higher metabolic rate could result in the accumulation of more acidic metabolic byproducts (e.g. alanine, succinate, carbon dioxide) and thereby cause a lower hemolymph pH than an organism with the same metabolic pathways but with a lower metabolic rate (potential mechanisms #1 and #3). Additionally, some bivalves can reduce their metabolic rate, shown through lowering their oxygen consumption as well as ATP requirements when exposed to hypoxia (Shumway and Koehn, 1982; de Zwaan *et al*., 1983; Willson and Burnett, 2000). This hypoxic-induced metabolic depression has been studied in *C. virginica*, but not in *C. ariakensis*. Therefore, if *C. ariakensis* either did not exhibit a hypoxic-induced metabolic depression or exhibited a less substantial depression, then this could explain the difference in acid accumulation (potential mechanism #2).

Mechanism #4 (byproducts) could explain the difference in metabolic rate, if the byproducts produced by *C. virginica* are less acidic than those produced by *C. ariakensis*. This is a possible mechanism as Harlan (2007) emersed and clamped *C. virginica* and *C. ariakensis* and found that after two days *C. ariakensis* accumulated significantly more aspartate and less alanine than *C. virginica.* Therefore, Harlan (2007) proposed that the species may engage in different anaerobic pathways, which may influence acid-base chemistry and could alter hemolymph pH. However, further research is needed to test this potential mechanism.

The fifth potential explanation involves different mechanisms between species to regulate acid-base balance within the tissues. To maintain acid-base homeostasis some mollusc species can counteract acidosis by mobilizing calcium carbonate from their valves to act as a buffer (Dugal, 1939; Akberali and Trueman, 1985; Byrne and McMahon, 1994; Dwyer and Burnett, 1996; Michaelidis *et al*., 2005). Metabolites can accumulate whenever gaping is prevented during such times as emersion, predator attack, hypoxia, or pollution inducing acidosis within tissues (Widdows *et al*., 1979; Akberali and Black, 1980; Akberali and Trueman, 1985). Therefore, it is possible that the difference in hemolymph pH between species (Chapter 3) can be explained not only by different rates of accumulating carbon dioxide and other acidic products, but also different rates of calcium carbonate mobilization into the hemolymph during clamped emersion. This theory is supported by Michaelidis *et al*., (2005) who found that juvenile Mediterranean mussels, *Mytilus galloprovincialis*, immersed in high carbon dioxide and low pH seawater exhibited a permanent acidic shift in their hemolymph, but that acidosis was partially mitigated by increased levels of hemolymph bicarbonate. Additionally, Byrne *et al*, (1989) found during 120 hours of emersion at 25°C that the calcium concentration in the hemolymph of the freshwater clam, *Corbicula fluminea*, increased over 300% and data suggest that hemolymph calcium concentration began to change within the first 24 hours. Thus, the difference in hemolymph pH may not reflect different metabolic rates (aerobic or anaerobic) or different types or quantities of byproducts accumulating in the hemolymph, but rather different calcium buffering abilities between species.

While buffering is likely a passive process, it is possible that the shell dissociation potential differs between species as Lombardi *et al.* (unpublished data) and Newell *et al.* (2007) found these species exhibited different shell characteristics. In particular it is possible that these species may have different proportions of foliated sheets to chalky deposits (Lombardi *et al*., unpublished data). These differences in shell properties may have different resistances to dissolution, thus affecting the rate of calcium which is dissolved into the tissues thereby mitigating acidosis. Further, Miller *et al.* (2009), found when under high carbon dioxide environments (pCO_2: 280-800μatm) *C. ariakensis* shell calcification or growth rates did not change, while *C. virginica* shell area decreased 16% and calcium content deceased by 42%. Although shell composition varies by between larval and adult oysters, the findings of Miller *et al*. (2009) demonstrate that during ontogeny shell characteristics varied between species and provide further support for potential differences in adult shell dissociation potentials.

Based on the results of Chapter 2, potential mechanism #1 (basal aerobic metabolic rate), can be rejected; however, the mechanism for the difference in hemolymph pH differences between species remain unknown. Therefore, further research should be conducted testing mechanisms #2 (hypoxic-induced), #3 (anaerobic), #4 (byproducts), and #5 (buffering).

In Chapter 3 differences in the gaping response and tissue acidity during hypoxia and clamping were observed. It was hypothesized that gaping could be a direct result of hypoxia or the resulting low pH environment. The findings of Chapter 4 (muscle contraction strength and speed) suggest differences in hypoxic gaping between *C.*

ariakensis and *C. virginica* may not be due to physiologically-induced muscle failure, but rather may be a behavioral response, possibly to prevent acidosis. If gaping was a physiological response, it could be due to oxygen concentration and/or carbon dioxide concentration/pH (see Equation 1.1 for the relationship between carbon dioxide and pH) directly affecting muscle function or indirectly affecting muscle function via impairing neurological processing or impulse transmission. In either case, if gaping was solely due to muscle failure, then one would expect a change in muscle function and control shown through changes in contraction strength. However, the results of Chapter 4 suggest that early hypoxic exposure does not induce a forced valve opening, nor impair muscle function, as the strength of contraction of the adductor muscle was not compromised by exposure to low oxygen, high carbon dioxide/acidic, or a combination of these environments over a 12 hour period. Since intact oysters, and not isolated muscles, were used, the difference in gaping between species (Chapter 3) is not likely due to a purely physiological response (muscle or neurologically controlled); thereby suggesting gaping may be a behavioral response induced by hypoxia, which differs between species. However, while the strength of closure (sqrt transformed) did not vary based on environment, oysters in combined low oxygen and hypercapnic environments closed their valves more slowly than oysters in the other environments. Therefore, the combined hypoxic and hypercapnic environment may be more stressful than these environmental conditions individually, and may have impact on adductor function. While there was no difference in valve closure strength or speed, *C. ariakensis* gaping response (width and frequency) was more drastic than that of *C. virginica* (Chapter 3). These findings indicate the species may engage in different behavioral gaping responses to low oxygen

which may impact their survival and lead to differing hypoxia tolerances. Gaping early in low oxygen may be adverse during hypoxia, as it permits hypoxic intrusion into the tissues (i.e. organismal hypoxia resulting from environmental hypoxia), which could lead to acidosis earlier than if the valves remained closed utilizing the oxygen contained in the water in the pallial fluid and tissues.

Understanding the mechanisms for gaping and hemolymph acid-base balance differences between species may help better understand survival of these species during hypoxic stress. For instance, elevated basal metabolic rates (potential mechanisms #1-4) can be detrimental when resources are limited, as elevated metabolic rate requires additional energy which may be difficult to obtain if phytoplankton resources are scarce. Further, if there was a difference in buffering (potential mechanism #5), it may translate into differences in survival strategies between species. Calcium concentration in the hemolymph has been shown to increase during both short-term and prolonged stress, and this carbonate mobilization can lead to a reduction in both shell mass and shell strength (Akberali *et al*., 1983; Akberali and Trueman, 1985). Thereby, calcium carbonate buffering may reflect a trade-off between future and immediate survival, as utilizing shell carbonate may aid in surviving acidosis but may reduce predator protection in the future (Akberali and Trueman, 1985). Understanding which of these mechanisms (#1-5) is responsible for the difference in pH could yield insight into differences in survival between these species as well as may help understand mechanisms for hypoxic adaptations of these species. This in turn may help better understand hypoxic adaptations in other species. Further, valve closure following gaping is an important part of predator avoidance (Tomkins, 1947; Loosanoff, 1956; Menzel and Niche, 1958; Carriker, 1996),

as closed calcium carbonate valves act as a shield providing protection from outside forces such as predators and environmental stressors including salinity changes, desiccation, anoxic or contaminated water, as well as provide space for internal organs (Carriker, 1996). However, when an oyster gapes, thereby exposing soft internal tissues to the external environment, it becomes vulnerable to predators and environmental stressors (Menzel and Nichy, 1958; White and Wilson, 1996). Thus, oyster shells are a mechanical defense; the integrity of the shell and the oyster's ability to remain closed are vital for protection from predators, particularly from predators such as crabs, rays and oyster drills which penetrate the shell to access the internal tissues (Carriker, 1996; White and Wilson, 1996). Identifying mechanisms responsible for hypoxic-induced gaping and acid-base balance aids in understanding oyster survival during hypoxia, and survival during the subsequent lack of energy and acidosis induced by hypoxia, as well as during predation attempts and environmental stress.

While environmental hypoxia likely selected for metabolic adaptations enabling survival during low oxygen, it is likely not the only selective pressure. When oysters detect a predator they close their valves and due to catch muscle they can remain closed for long periods of time with little to no energetic input (Millman, 1964**;** Morrison, 1996; Schmidt-Neilson, 1997). This isolates the oysters from the external environment, thereby, protecting them from an array of predators (Carriker, 1996); however, it also limits respiratory exchange and can lead to hypoxic, hypercapnic, and acidic environments (Crenshaw and Neff, 1969; Moon and Pritchard, 1970; Widdows *et al*., 1979; Akberali and Black, 1980; Booth *et al*., 1984; Akberali and Trueman, 1985; Truchot, 1990). Consequently, as a means to survive predation attempts oysters may

frequently undergo organismal hypoxia, hypercapnia, and acidosis. Therefore, mechanisms may have evolved to maintain homeostasis and tolerate these resulting secondary stressors. As a result, assessing gaping responses (Chapter 3), hemolymph homeostasis (Chapter 3) and adductor muscle function (Chapter 4) may not only contribute to better understanding mechanisms associated with survival during environmental hypoxia, but may also better illuminate adaptations enabling predator avoidance.

The findings of this research can be used to further the scientific understanding of the physiological ecology *C. ariakensis* and *C. virginica,* while contributing to the overarching field of comparative physiology and even biomedical research. Invertebrates have long been used as research subjects for biomedical research, with published articles dating back to the 1800s (Wilson-Sanders, 2011). Invertebrates are used for a variety of reasons including they can posses properties or structures not exhibited by humans or other vertebrates, and they often exhibit simpler processes than vertebrates, thereby aiding in the isolation and identification of specific processes and mechanisms (Andrews, 2011). Thus, studies on invertebrates, such as oysters, can provide valuable information for comparative studies across many taxa and can inform and direct biomedical research. Studying hypoxic adaptations of oysters could have applications to medical fields by 1) identifying novel compounds enabling tissue survival; 2) by identifying mechanisms to reduce metabolic rate thereby limiting energetic requirements; or 3) by identifying means of minimizing or mitigating metabolic acidosis. Research into these fields is important as the two main problems associated with tissue function and survival during hypoxia are metabolic acidosis and energy deficits (Hochachka, 1986; Wood, 1991). Therefore,

further comparative research which could identify substrates and compounds associated with survival during low oxygen and acidosis could be valuable contributions to pharmaceutical and other biomedical research.

This research along with the findings of Harlan (2007), North *et al.* (2006), and Matsche and Barker (2006) demonstrate that *C. ariakensis* may not exhibit as robust hypoxic adaptations and tolerance as *C. virginic*a. This suggests that *C. ariakensis*, despite its accelerated growth rate (Calvo *et al*., 2001; Paynter *et al*., 2008), and resistance and resilience to *P. marinus* (dermo) (Calvo *et al*., 2001), may not be a viable candidate for introduction as a means to enhance oyster populations for either commercial exploit or restoration of ecosystem services in areas prone to hypoxia. Therefore, this research may help inform restoration and management decisions in multiple areas of the Atlantic Coast of United States in particular Chesapeake Bay and North Carolina which have considered augmenting native oyster populations with *C. ariakensis* (NRC, 2004).

By comparing metabolic rate and hypoxic responses of the Asian oyster, *Crassostrea ariakensis*, with that of the Eastern oyster, *Crassostrea virginica*, I have identified different responses between these oysters. Specifically, I found that *C. ariakensis* spat exhibited a higher basal aerobic metabolic rate than *C. virginica* spat; *C. ariakensis* adults gaped more frequently and wider than *C. virginica*; that gaping was associated with acidification of the external environment; and when gaping was inhibited *C. ariakensis* had more acidic hemolymph than *C. virginica*. This suggests that gaping may help prevent acidosis by releasing acids to the external environment, that *C.*

ariakensis and *C. virginica* responses to hypoxia differ, and these differences may be associated with different responses to mitigate or prevent acidosis. Further, gaping was not likely a direct physiological response to hypoxic and/or hypercapnic (or associated acidic) environments, as the strength of adductor muscle contraction did not vary during normal, hypoxic, or hypercapnic conditions. These data yield insight into hypoxic and anoxic adaptations of *Crassostrea* oysters which may be applied to ongoing restoration efforts of *C. virginica*, particularly in areas which have considered introducing *C. ariakensis*, as well may contribute to comparative biology and hypoxia research.

References

Agutter, P.S., Wheatley, D.N. 2004. Metabolic scaling: consensus or controversy. Theor Biol Medical Model 1:13

Akberali, H.B., Black, J.E. 1980. Behavioural responses of the bivalve *Scrobicularia plana (*da Costa) subjected to short-term copper (Cu II) concentrations. Mar Environ Research 4(2):97-107

Akberali, H.B., Trueman, E.R. 1985. Effects of environmental stress on marine bivalves molluscs. Pp. 101-198 in J.H.S. Blaxter, A.J. Southward, and C.M. Yonge, eds. Advances in Marine Biology Vol 22. Academics Press, New York, San Francisco, London.

Andrews, P.L.R. 2011. Laboratory invertebrates: only spineless, or spineless and painless? Ilar J 52:121-125

Banavar, J.R., Maritan, A., Rinaldo, A. 1999. Size and form in efficient transportation networks. Nature 99: 130-132

Benedict, F.G. 1938. Vital Energetics: A Study in Comparative Basal Metabolism. Carnegie Institute, Washington DC

Bennett A.F. 1985. Temperature and muscle. J Exp Biol 115:333-344

Bing, O.H.L., Brooks, W.W., Messer, J.V. 1973. Heart muscle viability following hypoxia: Protective effect of acidosis. Science 180(4092):1297-1298

Blaxter, K.L. 1989. Energy metabolism in animals and man. Cambridge: Cambridge University Press

Bokma, F. 2004. Evidence against universal metabolic allometry. Funct Ecol 18:184–187

Booth, C.E., McDonald, D.G., Walsh, P.J. 1984. Acid-base balance in the sea mussel, *Mytilus edulis*. Effects of hypoxia and air-exposure on hemolymph acid-base status. Mar Biol Lett 5:347-358

Breitburg, D.L. 1992. Episodic hypoxia in Chesapeake Bay: Interacting effects of recruitment, behavior, and physical disturbance. Ecol Monogr 62(4):525-546

Breitburg, D. 2002. Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. Estuaries 25:767-781

Brody, S., Proctor, R. C. 1932. Relation between basal metabolism and mature body weight in different species of mammals and birds. University of Missouri Agricultural Experiment Station Research Bulletin 116:89–101.

Brown, J. H., Enquist, B. J., West, G. B. 1997. Allometric scaling laws in biology. Science 278:373

Burnett, L.E. 1997. The challenges of living in hypoxic and hypercapnic aquatic environments. Amer Zool 37: 633-640

Burnett, L.E., Stickle, W.B. 2001. Physiological Responses to Hypoxia. Pp. 101-114 in N.N. Rabalais and R.E. Turner (eds.) Coastal Hypoxia: Consequences for Living Resources and Ecosystems. American Geophysical Union. Washington, DC

Burreson, E.M. 2009. Infection with *Perkinsus marinus*. Pp. 342-353 in Manual of Diagnostic Tests for Aquatic Animals. 6th Edition: Office internationaldes epizooites. Paris

Burton, R.F. 1978. Intracellular buffering. Resp Physiol 33(1):51-58

Byrne, R.A., McMahon, R.F. 1994. Behavioral and physiological responses to emersion in freshwater bivalves. Amer Zool 34(2):194-204

Byrne, R. A., McMahon, R.F., Dietz, T.H. 1989. The effects of aerial exposure and subsequent reimmersion on hemolymph osmolality, ion composition and ion flux in the freshwater bivalve, *Corbiculajluminea.* Physiol ool 62:1187-1202

Byrne, R.A., Gnaiger E., McMahon, R.F., Dietz T.H. 1990. Behavioral and metabolic responses to emersion and subsequent reimmersion in the freshwater bivalve, *Corbicula fluminea*. Biol Bull 178:251-259

Byrne, R.A., Shipman, B.N., Smaresk, N.J., Dietz, T.H., McMahon, R.F. 1991. Acid-base balance during emergence in the freshwater bivalve Corbicula fluminea. Physiol Zool 64:748-766

Calder, W. A. (1987). Scaling energetics of homeothermic vertebrates: an operational allometry. Ann Rev of Physiol 49:107–120

Calow, P., Sibly, R.M. 1990. A physiological basis of population processes: ecotoxicological implications. Funct Ecol 4:283-288

Calvo, G. W., Luckenbach, M. W., Allen S. K. Jr., Burreson, E. M. 2001. A comparative field study of *Crassostrea ariakensis* (Fujita 1913) and *Crassostrea virginica* (Gmelin 1791) in relation to salinity in Virginia. J Shellfish Res 20:221–229

Carriker, M.R. 1996. The Shell and Ligament. Pp. 75-168, Chapter 3 in V.S. Kennedy, R.I.E. Newell, and A. Eble (eds.) The Eastern Oyster, *Crassostrea virginica*. Maryland Sea Grant Publication

Carriker, M.R., Gaffney, P.M. 1996. A Catalogue of selected species of living oysters (Ostreacea) of the world. Pages 1-15, Chapter 3 in V.S. Kennedy, R.I.E. Newell, and A. Eble (eds.). The Eastern Oyster, *Crassostrea virginica*. Maryland Sea Grant Publication.

Castellini, M.A., Somero, G.N. 1981. Buffering capacity of vertebrate muscle: Correlations with potentials for anaerobic function. J Comp Physiol 143:191-198

CENR (Committee on Environment and Natural Resources). 2010. Scientific Assessment of Hypoxia in U.S. Coastal Waters. Interagency Working Group on Harmful Algal Blooms, Hypoxia, and Human Health of the Joint Subcommittee on Ocean Science and Technology. Washington, DC

Chantler, P.D., Lakatta, E.G. 2009. Role of body size on cardiovascular function: can we see the meat through the fat. Hypertension 54:459-461

Chown, S., Marais, E., Terbalnche, J., Klok C., Lighton, J., Blackburn, T. 2007. Scaling of insect metabolic rate is inconsistent with the nutrient supply network model. Funct Ecol 21(2):282-290

Cochran, R.E., Burnett, L.E. 1996. Respiratory responses of the salt march animals, *Fundulus heteroclitus, Leiostomus xanthurus,* and *Palaemonetes pugio* to environmental hypoxia and hypercapnia and to the organophosphate pesticide, azinphosmethyl. J Exp Mar Biol Ecol 195:125-144

Collicutt, J.M., Hochachka, P.W. 1977. The anaerobic oyster heart: coupling of glucose and aspartate fermentation. J Comp Physiol 115:147–157

Crenshaw, M.A., Neff, J.M. 1969. Decalcification at the mantle-shell interface in molluscs. Integr Comp Biol 9(3):881-885

Dauer, D.M., Llanso, R.J., Lane, M.F. 2008. Depth-related patterns in benthic community condition along an estuarine gradient in Chesapeake Bay, USA. Ecol Indic 8(4):417-424

Davison, J. 1955. Body weight, cell surface, and metabolic rate in anuran Amphibia. Biol Bull 109:407–419

Davison, J. 1956. An analysis of cell growth and metabolism in the crayfish (*Procambarus alleni*). Biol Bull 110:264–273

Dewey, F.E., Rosenthal, D., Murphy, D.J. Jr., Froelicher, V.F., Ashley E.A. 2008. Does size matter?: Clinical applications of scaling cardiac size and function for body size. Circulation 117:2279-2287

Diaz, R. 2001. Overview of hypoxia around the world. J Environ Qual 30:275-281

Diaz, R.J., Rosenberg, R. 1995. Marine benthic hypoxia: A review of its ecological effects and the behavioural responses of benthic macrofauna. Mar Biol Annu Rev 33:245-303

Diaz, R., Rosenberg R. 2008. Spreading dead zones and consequences for marine ecosystems. Science 321(5891):926-929

Dmi'el R. 1972. Relation of metabolism to body weight in snakes. Copeia *1972:*179-81.

Dodds, P.S., Rothman, D.H., Weitz, J.S. 2001. Re-examination of the "3/4-law" of metabolism. J of Theor Biol 209(1):9-27

Downing, S.E, Talner, N.S., Gardner, T.H. 1965. Cardiovascular responses to metabolic acidosis. Amer J Physiol 208:237-242

Dugal, L-P. 1939. The use of calcareous shell to buffer the product of anaerobic glycolysis in *venus mercenaria*. J Cell Comp Physiol 13(2):235-251

Dwyer, J.J., Burnett, L.E. 1996. Acid-base status of the oyster, *Crassostrea virginica*, in response to air exposure and to infections by *Perkinsus marinus*. Biol Bull 190:139-147

Earle, A.M. 2009. Home Life in Colonial Days. Echo Library. Middlesex, UK

Earle, C. 1979. Environment, disease and mortality in early Virginia. J Hist Geogr 5(4):365-390

Eble, A.F., Scro, R. 1996. General Anatomy. Pages 19-73, Chapter 2 In: V.S. Kennedy, R.I.E. Newell, and A. Eble (eds.). The Eastern Oyster, *Crassostrea virginica*. Maryland Sea Grant Publication

Eberlee, J.C., Storey, K.B. 1984. Buffering capacities of the tissues of marine molluscs. Physiol Zool 57(5):567-572.

Eberlee, J.C., Storey, J.M., Storey, K.B. 1983. Anaerobiosis, recovery from anoxia, and the role of strombine and alanopine in the oyster *Crassostrea virginica*. Can. J. Zool. 61:2682–2687

Elliott, M., McLusky, D.S. 2002. The need for definitions in understanding estuaries. Estuar Coast Shelf Sci 55:815–827

Elliott, M., Quintino, V. 2007. The estuarine quality paradox, environmental homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. Marine Pollut Bull 54:640-645

EPA. 1996. Ecological Effects Test Guidelines. Oyster Acute Toxicity Test (Shell Deposition). OPPTS 850.1025. EPA 712–C–96–115

Erickson, J.C., Hollopeter, G., Palmiter, R.D. 1996. Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. Science 274(5293):1704-1707

Foighil, D.Ó., Gaffney, P.M., Hilbish, T.J. 1995. Differences in the mitochondrial 16S ribosomal gene sequences allow discrimination among America [*Crassostrea*

virginica (Gmelin)] and Asian [*C. gigas* (Thunberg) *C. ariakensis* Wakiya] oyster species. J Exp Mar Biol Ecol 192(2):211-220

Galvão, P.E., Tarasantchi, J., and Guertzenstein, P. 1965. Heat production of tropical snakes in relation to body weight and body surface. Amer J Physiol 209:501-6

Gatsoff, P.S. 1964. The American oyster, *Crassostrea virginica* Gmelin. Fishery Bull Fish Wild Serv U.S 6:1-480

Geiser, F. 2004. Metabolic rate and body temperature reduction during hibernation and daily torpor. Annu Rev Physiol 44:239-274

Glazier, D.S. 1991. Separating the respiration rates of embryos and brooding females of *Daphnia magna*: implications for the cost of brooding and the allometry of metabolic rate. Limnol Oceanogr 36:354-362

Glazier, D.S. 2005. Beyond the '3/4-power law': variation in the intra-and interspecific scaling of metabolic rate in animals. Biol Rev 80: 611-662

Glazier, D.S. 2006. The 3/4-power law is not universal: evolution of isometric, ontogenetic metabolic scaling in pelagic animals. BioScience 56: 325-332

Glazier, D.S. 2010. A unifying explanation for diverse metabolic scaling in animals and plants. Biol Rev 85:111-138

Glazier, D.S., Butler, E.M., Lombardi, S.A., Deptola, T.J., Reese, A.J., Satterthwaite E.V. 2011. Ecological effects on metabolic scaling: amphipod responses to fish predators in freshwater springs. Ecol Monogr 81(4):599–618

Gosling, E. 2003. Bivalve molluscs: Biology, Ecology and Culture. Fishing News Books, Oxford, UK

Gray, J.S., Wu, R.S., Or, Y.Y. 2002. Effects of hypoxia and organic enrichment on the coastal marine environment. Mar Ecol Prog Ser 238:249-279

Guiot, C., Degiorgis P.G.,. Delsanto, P.P., Gabriele, P., Deisboeck, T.S. 2003. Does tumor growth follow a "universal law"? J. Theor Biol 225:147–151

Hagy, J.D., Boynton, W.R., Keefe, C.W., Wood, K.V. 2004. Hypoxia in Chesapeake Bay, 1950-2001: Long-term change in relation to nutrient loading and river flow. Estuaries 27(4):634-658

Hamburger, K., Møhlenberg, F., Randlùv, A., Riisgård, H.U. (1983). Size, oxygen consumption and growth in the mussel *Mytilus edulis*. Mar. Biol 75:303-306

Hammer, K.M., Kristiansen, E., Zachariassen, K.E. 2011. Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius, 1779) (Bivalvia; Limidae). Mar Environ Res 72:135-142

Hargis, W.J., Haven, D.S. 1999. Chesapeake oyster reefs, their importance, destruction and guidelines for restoring them. In: M.W. Luckenbach, R. Mann. J.A. Wesson (eds.) Oyster reef habitat restoration: A synopsis and synthesis of approaches. Virginia Institute of Marine Science Press, Gloucester Point, VA

Harlan, N.P. 2007. A comparison of the physiology and biochemistry of the Eastern oyster, *Crassostrea virginica,* and the Asian oyster, *C. ariakensis*. Masters Thesis. University of Maryland, College Park, Maryland, USA

Haugan, P.M., Drange, H. 1996. Effects of CO_2 on the ocean environment. Energ Convers Manage 37(6-8):1019-1022

Hawkins, B.A. Albuquerque, F.S. Araújo, M.B., Beck, J., Bini L.M., Cabrero-Sañudo, F.J., Castro-Parga, I., Diniz-Filho JA., Ferrer-Castán, D., Field, R., Gómez, J.F.,

Hortal, J., Kerr, J.T., Kitching, I.J., León-Cortés, J.L., Lobo, J.M., Montoya, D., Moreno, J.C., Olalla-Tárraga, M.Á., Pausas, .JG., Qian, H., Rahbek, C., Rodrígues, M.Á., Sanders N,J., Williams P. 2007. A global evaluation of metabolic theory as an explaination for species richness gradients. Ecology 88(8):1877-1888

Hayssen, V., Lacy, R.C. 1985. Basal metabolic rate in mammals: allometry of BMR and body mass. Comp Biochem Physiol A. 81(4):741-754

Heath, A.G. 1995. Water pollution and Fish Physiology. 2nd ed. Boca Raton: CRC Press, Inc.

Hemmingsen, A. M. (1960). Energy metabolism as related to body size and respiratory surfaces, and its evolution. Reports of the Steno Memorial Hospital and the Nordisk Insulin Laboratorium (Copenhagen) 9:1–110

Hochachka, P.W. 1980. Living without Oxygen. Cambridge: Harvard UP. pp. 27-41.

Hochachka, P.W. 1986. Defense strategies against hypoxia and hypothermia. Science 231(4735):234-241

Hochachka, P.W., Mustafa, T. 1972. Invertebrate facultative anaerobiosis. Science 178: 1056-1060

Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., Harvell, C.D., Sale, P.F., Edwards, A.J., Caldeira, K., Knowlton, N., Eakin, C.M, Iglesias-Prieto, R., Muthiga, N., Bradbury, R.H., Dubi, A., Hatziolos, M.E. 2007. Coral reefs under rapid climate change and ocean acidification. Science 318:1737-1742

Hubertz, E., Cahoon, L. 1999. Short-term variability of water quality parameters in two shallow estuaries of North Carolina. Estuar Coasts 22, 814-823

Hui, Y.H. 2006. Handbook of food science, technology, and engineering, Volume 1. Boca Raton: CRC press

IPCC. 2010. Intergovernmental panel on climate change data distribution centre. Carbon dioxide: Projected emissions and concentrations. Data from Atmospheric CO_2 concentrations as observed at Mauna Loa from 1958 to 2008. http://www.ipcc-data.org/ddc_co2.html

Isaac, N.J., Carbone, C. 2010. Why are metabolic scaling exponents so controversial? Quantifying variance and testing hypotheses. Ecol Letters 13(6):728-735

Janssen, H.C.J.P, Samson M.M., Verhaar H.J.J. 2002. Vitamin D deficiency, muscle function and falls in elderly people. Am J Clin Nutr 75:611-615

Jones, T., Bourne, N.F., Bower, S.M., Iwama, G.K. 1993. Effect of repeated sampling on haemolymph pH, PO_2 and haemocyte activity in the Pacific oyster, *Crassostrea gigas* (Thunberg). J Exp Mar Biol Ecol 167(1):1-10

Karlson, K., Rosenberg, R., Bonsdorff, E. 2002. Temporal and spatial large-scale effects of eutrophication and oxygen deficiency on benthic fauna in Scandinavia and Baltic waters: A review. Oceanogr Mar Bio. Annu Rev. 40, 427:489

Kaiyala, K.J., Morton, G.J., Leroux, B.G., Ogimoto, K., Wisse, B., Schwartz, M.W. 2010. Identification of body fat mass as a major determinant of metabolic rate in mice. Diabetes 59(7):1657-1666

Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde,

E.D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M., Stevenson, J.C. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. Mar Ecol Prog Ser 303:1-29

Kerkhoff, A.J., Enquist, B.J. 2009. Multiplicative by nature: Why logarithmic transformation is necessary in allometry. J Theor Biol 257:519-521.

Killen, S.S., Atkinson, D., Glazier, D.S. 2010. The intraspecific scaling of metabolic rate with body mass in fishes depends on lifestyle and temperature. Ecol Letters 13(2):184-193

Kingsley-Smith, P., Luckenbach, M.W. 2008. Post-settlement survival and growth of the Suminoe oyster, *Crassostrea ariakensis*, exposed to simulated emersion regimes. J Shellfish Res 27:609-618

Kleiber, M. 1932. Body size and metabolism. Hilgardia 6:315–353

Kozlowski, J., Konarzewski, M. 2004. Is West, Brown, and Enquist's model of allometric scaling mathematically correct and biologically relevant. Funct Ecol 18 283-289

Landrum, K., Ache, B. 2000. The economic impact of oyster closures and their implications for the shellfish challenge in the Baratariaterrebonne national estuary, Louisiana. Proceedings of the Water Environment Federation. 12:796-807

Lanning, G., Eilers, S., Pörtner, H.O., Sokolova, I.M., Bock, C. 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas* -- Changes in metabolic pathways and thermal response. Mar Drugs 8:2318-2339

Loosanoff, V.L. 1956. Two obscure oyster enemies in New England waters. Science 123:1119–1120

Lopes, J., Russell D.M.C.R., Whitwell, J., Jeejeebhoy, K.N. 1982. Skeletal muscle function in malnutrition. Am J Clin Nutr 36:602-610

Machado, J., Coimbra, J., Sa, C., Cardoso, I. 1988. Shell thickening in *Anodonta cygenea* by induced acidosis. Comp Biochem Physiol A 91(4):645-651

Mackiernan, G.B., Flemer, D.A., Nehlsen, W., Tippie, V.K. 1983. State of the bay. Pp. 15-35 in D.A. Barker, M. Manganello, D. Pawlowicz, S. Katsonos (eds.) Chesapeake Bay: a framework for action. United States Environmental Protection Agency, Chesapeake Bay Program, Annapolis, Maryland, USA

Malanga, C.J., Aiello, E.L. 1972. Succinate metabolism in the gills of the mussels *Modiolus demissus* and *Mytilus edulis.* Comp Biochem Physiol B 43(15):795-806

Marsden ,I.D., Weatherhead, M.A. 1998. Effects of aerial exposure on oxygen consumption by the New Zealand mussel *Perna canaliculus* (Gmelin, 1791) from an intertidal habitat. *J Exp Mar Biol Ecol* 230:15-29

Matsche, M., Barker, L. 2006. Juvenile oyster mortality following experimental exposure to anoxia/hypoxia—*C. ariakensis* vs. *C. virginica*. Report to the Maryland Department of Natural Resources

May, R.C., Kelly, R.A., Mitch, W.E. 1987. Metabolic acidosis stimulates protein degradation in rat muscle by Glucocorticoid-dependent mechanisms. J Clin Invest 79(4): 1099-1103

McLusky, D.S., Elliott, M. 2007. Transitional waters: a new approach, semantics or just muddying the waters? Estuar Coast Shelf Sci 71:359–363

McMahon, R.F. 1983. Desiccation resistance and the use of mantle cavity for aerial respiration in the mangrove bivalve mollusc, *Geloina erosa*. Amer Zool 23:938

McKechnie, A. E., Freckleton, R. P. Jetz, W. 2006. Phenotypic plasticity in the scaling of avian basal metabolic rate. P Roy Soc B-Biol Sci 273:931-937

Menzel, W.R., Nichy, F.E. 1958. Studies of the distribution and feeding habits of some oyster predators in Alligator Harbor, Florida. B Mar Sci 8(2):125-145

Metzger, J.M., Fitts, R.H. 1987. Role of Intracellular pH in muscle fatigue. J Appl Physiol 42(4):1392-1397

Michaelidis, B., Haas, D., Grieshaber M.K. 2005a. Extracellular and intracellular acid-base status with regard to energy metabolism in the oyster *Crassostrea gigas* during exposure to air. Physiol Biochem Zool. 78(3):373-383

Michaelidis, B., Ouzounis, C., Palera, A., Pörtner H.O. 2005b. Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser. 293:109-118

Miller, A.W., Reynolds, A.C., Sobrino, C., Riedel, G.F. 2009. Shellfish face uncertain future in high CO_2 world: Influence of acidification on oyster larvae calcification and growth in estuaries. PLoS One 4(5):e5661

Milliman, C.M. 1970. A comparison of the adductor muscles of several pelecypods (Phylum Mollusca). Doctoral dissertation. Dalhousie University, Halifax, Nova Scotia, Canada

Millman, B.M. 1964. Contraction in the opaque part of the adductor muscle of the oyster (*Crassostrea angulata*). J Physiol 173:238-262

Mitch, W.E., Medina, R., Grieber, S., May, R.C, England, B.K., Price, S.R., Bailey, J.L., Goldberg, A.L. 1994. J Clin Invest 93(5):2127-2133

Mongin, P. 1968. Role of acid-base balance in the physiology of egg shell formation. World Poultry Sci J 24:200-230

Moon, T.W., Pritchard, A.W. 1970. Metabolic adaptations in vertically-separated populations of *Mytilus californianus* Conrad. J Exp Mar Biol Ecol 5(1):35-46

Morrison, C.M. 1996. Adductor and Mantle Musculature. Pages 169-184, Chapter 4 In: V.S. Kennedy, R.I.E. Newell, and A. Eble (eds.). The Eastern Oyster, *Crassostrea virginica*. Maryland Sea Grant Publication

Mueller, P., Diamond, J., 2001. Metabolic rate and environmental productivity: Well-provisioned animals evolved to run and idle fast. Proc Nat Acad Sci USA 98(22):12550-12554

Murphy, R.R., Kemp, W.M., Ball, W.P. 2011. Long-term trends in Chesapeake Bay seasonal hypoxia, stratification, and nutrient loading. Estuar Coasts 34:1293-1309

Naylor, C., Maltby, L., Calow, P. 1989. Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. *Hydrobiologia* 188/189:517-523

Newell, R.I.E. 1988. Ecological changes in Chesapeake Bay: Are they the result of overharvesting the American oyster, *Crassostrea virginica*? Understanding the Estuary: Advances in Chesapeake Bay Research. Proceedings of a Conference. 29-31. Chesapeake Research Consortium Publication 129

Newell, R.I., Kennedy, V.S., Shaw, K.S. 2007. Comparative vulnerability to predators, and induced defense responses, of eastern oysters *Crassostrea virginica* and non-native *Crassostrea ariakensis* oysters in Chesapeake Bay. Mar Biol 152: 449-460

Nicastro, K.R., Zardi G.I., McQuaid, C.D., Stephens, L., Radloff S., Blatch G.L. 2010. The role of gaping behavior in habitat partitioning between coexisting intertidal mussels. BMC Ecology 10(1):17

North, E. W., Schlag, Z., Hood, R.R., Zhong, Li, M., Gross, T. 2006. Modeling dispersal of *Crassostrea ariakensis* oyster larvae in Chesapeake Bay. Final Report to Maryland Department of Natural Resources, July 31, 2006. 55 p

NRC. 2004. Nonnative Oysters in the Chesapeake Bay in O. S. B. Committee on Nonnative Oysters in the Chesapeake Bay, editor. National Research Council, National Academy of Sciences, Washington, DC

Orchard, C.H., Kentish, J.C 1990. Effects of changes of pH on the contractile function of cardiac muscle. Am J Physiol – Cell PH 258(6):967-981

Packard, G.C., Boardman, T.J. 1987. The misuse of ratios to scale physiological data in data that vary allometrically with body size. Pp 316-349 in M.E. Feder, A.F. Bennet, W.W Burggren, and RB Huey (eds). New Directions in Ecological Physiology. Cambridge University Press. Cambridge

Paynter, K.T. 1996. The effects of *Perkinsus marinus* infection on physiological processes in the eastern oyster, *Crassostrea virginica*. J Shellfish Res 15(1):119-125

Paynter, K.T., Goodwin, J.D., Chen, M.E., Ward, N.J, Sherman, M.W, Merritt, D.W, Allen, S.K. 2008. *Crassostrea ariakensis* in Chesapeake Bay: Growth, disease and mortality in shallow subtidal environments. J Shellfish Res 27(3):601-608

Paynter, K.T., Politano, V., Lane, H.A., Allen, S.M., and Meritt, D. 2010. Growth Rates and Prevalence of *Perkinsus marinus* in Restored Oyster Populations in Maryland. J Shellfish Res 29(2):309-317

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., Collins, F. Effects of the obese gene product on body weight regulation in ob/ob mice. Science. 269(5223):540-543

Pérez, H.M., Janssoone, X., Côte C., Guderley H. 2009. Comparisons between *in vivo* force recordings during escape responses and *in vitro* contractile capacities in the sea scallop *Placopecten magellanicus.* . J Shellfish Res 28(3):491-495

Piiroinen, S., Lindström, R., Lyytinen, A. 2010. Resting metabolic rate can vary with age independently from body mass changes in the Colorado potato beetle, *Leptinotarsa decemlineata*. J Insect Physiol. 56:277-282

Poehlman, E.T. and Toth, M.J. 1995. Mathematical ratios lead to spurious conclusions regarding age- and sex- related differences in resting metabolic rate. Am J Clin Nutr 61(3):482-485

Pokras, M.A, Zeman, A., Kirkwood, J., Sedgwick, C.J. 1993. An introduction to allometric scaling and its uses in raptor medicine. Pp. 211-224. in P.T. Redig, J.E. Cooper., J.D. Remple D.B. Hunter (eds). Raptor Biomedicine. University of Minnesota Press. Minneapolis

Porter, E.T., Cornwell, J.C., Sanford, L.P. 2004. Effect of oysters *Crassostrea virginica* and bottom shear velocity on benthic-pelagic coupling and estuarine water quality. Mar Ecol Prog Ser 271:61-75

Rabalais, N.N., Turner, R.E., Wiseman, W.J.Jr. 2002. Gulf of Mexico hypoxia, a.k.a. "The dead zone". Annu Rev Ecol Syst 33:235-263

Raven, P.H., Johnson, G.B. 2002. Biology: sixth edition. McGraw-Hill Companies, Inc., New York, NY pp. 141-204

Ray, S.M. 1952. A culture technique for the diagnosis of infections with *Dermocystidium marinum* Makin, Owen and Collier in oysters. Science 116:360-361

Ray, S.M. 1966. A review of the culture method for detecting *Dermcystidium marinum*, with suggested modifications and precautions. Proc Nat Shellf Association 54:55-69

Rayne, S., Forest, K. 2009. A new class of perfluorinated acid contaminants: Primary and secondary substituted perfluoroalkyl sulfonamides are acidic at environmentally and toxicologically relevant pH values. J Environ Sci Heal A 44:1388-1399

Reaka, M.L., Lombardi, S.A. 2011. Hotspots on global coral reefs. In: Zachos FE, Habel JC (eds.), Biodiversity Hotspots, Springer Pub, NY

Refinetti, R. 1989. Body size and metabolic rate in the laboratory rat. Exp Biol 48, 291–294

Reece, K.S., Cordes, J.F., Stubbs, J.B., Hudson, KL., Francis, E.A. 2008. Molecular phylogenies help resolve taxonomic confusion with Asian *Crassostrea* oyster species. 2008. Mar Biol 153:709-721

Riisgård, H.U., Randløv, A., Hamburger, K. 1981. Oxygen consumption and clearance as a function of size in *Mytilus edulis* L. veliger larvae. Ophelia 20: 179-183

Riisgård, H.U. 1998. No foundation of a "3/4 power scaling law" for respiration in biology. Ecol Letters 1: 71-73

Riisgård, H.U., Randløv, A., Kristensen, P.S. 1980. Rates of water processing, oxygen consumption and efficiency of particle retention in veligers and young post-metamorphic *Mytilus edulis.* Ophelia 19: 37-47

Ringwood, A.H., Keppler, C.J. 2002. Water quality variation and clam growth: is pH really a non-tissue in estuaries. Estuaries 25:901-907

Rodney, W.S., Paynter, K.T. 2006. Comparisons of macrofaunal assemblages on restored and non-restored oyster reefs in mesohaline regions of Chesapeake bay in Maryland. J Exp Mar Biol Ecol 335:39-51

Rognum, T.O., Saugstad, O.D. 1991. Hypoxanthine levels in Vitreous Humor: Evidence of Hypoxia in Most Infants Who Died of Sudden Infant Death Syndrome. Pediatrics 87(3):306-310

Rognum, T.O., Saugstad, O.D., Øyasæter, S., Olaisen, B. 1988. Elevated levels of hypoxanthine in vitreous humor indicate prolonged cerebral hypoxia in victims of sudden infant death syndrome. Pediatrics 82:615-618

Rome, L.C. 1990. Influence of temperature on muscle recruitment. Am J Physiol-Reg I 259(2): R210-R222

Rubner, M. 1883. Über den Einfluss der Körpergrosse auf Stoffund Kraftwechsel. *Zeitschrift für Biologie* (Munich) 19:535–562

Schmidt-Nielsen, K. 1984. Scaling: Why is animal size so important? Cambridge. Cambridge University Press

Schmidt-Nielson, K. 1997. Animal Physiology: Adaptation and environment. 5th ed. Cambridge: Cambridge University Press

Seibel, B.A. 2006. On the depth and scale of metabolic rate variation: scaling of oxygen consumption rate and enzymatic activity in the Class Cephalopoda (Mollusca). J Exp Biol 210:1-11

Semenza, G., Agani, F., Feldser, D., Lyer, N., Kotch, L., Laughner, E., Yu, A. 2002. Hypoxia, HIF-1, and the pathophysiology of common human diseases. Adv Exp Med Biol 475:123-130

Shumway, S.E., Koehn, R.K. 1982. Oxygen consumption in the American oyster, *Crassostrea virginica*. Mar Ecol Prog Ser 9:59-68

Singhal, A., Davie, R., Sahota, A., Thomas, P.W., Serjeant, G.R. 1993. Resting metabolic rate in homozygous sickle cell disease. Am J Clin Nutr 54:32-34

de Souza, S.C.R., de Carvalho, J.E., Abe, A.S., Bicudo, J.E.P.W., Bianconcini, M.S.C. 2004. Seasonal metabolic depression, substrate utilization and changes in scaling patterns during the first year cycle of tegu lizards (*Tupinambis merianae).* J Exp Biol 207:307-318

Stickle, W.B., Kapper, M.A., Liu, L., Gnaiger, E., Wang, S.Y. 1989. Metabolic adaptations of several species of crustaceans and molluscs to hypoxia: Tolerance and microcalorimetric studies. Biol Bull 177:303-312

Stokes, T.M., Awapara, J. 1968. Alanine and succinate as end products of glucose degradation in the clam *Rangia cuneata.* Comp. Biochem. Physiol. 25: 883-892

Storey, K. B. 1993. Molecular mechanisms of metabolic arrest in mollusks. Pp. 253-269 in P. W. Hochachka, P. L. Lutz, T. Sick, M. Rosenthal, G. van den Thillart (eds.) Surviving Hypoxia: Methods of Control and Adaptation. CRC Press, Boca Raton

Strachey, W., Major, R.H. 1849 The historie [SIC] of travaile [SIC] into Virginia Britannia. Hakluyt Society, London

Sutton, R.A.L., Wong, N.L.M., Dirks, J.H. 1979. Effects of metabolic acidosis and alkalosis on sodium and calcium transport in the dog kidney. Kidney Int 15:520-533

Taggart, M.J., Wray, S. 1998. Hypoxia and smooth muscle function: key regulatory events during metabolic stress. J Physiol 502(2):315-325

Taft, J.L., Taylor, W.R., Hartwig, E.O., Loftus R. 1980. Seasonal oxygen depletion in Chesapeake Bay. Estuaries 3(4):242-247

Thessen, A.E., Soniat, T.M., Dortch, Q., Doucette, G.J. 2010. Crassostrea virginica grazing on toxic and non-toxic diatoms. Toxicon 55(2-3):570-579

Tomanek, L., Zuoq, M.J., Ivanina, A., Beniash, E., Sokolova, I.M. 2011. Proteomic response to elevated P_{Co2} in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress J Exp Biol 214:1836-1844

Tomkins, I.R. 1947. The oyster-catcher of the Atlantic coast of North America and its relation to oysters. Wilson Bull 59(4):204-208

Tracy, C.R., Sugar, J. 1989. Potential misuse of ANCOVA: comment on Packard and Boardman. Physiol Zool 62(4):993-997

Tran, D., Boudou, A., Massabuau, J.C. 2000. Mechanism for maintaining oxygen consumption under varying oxygenation levels in the freshwater clam *Corbicula fluminea*. Can J Zool 78: 2027-2036

Truchot, J.P. 1990. Respiratory and ionic regulation in invertebrates exposed to both water and air. Annu Rev Physiol 52:61-76

Ueno, S., Yokoyama, K., Nakagawa, M., Araki, S. 2002. Effects of pH and temperature on force and stiffness of skeletal muscle fibers during contraction and relaxation in relation to musculoskeletal disorders. Ind Health 40:362-369

Vaupel, P., Schlenger, K., Knoop, C., Höckel, M. 1991. Oxygenation of human tumors: Evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. Cancer Res 51:3316-3322

Wang, H., Gui, X., Zhang, G., Zhang, F. 2004. Classification of jinjiang oysters *Crassostrea rivularis* (Gould, 1861) from China, based on morphology and phylogenetic analysis. Aquaculture 242:137-155

Weisburg, S.B., Ranasinghe, J.A., Dauer, D.M., Schaffner, L.C., Diaz, R.J., Frithsen, J.B. 1997. An estuarine benthic index of biotic integrity (B-IBI) for Chesapeake Bay. Estuar Coasts 20(1):1559-2731

West, G. B., Brown, J.H., Enquist, B. J. 1997. A general model for the origin of allometric scaling laws in biology. Science 276:122–126

White, C. 2003. Allometric Analysis beyond Heterogeneous Regression Slopes: Use of the Johnson-Neyman Technique in Comparative Biology. Physiol Biochem Zool 76(1):135-140

White, C. 2011. Allometric estimation of metabolic rates in animals. Comp Biochem Physiol B 158(3):346-57

White, M.E., Wilson, E.A. 1996. Predators, pests, and competitors. Pages 559-580, Chapter 16 In: V.S. Kennedy, R.I.E. Newell, and A. Eble (eds.). The Eastern Oyster, *Crassostrea virginica*. Maryland Sea Grant Publication

Wickes, M.A., Morgan, R.P.II. 1976. Effects of salinity on three enzymes involved in amino acid metabolism from the American oyster, *Crassostrea virginica*. Comp Biochem Physiol B 53(3):339-343

Widdows J., Bayne, B.L., Livingstone, D.R., Newell, R.I.E., Donkin, P. 1979. Physiological and biochemical responses to bivalve molluscs to exposure to air. Comp Biochem Physiol A 6(2):301-308

Widdows, J., Nasci, C., Fossato, V. 1997. Effects of pollution on the scope for growth of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy. Mar Environ Research 43(1-2):69-79

Willson, L.L., Burnett, L.E. 2000. Whole animal and gill tissue oxygen uptake in the Eastern oyster, *Crassostrea virginica*: Effects of hypoxia, hypercapnia, air exposure, and infection with the protozoan parasite *Perkinsus marinus*. J Exp Mar Biol Ecol 246:223-240

Wilson-Sanders, S.E. 2011. Invertebrate models for biomedical research, testing, and education. Ilar J 52:126-152

Wood, S.C. 1991. Interactions between hypoxia and hypothermia. Annu Rev Physiol 53:71-85.

Zhang X., Qi, Z., Li, J., Ma, X., Wang, Z., Huang, X., Zhuang, Q. 1960. Bivalves of South China Sea (Nanhai). Beijing: Science Press. pp 274

Zhong, H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L, Simons, J.W. 1999. Overexpression of hypoxia-inducible Factor 1α in common human cancers and their metastases. Cancer Res 59:5830-5835

Zhou, M., Allen, S.K. 2003. A review of published work on *Crassostrea ariakensis*. J Shellfish Res 22(1):1-20

de Zwaan, A. 1983. Carbohydrate catabolism in bivalves. Pp 137-175 in P. W. Hochachka, ed: The Mollusca, Vol.1. Metabolic Biochemistry and Molecular Biomechanics. Academic Press, New York

de Zwaan, A., Zandee, D.I. 1972. The utilization of glycogen and accumulation of some intermediates during anaerobiosis in *Mytilus edulis L.* Comp. Biochem. Physiol B 43(1):47-54

de Zwaan, A., Wijsman, T.C.M. 1976. Anaerobic metabolism in bivalvia (Mollusca) Characteristics of anaerobic metabolism. Comp Biochem Physiol 54(3):313-323.

de Zwaan, A, Marrewijk, W. van. 1973. Anaerobic glucose degradation in the sea mussel *Mytilus edulis* L. Comp. Biochem. Physiol. B. 44(2):429-39

de Zwaan, D.A., Cortesi, P., Van Den Thillart, G., Roos, J., Storey, K.B. 1991. Differential sensitivities to hypoxia by two anoxia-tolerant marine molluscs: a biochemical analysis. Mar Biol. 111:343–351