ABSTRACT

Title of Document: A COMPARISON OF THE PHYSIOLOGY
                                            AND BIOCHEMISTRY OF THE EASTERN
                                            OYSTER, *CRASSOSTREA VIRGINICA*, AND
                                            THE ASIAN OYSTER, *CRASSOSTREA
                                            ARIAKENSIS*

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The Chesapeake Bay Foundation, US Environmental Protection Agency, and
the Chesapeake Bay Commission, requested research on the introduction of the Asian
oyster, *Crassostrea ariakensis*, to help restore the fishery and ecosystem function of
the native Eastern oyster, *Crassostrea virginica*. In order to augment the role of *C.
virginica* in Chesapeake Bay, *C. ariakensis* will likely require tolerances to low
dissolved oxygen similar to that of the native oyster. This research showed that
triploid and diploid *C. virginica* lived significantly longer than *C. ariakensis* under
anoxic conditions, although the oxygen consumption rates of diploid oysters did not
differ between species. Free amino acid pools in the gill tissue of oysters exposed to
normoxia or hypoxia were analyzed. Alanine increased in both species during
hypoxia, indicating the use of alternative metabolic pathways. Aspartate was
consumed by *C. virginica* during hypoxia, confirming the use of a pathway coupling
glucose and aspartate fermentation. Differences in the free amino acid pools of these
two species suggest an explanation for the disparity in anaerobic metabolism between
C. ariakensis and *C. virginica*.

A COMPARISON OF THE PHYSIOLOGY AND BIOCHEMISTRY OF THE
EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, AND THE ASIAN OYSTER,
CRASSOSTREA ARIAKENSIS

By

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Chapter 1: Overview

Organisms have adapted to life in diverse environments on Earth, including environments without oxygen. Perhaps because so many organisms, including humans, cannot live without oxygen, scientists have shown an interest in studying organisms that cope with anoxic environments. Anaerobiosis, or "life without oxygen," challenges organisms in the deep ocean, bacteria and worms in the human intestine, marine organisms exposed to air during tidal cycles, and organisms performing strenuous exercise. Whether in marine sediments or the human gut, organisms have developed unique mechanisms for dealing with anoxia.

Oysters are found around the world in diverse marine and estuarine environments. They are frequently exposed to anoxic conditions, whether through air exposure during emersion in the intertidal zone, or during the summer when water temperatures drive dissolved oxygen in the bottom waters to low levels (Widdows et al., 1979; Lenihan et al., 1995). In the Chesapeake Bay, hypoxia has been driven by anthropogenic sources, including farming runoff from fertilization and animal feces and wastewater treatment plants (Kemp et al., 2005; Fisher et al, 2006).

In most aquatic systems, oxygen from the air exchanges with the water at the air-water interface, and this oxygen mixes with the bottom waters, supplying benthic organisms like oysters with the oxygen they require for survival. The conditions necessary for the development of a "dead zone" of hypoxic water are stratification of the water column, which prevents mixing of the oxygenated surface layer with the oxygen-poor bottom waters, and the breakdown of organic matter in the bottom waters. Bacterial decomposition of organic matter uses up the oxygen in the already

oxygen depleted bottom waters, and stratification prevents replenishing bottom waters with oxygen from the surface layers (Taft et al., 1980; Nixon, 1995; Fisher et al., 2006).

Sources of organic matter in the benthos may be natural, including pseudofeces production by oysters, phytoplankton die-off, and death of other aquatic organisms. However, in recent years, anthropogenic causes have increased organic matter in the benthos, driving oxygen consumption and lowering benthic dissolved oxygen levels (Seliger and Boggs, 1988; Officer et al., 1984; Kemp et al., 2005). Eutrophication, excessive plant growth as a result of increased nutrient inputs, is caused by agriculture, industry, and human development of watersheds. Nutrient inputs to estuaries from fertilization, agricultural run-off, and wastewater treatment plants stimulate the growth of phytoplankton, which are typically nitrogen-limited (Anderson et al., 2002). The resulting die-off of these organisms feeds bacteria that consume oxygen in the process.

The subsequent hypoxia, or low dissolved oxygen, affects estuarine organisms differently. In this paper anoxia is considered less than 1.0 mg/L O_2, hypoxia 1.0-3.0 mg/L O_2, and normoxia above 3.0 mg/L O_2. The Eastern oyster, *Crassostrea virginica*, is capable of surviving hypoxia for three weeks at $25^{\circ}C$ (Hochachka, 1980). In *Crassostrea* species and other bivalve species, including mussels, physiological changes result that allow the oyster to persist in anoxic conditions, especially upon emersion. This has been termed habitat-dependent or environmental anaerobiosis (Storey and Storey, 1990). The goal of metabolism, whether aerobic or anaerobic, is to break down carbohydrates, lipids, and proteins to release and store energy and to

break down and assemble these building blocks to provide for the organism's growth and reproduction. Adenosine triphosphate (ATP), the cellular currency of energy, is required to power such processes including muscular activity, including contraction of the adductor muscle to shut the oyster shell.

When acting aerobically, oysters require oxygen to create sufficient ATP to drive cellular processes. Under conditions of normal oxygen levels, the aerobic pathway for carbohydrate breakdown begins with a six-carbon glucose molecule, which is split into two three-carbon molecules of pyruvate. This process, called glycolysis, occurs in the cytoplasm and requires two molecules of ATP, and creates four molecules of ATP and two molecules of NADH.

These three-carbon pyruvate molecules are shunted into the citric acid cycle, which takes place in the mitochondria. The citric acid cycle produces two carbon dioxide molecules, reduces three nicotinamide adenine dinucleotide (NAD^+) and one flavin adenine dinucleotide (FADH), which are electron acceptors, and produces one molecule of guanosine triphosphate (GTP), a cellular equivalent of ATP. The citric acid cycle is performed twice for each molecule of glucose.

From the citric acid cycle, the electron carriers NADH and $FADH_2$ are oxidized in the electron transport chain in the inner mitochondrial membrane. Oxygen acts as the terminal electron acceptor in the electron transport chain, where it is reduced to water. Oxidative phosphorylation produces ATP through the transfer of electrons in the electron transport chain by chemiosmosis. Protons from the inner mitochondria are shuttled into the intermembrance space, setting up a proton gradient between the membranes. Protons that have built up on in the intermembrane space

flow down the gradient, and this energy is harnessed to drive the action of an ATP synthase pump. For each molecule of NADH, 2.5 ATPs are created. $FADH_2$, with a lower energetic yield, will produce 1.5 molecules of ATP. The net energy yield for complete oxidation of glucose to CO_2 and H_2O is approximately 30 ATP.

When there are not enough oxygen molecules present to act as terminal electron acceptors in cells, the cells cannot create sufficient energy to maintain normal physiological function. Louis Pasteur first noted in yeast that an effort to produce energy in a usable form, ATP, in the absence of oxygen causes the cell to ramp up its rate of glycolysis. Two ATP are produced from each molecule of glucose by breaking it into two molecules of pyruvate, which are then converted into lactate by lactate dehydrogenase (LDH). This metabolic compensation process is called the Pasteur effect. This pathway produces only 2 ATP for each glucose molecule, and even performed at a high rate it is relatively inefficient compared to aerobic metabolism, which produces approximately 30 ATP per glucose. Furthermore, lactate acidifies the cell, and acidification changes the balance of ions in the cell. When the oxidation-reduction (redox) balance of the cell is disrupted, pH dependent enzymes lose activity, the redox potential of cellular processes is reversed, and apoptosis, or programmed cell death, may be induced. If this happens in enough cells of an aerobic organism, this results in massive cell death, tissue death, and eventually death of the organism.

Organisms whose tissues are deprived of oxygen undergo substrate-level phosphorylation. A high-energy phosphorylated intermediate such as phosphoenolpyruvate (PEP) or 1,3 bisphosphoglycerate reacts with ADP and creates

pyruvate or 3-phosphoglycerate and ATP. This direct transfer of a phosphate group is catalyzed by pyruvate kinase or phosphoglycerate kinase. Substrate-level phosphorylation occurs twice in oxidative cellular respiration, during glycolysis. However, this process is difficult to sustain in oxygen-deprived cells for a number of reasons. First, substrate-level phosphorylation produces only 2 ATP for each glucose molecule, while the electron transport chain can produce approximately 30 ATP per glucose, so it is relatively inefficient. Second, in order to continue to undergo glycolysis, the cell must have sufficient $NAD+$, so this molecule must be regenerated from NADH elsewhere in the cell. These molecules couple reactions within the cell. Once the cell has undergone glycolysis and produced ATP, pyruvate remains. If pyruvate is converted into lactate, an acidic endproduct builds up, disrupts the redoxx balance, and may destroy the cell by triggering apoptosis. Therefore, the organism must be able to produce non-toxic endproducts during anaerobic respiration.

Marine molluscs have four major mechanisms that allow them to surmount these biochemical obstacles. First, molluscs couple the breakdown of glycogen and aspartate and accumulate succinate and alanine as the end products of this pathway (Figure 1). This reaction produces $NAD+$, thereby allowing substrate-level phosphorylation to continue. Aspartate fermentation to succinate produces one mole of ATP for each mole of aspartate and three moles of ATP for each glucose created from glycogen (Collicutt and Hochachka, 1977). Aspartate is an immediate energy source for the bivalve cell after hypoxia begins (Collicutt and Hochachka, 1977; Ellington, 1981; Ebberink et al., 1979).

Second, alanine and succinate are less acidic than lactate, and therefore have a better buffering capacity, allowing the mollusc to store these end products without damage to the cell. The enzyme lactate dehydrogenase (LDH) reduces pyruvate into lactate through the oxidation of NADH. However, LDH has low activity within bivalve cells, which prevents the buildup of an acidic endproduct. Redox regulation in the cell is accomplished by malate dehydrogenase (MDH) and alanopine dehydrogenase (ADH).

Third, in addition to the fermentation of glycogen and aspartate, ATP may be produced through other pathways that synthesize organic acids. The fixation of CO_2 to phosphoenolpyruvate (PEP) to produce oxaloacetate (OAA) is catalyzed by PEP carboxykinase (PEPCK) and produces one molecule of GTP. Alanopine is created from alanine and pyruvate. The creation of 2 molecules of alanopine from glucose and two alanine molecules yields 3 ATP (Gnaiger, 1977).

Last, the mollusc may lower its metabolic rate during anoxic stress in order to conserve available fuel sources. Like most facultative anaerobes, the Eastern oyster does not undergo the Pasteur effect. In fact, in some bivalves, glycolysis actually slows after anoxic exposure (de Zwaan, 1983). Metabolic regulation may occur over short periods of time, such as a tidal cycle, intermediate periods of time, including seasonal changes, and long periods of time, the case of evolutionary adaptation (Russell and Storey, 1995; Greenway and Storey, 1999).

Over the past 100 years, overharvesting and two oyster diseases, MSX (*Haplosporidium nelsoni*) and Dermo (*Perkinsus marinus*), have decreased the native oyster population to 1% of its historical levels (Newell, 1988). The introduction of *C.*

ariakensis into the Chesapeake Bay has been proposed as a means of rehabilitating the ecosystem and commercial oyster fishery. The Asian oyster has been chosen because of its resistance to MSX and Dermo as well as its high growth rate (Paynter et al., 2006). Studies to date have shown that *C. ariakensis* has greater survival than the native oyster, even than native strains selected for resistance to MSX and Dermo. When cultured in South China seas, the oysters are harvested within two to three years for a 10 to 15 cm shell height (Guo et al., 1999). When triploid *C. ariakensis* were deployed in the Chesapeake Bay, they grew 2.6 mm/month in <15 psu, 4.9 mm/month in 15-25 psu, and 6.2 mm/month in >25 psu, on average (Calvo et al., 2001).

The Asian oyster is found along the coast of China and in Southern Japan in subtidal regions where water temperatures range from 14 to 31.8^{o}C and salinities from 7.5 to 30.2 psu (Cai et al., 1992). *C. ariakensis* has also been reported in Korea, Maylasia, and the Phillipines, and may extend into India and Pakistan. It has been cultured in China and Japan for 300 years in both intertidal and subtidal areas (Cai et al., 1992; Guo et al., 1999). Information on the range of the Suminoe oyster is confounded by taxonomic confusion about the species. On more than one occasion, this oyster has been mistaken for *Ostrea rivularis*, *C. rivularis*, *C. discoidea*, or *C. paulucciae*.

Understanding the hypoxic tolerance and physiological abilities of the native oyster is of increased importance since the proposed introduction of a nonnative Asian oyster, *Crassostrea ariakensis*. If the Asian oyster is introduced, it should be placed in areas of the Chesapeake Bay where it will have the best survival, providing

good filtration and growing to harvest size. Among the requirements for *C. ariakensis* to be a successful supplement to the native oyster, the Asian oyster must exhibit similar tolerances to fluctuations in temperature, salinity, and dissolved oxygen. The latter is especially important, as hypoxic and anoxic zones extend down the mainstem during summer months and may drop as low as 1 mg/L O_2 for three days (MacKiernan, 1987; Officer et al., 1984, Seliger and Boggs, 1988).

To fulfill the needs of the industry and ecosystem, *C. ariakensis* must be able to live in the Bay long enough to grow to market size, and it must occupy a similar functional role within the ecosystem. While *Crassostrea virginica's* ability to endure anoxic stress is well studied, little is known about the hypoxic tolerance of *Crassostrea ariakensis*.

Early reports of *C. ariakensis* stated that oysters delivered on ice to restaurants were gaping by their arrival, and chefs refused delivery, since they appeared dead. Another incident occurred in which four hundred oysters of each species were held in flow-through water systems at the Chesapeake Biological Laboratory in Solomons, MD. The water was accidentally turned off for three days, and when the researchers returned, the water was fouled and the dissolved oxygen level was less that 1.0 mg/L. Four days after flow to the tanks was restored, five of 376 native oysters (1.3%) died, while 79 of 388 *C. ariakensis* (19.5%) died. Forty-three days after the incident, 28 more *C. ariakensis* died, while only 4 more *C. virginica* died (Paynter, unpublished data).

This incident prompted the first experiment in this study to determine whether there was a significant difference between species in time to mortality during anoxia.

To replicate the conditions of the tanks, individual oysters were placed in jars, and the oxygen sparged from the containers with nitrogen gas. Oysters were held in these chambers until death occurred. The experiment was performed twice. Both times, *C. ariakensis* died significantly sooner than *C. virginica*. *C. ariakensis* also showed behavioral differences, gaping almost immediately upon reimmersion to water.

The next set of experiments was designed to determine the cause of the dissimilarity in mortality times of the two species. If the Asian oyster has a greater oxygen demand, it would explain its inability to survive in low dissolved oxygen environments. Therefore, I calculated the oxygen consumption rate, which is a measure of an organism's aerobic respiration. This rate is only a portion of the total metabolic rate, which also includes the anaerobic respiration (Hammen, 1983). I placed oysters in sealed containers of oxygenated water and monitored the dissolved oxygen level over eighteen hours. From the change in dissolved oxygen level I calculated the weight-specific oxygen consumption rate. This experiment was repeated at two temperatures and three salinities with three oysters in each treatment. There was no significant difference between the oxygen consumption rates of the two species at 10 and $20^{o}C$ and salinities of 5, 15, and 25.

If oxygen consumption demands did not differ between species, the metabolic response during hypoxia must be responsible for their differential mortality times. Most bivalve species survive hypoxia because of their ability to downregulate their metabolism in addition to using non-glycolysis ATP-producing pathways, but an inability to *C. ariakensis* to perform these processes could explain their death rates in anoxia. In order to investigate whether or not the oysters are fermenting glycogen

and aspartate, producing less acidic end products, and creating ATP in conjunction with organic acids, contents of the free amino acid pools in the gill tissue can be quantified.

Free amino acid pools are collections of amino acids available for metabolism and osmoregulation. These amino acids may be used to make proteins, produce energy, or regulate osmotic tolerance. Because some of these amino acids are intermediates and end products in energy producing pathways, they can be used to determine what pathways an organism uses. I measured the levels of free amino acids (FAA) present in the gill tissue of normoxic oysters of both species as well as the FAA levels in oysters exposed to hypoxia for 48 hours. I performed this experiment twice.

If glycogen and aspartate are broken down to produce energy, there should be lower amounts of aspartate after anoxic exposure, and high levels of alanine. If the Asian oyster's hypoxic tolerance is lower because it does not use these hypoxic pathways as readily, it may show more aspartate and less alanine after hypoxic exposure than a typical bivalve mollusc. In ribbed mussel (*Modiolus demissus*) gill tissue, glycine is important in osmoregulation, increasing with high salinities, and decreasing in low salinities (Ellis et al., 1985). Serine and glycine interconvert readily and act as intermediates in many metabolic pathways. At high salinites, serine can be converted to pyruvate and alanine (Ellis et al., 1985).

Results from our experiment showed that after hypoxic exposure, alanine increased in both species, though it increased 40% more in *C. virginica* than in *C. ariakensis*. Aspartate increased 70% more in the Asian oyster than in the native

oyster. Glycine decreased in *C. virginica* and increased in *C. ariakensis*. Its similarity to alanine means that alanopine dehydrogenase can use glycine as a substrate as well, which may explain the decrease in glycine levels. Overall, the differences in FAA levels within the gill tissues of these species give a possible explanation for the disparity in hypoxic tolerances.

In addition to providing an explanation for the difference in hypoxic tolerance, it may be significant that two closely related species use different metabolic pathways and might point to divergent evolution. Nicchitta and Ellington (1983) found differences in metabolic responses to air exposure in two species of mussel and speculated that this divergence were due to adaptation to microhabitats. It is possible that *C. ariakensis* and *C. virginica*, which live in dissimilar environments, have also evolved very different mechanisms for coping with anoxia.

These experiments indicate a disparity in the hypoxic tolerance of the two species as well as evidence of differences in the anaerobic pathways used by the species. Further evidence that a metabolic difference exists between the species is that the Asian oyster grows significantly faster than the native oyster (Paynter, unpublished data). Determining whether or not this disparity exists, and if so, the cause of the discrepancy, is crucial to the decision to seed nonnative oysters into the Chesapeake Bay. If *C. ariakensis* has a low hypoxic tolerance, it may not be a feasible for the native oyster in the Chesapeake Bay.

Chapter 2: Comparison of Mortality Times of *Crassostrea ariakensis* and *Crassostrea virginica* during Exposure to Anoxic Stress

Introduction

The decline of the native oyster, *Crassostrea virginica*, due to overharvesting, disease, and habitat degradation has led State agencies in Maryland and Virginia to propose to replace or augment the native population with the non-native Asian or Suminoe oyster, *Crassostrea ariakensis*. At the time of the proposal, the stated goal of these agencies was to rehabilitate the ecosystem and oyster industry of the Chesapeake Bay. In addition to providing a valuable fishery, oysters create reefs of hard substrate that become a habitat for diverse populations of fish and invertebrates (Rodney and Paynter, 2006). However, the success of the Asian oyster will depend on its tolerance to the many highly variable estuarine conditions, including hypoxic and anoxic events, in the Chesapeake Bay.

Prior to its precipitous decline in the 1950s, the Eastern oyster had a wide geographic range and was very successful in the intertidal zone of the North American Southeast. This success was largely based on its ability to survive emersion and rapid environmental changes including temperature and salinity fluctuations and periods of seasonal hypoxia. While it is widely known that the Eastern oyster is an excellent facultative anaerobe (Hochachka, 1980; de Zwaan, 1983), little is known about the response of the Asian oyster to low dissolved oxygen.

Hypoxia in the Chesapeake Bay appears to be a relatively recent phenomenon, with seasonal hypoxia occurring regularly in the past fifty years (Seliger and Boggs, 1988; Kemp et al., 2005). Both physical and biological processes are responsible for

the development of hypoxic conditions in the Chesapeake Bay (Taft et al. 1980,

Kemp et al. 1992). In the springtime, hypoxic events are controlled by freshwater

inflow to the Bay, which is responsible for stratification in the water column. The

later summer oxygen depletion is caused by eutrophication (Hagy et al. 2004). As

increased nitrogen and phosphorus inputs drive algal blooms, the consequent die-off

promotes bacterial use of dissolved oxygen, thereby lowering the oxygen level in the

water. Despite a good tolerance for hypoxia, long-term events may severely affect

oysters. Lenihan and colleagues documented an 18-day hypoxic event that killed all

oysters below 5 m in the Neuse River in North Carolina (1995).

Many bivalve species can survive long periods of hypoxia, some longer than

three weeks at $25^{\circ}C$ (Hochachka, 1980). Changes in the pallial cavity characterize

the oyster's transition to an anoxic state. Oxygen tension and pH decrease within the

shell and stay at these levels for the three weeks the oyster can stay alive.

Crassostrea virginica has been shown to survive anoxia from 3 days to more than 28

days of exposure, and anoxic and hypoxic tolerance were found to be temperature

dependent (Stickle et al., 1989). Other bivalve species show similar patterns of

decreasing tolerance to hypoxia as temperature increases. The brown mussel, *Perna*

perna (Linnaeus), can tolerate a PO_2 of 4 kPa (approximately 1.25 mg/L O_2) at $15^{\circ}C$,

but only 6 kPa at $20^{\circ}C$ (Hicks and McMahon, 2005). Survival and feeding of oyster

larvae is severely limited by anoxic stress (Widdows et al., 1989).

The massive *C. ariakensis* die-off at the Chesapeake Biological Laboratory

mentioned in the literature review prompted me to determine if the interspecies

difference in hypoxic tolerance could be replicated under laboratory settings. I chose

to test triploid and diploid oysters of both species since the introduction of both has been suggested. Triploid oysters have been shown to grow faster than diploids in many environments, indicating potential metabolic differences in ploidy (Ruiz-Verdugo, 2001). Scientists have proposed many theories to explain this growth difference: 1) sterility allows the organism to shunt more energy into its growth instead of reproduction (Stanley et al., 1984; Allen and Downing, 1986; Shpigel et al., 1992), 2) cells of triploid organisms are larger (Guo and Allen, 1994), 3) triploids may have greater numbers of alleles, inducing heterosis effects (Hedgecock et al., 1996), 4) more copies of genes allows for faster transcription (Magoulas et al., 2000), and 5) triploids tolerate stress better because they can shunt more energy into survival when stressed (Hawkins and Day, 1996). Heterosis is commonly known as "hybrid vigor" and is the theory that outbreeding that leads to heterozygous organisms will lead to higher fitness of the offspring. Despite these suggestions, some have questioned the performance of triploids under suboptimal conditions (Stanley et al., 1984).

Triploid molluscs have different amounts of energy sources than diploids, which could potentially affect their survival in hypoxic conditions. Triploid clams have significantly higher levels of carbohydrates (Akashige, 1990), and triploid bay scallops have higher glycogen contents within the tissues than diploids (Tabarini, 1984). Because glycogen is a crucial energy store during hypoxic conditions, triploids may be better equipped to deal with hypoxia than diploids.

In this experiment, oysters of both species were left in hypoxic conditions, simulated by sparging the water with nitrogen gas, and the time until mortality was

recorded. On average, triploid *C. ariakensis* died after 4 days of exposure to anoxic stress, while triploid *C. virginica* died after an average of 15 days. When the experiment was repeated with diploid oysters, *C. ariakensis* succumbed after an average of 13 days, and *C. virginica* persisted on average for 17 days.

Materials and Methods

In order to determine if there is a difference in the response of the two species to anoxic stress, we placed oysters in normoxic (6.5 mg/L O_2) and anoxic (<1.0 mg/L O_2) water at 20^oC and measured the number of days until mortality occurred.

Experiment 1: Triploids

In March 2005, triploid *Crassostrea ariakensis* and *Crassostrea virginica* were cultured at the Horn Point Laboratory in Cambridge, Maryland, and transported to College Park on ice. They were approximately 3 years old with shell heights ranging from 70 to 85mm. Prior to experimentation, the oysters remained for 1 week in artificial seawater (ASW) at 20^oC and 12.5 psu without food to allow them to clear their gut.

One-liter Mason jars containing a single oyster were filled with artificial seawater at 12.5 psu subsequently was sparged with nitrogen gas until the dissolved oxygen level was 0.5 mg/L. Dissolved oxygen was measured using an YSI 5000 bench probe. The jars were quickly sealed. Several test jars containing only seawater and were opened at 1, 3, 5, and 15 days to confirm the low dissolved oxygen level during the course of the experiment.

Mortality of the oysters was determined by observing gaping behaviors approximately every four hours. If the oyster gaped, the jar was shaken. Live oysters responded to the disturbance by closing their valves, while dead or nearly dead oysters did not. The unresponsive oysters were removed from their jars and placed in normoxic water.

Upon removal from the jar, the dissolved oxygen level was measured to confirm that the dissolved oxygen level remained below 0.5 mg/L. In normoxic water, if the oyster closed when disturbed, it was judged alive and removed from the experiment. If the oyster remained open, its time to mortality was recorded. Wet and dry tissue weights and shell heights were recorded. Dry weight was measured by placing oyster tissue in a drying oven at 60^oC for 72 hours. The time to mortality was compared between species, and results were analyzed using analysis of variance (ANOVA). To ensure that oyster weight did not affect response to hypoxia, dry weights were recorded and tested by ANOVA to ensure that the mean dry weights did not differ significantly from 1 g. Oyster weights ranged from 0.9-1.1 g, making it unnecessary to use weight as a covariate in the statistical analysis.

Experiment 2: Diploids

The experimental design was a 2x2 factorial, with species and oxygenation as the two treatments. Twenty oysters of each species were randomly assigned to two treatments, normoxic or hypoxic. There were ten replicates for each treatment. In early January 2007, I collected diploid *C. ariakensis* from the Horn Point oyster hatchery. On January 30, divers harvested diploid *C. virginica* from the Glebe Bay managed reserve area of the Chester River. The temperature and salinity at the time

of collection were 1.7^{o}C and 7.7. Oysters were stored for 20 hours in a refrigerator at 4°C and were then transported to the Horn Point Laboratory in Cambridge, MD. The oysters were placed in 8°C artificial seawater (ASW) at a salinity of 7.7. At the conclusion of the experiment, *P. marinus* status of the native oysters was analyzed, and three oysters had very light infections, one had a light infection and the other six had no infection.

C. ariakensis were moved from conditioning tables in the quarantine room to the spawning table. The *C. ariakensis* had been acclimated two weeks before to 8°C at a salinity of 9.0. The *C. ariakensis* diploids were hatchery oysters of the same strain used in the second experiment.

The oysters were acclimated to 20°C on the spawning table in the quarantine room of the HPL oyster hatchery. We placed the oysters in containers of ASW made up to the correct salinity; these containers were placed in a flow-through water bath that surrounded the chambers but did not mix with the water inside. Oysters were acclimated to 20°C by increasing the temperature 2°C every day, with a two-day pause at 14°C. Oysters sat at 20°C for five days before we began the experiment. In this experiment, the diploids were not held at warm temperatures for long periods of time before the experiment was performed in order to limit their gonadal development.

Oysters were placed in containers filled with ASW at 20°C and a salinity of 7.7 for the *C. virginica* and 9.0 for *C. ariakensis*. There was one oyster per container. Containers were plastic Ziploc brand. I tested the containers for permeability by filling them with ASW that had been sparged with nitrogen to lower the DO level to

less than 0.5 mg/L. At the end of two weeks there was no significant change from the starting DO.

Oysters in the normoxic treatments were placed in open plastic tubs with an airstone. Hypoxic treatment oysters were held in Ziploc containers filled with ASW and sparged with nitrogen to lower the DO level to 0.5 to 0.75 mg/L. I placed the one oyster each in a container filled with water, and sparged the water with nitrogen gas, until the dissolved oxygen (DO) level reached 0.5-0.75 mg/L. DO level in the normoxic treatments ranged from 6.26 to 7.30 mg/L.

Oysters were then randomly placed on the spawning table to eliminate the effects of a possible temperature gradient caused by the output of water in one corner of the table. Every 24 hours oysters were observed for gaping behavior. If gaping was observed, I disturbed the oysters by shaking the container. To control for the effects of disturbance on the oysters, if I disturbed one oyster to test for mortality, I disturbed all oysters. If the oyster closed, it was placed back on the spawning table and considered alive. If the oyster did not close, it was left for fifteen minutes and disturbed again. If it did not close after being disturbed three times, the oyster was removed from the jar and placed in normoxic water for at least four hours. If the oyster closed after being disturbed in normoxic water, it was considered alive. If the oyster continued to gape after being disturbed during the normoxic exposure, it was dead and length, wet and dry weight were recorded. At the conclusion of the experiment, I recorded the DO level of the water in the container.

Data from the first experiment were analyzed with JMP using a one-way ANOVA with species as the explanatory factor and time to mortality as the response

variable (2003). Data from the second experiment were analyzed as a two-way

ANOVA using species and oxygenation as the two explanatory factors and time to

mortality as the response variable. The level of significance was set at 0.05.

Results

In the first experiment, mortality of triploid *C. ariakensis* occurred at an

average of 4 days, while *C. virginica* mortality occurred at an average of 15 days

(n=33, F=130.34, p<0.0001, Fig. 1). When the experiment was performed with

diploid oysters, the average number of days to mortality for *C. ariakensis* was 13

days, and the average number of days to mortality for *C. virginica* was 17 days (Table

2). No significant species by treatment interaction exists (F=0.2654, p=0.6096,

n=40). Treatment had a significant effect on time to mortality, with normoxic-treated

oysters persisting longer than hypoxic-treated oysters (F=7.0202, p=0.0019, n=40).

There is a significant species effect, with *C. virginica* living longer than *C. ariakensis*

(F=10.9690, p=0.0012, n=40).

When diploid and triploid oysters of both species were compared, a significant

interaction between ploidy and species was found (n=53, F=16.9724, p<0.0001, Table

2). In both experiments, differences in gaping behavior were evident. *C. ariakensis*

began gaping during the sparging process and continued this behavior throughout the

experiment, although they would close if disturbed. The non-native oyster gaped 5-

20 mm, while the native oyster only gaped more than 5 mm if it was dead. The native

oyster remained closed until 1-2 days before its death, at which point it would gape 2-

3 mm. Once *C. virginica* began to gape response times to disturbance were extremely

slow and labored. Despite wide gaping during most of the experiment, *C. ariakensis*

was very responsive to disturbance; they would close quickly after being shaken. After closing due to disturbance, however, they opened again in fifteen to thirty minutes and would remain open until the next disturbance. These differences indicate important physiological differences between these two species.

Discussion

I exposed native and non-native oysters to normoxic and anoxic conditions in order to determine if there is a difference in the behavioral and physiological response under anoxia. *C. ariakensis* mortality time was significantly shorter than and *C. virginica* mortality time during anoxic stress exposure at 20°C for both triploid and diploid oysters. Diploid *C. ariakensis* and *C. virginica* lived longer than their triploid cohorts in anoxic stress environments, indicating that triploids may not survive environmental insults as well as diploids, despite having larger glycogen stores and different allocations of energy (Tabarini, 1984; Akashige, 1990). Because the experiments on diploids and triploids were performed two years apart, the differences could have occurred because of the time. In the future, a study should compare triploids and diploids side by side.

Hochachka found a three-week mortality time for diploid *C. virginica* at 25°C in anoxic environments (1980). Another study showed that diploid *C. virginica* withstood four days of anoxia at 20°C (Eberlee et al., 1983). Diploid *C. virginica* in this experiment persisted for only fifteen days at 20°C. Length of acclimation period, season, and feeding regime could explain why oysters in this study lived one week shorter than in the previous study. Oysters in the study by Hochachka may have had

greater energy stores than the oysters used in this experiment, allowing them to survive longer in anoxia.

Differences in mortality time for juvenile (15 months since spat set) diploid *C. ariakensis* and *C. virginica* were reported by the Maryland Department of Natural resources (Matsche and Barker, 2006). Median mortality time for *C. ariakensis* was 3 days, compared to 6 days for *C. virginica* in 0% oxygen saturation. In treatments with declining oxygen saturation (0.75 to 0.45 mg/l O_2 and 1.5 to 1.0 mg/l O_2), median mortality times were for 6 day s and 7 days for *C. ariakensis*, lower than *C. virginica*'s 8 days and 11 days in the same treatments (Matsche and Barker, 2006). In this study, juvenile *C. ariakensis* had the same limited ability to survive anoxic stress as the older oysters. In both species, the older oysters lived longer than the juvenile oysters in this experiment. Perhaps greater energy stores in the larger oysters may facilitate their survival.

This study tells us about one of the tolerances of the non-native oyster so that we can determine where *C. ariakensis* are most likely to survive. We can therefore determine the areas of the Chesapeake Bay that are best suited for survival.

Definite behavioral differences exist between *C. ariakensis* and *C. virginica*. Gaping occurred in *C. ariakensis* almost immediately upon reimmersion in water, while *C. virginica* would remain closed until death occurred. *C. ariakensis* would gape a few millimeters three to five days before their death, but would still manage to close their shell after a disturbance. They would open again in approximately an hour. *C. virginica* rarely opened prior to their death, and were thus unable to close when disturbed. The method and location of energy production for remaining shut in

anoxic conditions are different from those used in a short term action like closing the shell. In the next chapter, these behavioral differences are quantified by measuring the time to gape after reimmersion for both species.

Chapter 3: Oxygen Consumption Rates of *Crassostrea ariakensis* and *Crassostrea virginica* at Two Temperatures and Three Salinities

Introduction

Results from the anoxic stress experiments indicate that there may be a physiological difference between *C. ariakensis* and *C. virginica*. One possible cause of this disparity is a difference in the basal metabolic rates of these two species. A higher metabolic rate could explain a greater demand for oxygen, and therefore a higher mortality rate at low dissolved oxygen levels. Furthermore, a higher metabolic rate indicates that more fuel must be consumed to meet the energetic requirements of the organism. If more energy is necessary to one species, and both have limited but equal energy stores, the species that requires more energy will die first. While metabolic rates of *C. virginica* have been studied and reported previously; no such data exist for *C. ariakensis*.

The metabolic rate is equal to both the aerobic and anaerobic respiration of the organism. I measured the oxygen consumption rate by measuring the change in dissolved oxygen in a sealed jar containing an oyster. The term oxygen consumption rate refers to the aerobic part of the oyster's metabolic rate. This experiment does not measure the anaerobic respiration rate. Since the oyster is in oxygen-saturated water, the anaerobic portion of metabolism should be minimal to none.

By measuring the oxygen consumption rate of both species, I can estimate the metabolic demand of both species and determine if one is higher than the other. If one species has a higher metabolic demand, then it may have a limited ability to survive anoxia. Oxygen acts as the terminal electron acceptor in the electron

transport chain, where the creation of a proton gradient drives the action of ATP synthase. The electron transport chain can generate approximately 26 ATP, compared to only 4 ATP gained from glycolysis and citric acid cycle reactions of the cell. Therefore, oxygen is crucial in generating enough energy for the cell.

Oxygen consumption rates of *C. virginica* have been reported as 0.233 ml O_2 g^{-1} h^{-1} at 10^oC and 14 psu and 0.461 ml O_2 g^{-1} h^{-1} at 20^oC and 14 psu (Shumway and Koehn, 1982). At 7 psu, oxygen consumption rates were 0.342 and 0.6155 233 ml O_2 g^{-1} h^{-1} at 10^oC and 20^oC, respectively, while at 28 psu, the rates were 0.132 and 0.241 ml O_2 g^{-1} h^{-1}at 10^oC and 20^oC (Shumway and Koehn, 1982). Prior to this experiment, oxygen consumption rates for *C. ariakensis* have not been studied.

Materials and Methods

Experiment 1: Triploid Oxygen Consumption Rate

I performed a preliminary experiment measuring the oxygen consumption rates of 6 triploid *C. ariakensis* and 4 triploid *C. virginica*. Triploid oysters were removed from reef trays in the Severn River during April 2005. These oysters came from the Virginia Institute of Marine Science and were reared at the Horn Point oyster hatchery until they were deployed in reef bags in the Severn River. Both species were three years old and were maintained in the same tank. Oysters were maintained in artificial seawater at 20^oC and 12.5 psu with diet supplementation with 1.5 ml of Reed Mariculture Shellfish Diet 1800 ® per animal until 1 week before the experiment, when they were placed in artificial seawater without food. I monitored feces and pseudofeces production to ensure that their gut was clear prior to

experimentation. Prior to the experiments, oysters were scrubbed clean and soaked in freshwater to remove any epiphytic organisms that might consume oxygen.

Environmental control chambers ensured a constant temperature throughout the experiment. I sealed each oyster in oxygen saturated artificial seawater in an airtight container, each with a hole in the top for the probe from a YSI 5000 benchtop model dissolved oxygen meter, were sealed. A stir ring magnet circulated seawater around the probe. The probe measured dissolved oxygen every second and logged the information in a computer. Oysters remained in the jar for 18 hours, or until all the dissolved oxygen was consumed. Gaping behaviors were observed and recorded throughout the experiment.

Oxygen consumed was calculated by inspection of the dissolved oxygen profile. Because the oyster remained shut for a short time prior to the experiment, the oyster accumulated a small oxygen debt, resulting in a sharp intake of oxygen after the oyster reopened inside the sealed container. This steep incline at the beginning of the oxygen consumption profile was not used in the oxygen consumption rate estimation. After this initial drop in dissolved oxygen, the oxygen consumption became relatively constant. I considered the initial dissolved oxygen concentration the start of this curve. The consumption of oxygen was measured for 45 minutes. If the initial dissolved oxygen concentration occurred below 3.0 mg/L of oxygen, we considered the oxygen consumption a "hypoxic rate."

After the experiment, the shell height, whole weight, wet tissue weight, and dry tissue weight were measured. The volume of water in the container was carefully

measured. The weight specific oxygen consumption rates were calculated using the following formula:

$$Oxygen\ consumed/(Time*Weight^b)$$

B is the "weight exponent," calculated by plotting the log of the oyster weights against the log of the oxygen consumptions and finding the slope of the line (as described by Widdows, 1985). No animals died during the measurement of oxygen consumption rate.

Experiment 2: Diploid Oxygen Consumption Rate

Diploid *C. ariakensis* and *C. virginica* oxygen consumption rates were measured at two temperatures and three salinities. Salinity, treatment, and species were the explanatory factors in a three by two by two factorial design. The temperatures (10 and 20°C) and salinities (5, 15, 25 psu) were fully crossed for a total of six treatments. Three replicates of each species were run for each treatment. Both species were maintained in the same tank in the same temperature and salinity regime.

As in other experiments, the oysters were cultured at the Horn Point Laboratory and were approximately three years of age, both species were approximately 1.0 g dry tissue weight. Oysters were randomly assigned to treatments and acclimated to the appropriate temperature and/or salinity over 1 to 2 weeks. The oysters were maintained in these conditions and fed for another three weeks, with water changes every four days. Prior to experimentation, the oysters were moved to artificial seawater without food for 1 week. Again, feces and pseudofeces production was monitored so that a basal oxygen consumption rate would be measured.

Oysters were randomly assigned to one of four oxygen consumption assemblies (as described above) containing oxygenated artificial seawater at the appropriate temperature and salinity. The container was sealed with the probe in the lid, and oxygen consumption was measured for 18 hours or until the oxygen was consumed completely. Each oyster's shell height, whole weight, wet weight, and dry weight were measured, and the volume of water in each container was measured and recorded. As in the first experiment, no animals died during the measurement of oxygen consumption rate.

The Q_{10} was calculated as the quotient of the weight-specific oxygen consumptions at $10^{o}C$ and $20^{o}C$:

$$Q_{10}=[O_2 \text{ Consumption at } 20^{o}C/O_2 \text{ Consumption at } 10^{o}C]$$

across all salinities.

Weight-specific oxygen consumption rates were calculated as described above. A multi-way ANOVA with 3 explanatory factors: species, temperature, and salinity, and oxygen consumption rate as the response variable was performed using JMP (2003). The time to gape after reimmersion was compared between species across temperatures and salinities using a one-way ANOVA with species as the explanatory variable. The level of significance was set at 0.05.

Results

Grouped across temperature and salinity treatments, average time to gape was 12 minutes for *C. ariakensis* and 1 hour and 53 minutes for *C. virginica* (ANOVA, $F=14.56$, $p=0.0005$, $n=36$). *C. ariakensis* gaped almost immediately upon immersion in the water, while *C. virginica* remained shut for a significantly longer period of time

before opening and beginning to consume oxygen. If *C. ariakensis* consumed all the oxygen in the container, they would remain open, while C. virginica would shut their valves if the DO level was 1.0 mg/L or less. Because the containers were aerated, this did not occur often enough to perform a statistical analysis of this behavior.

The mean oxygen consumption rates for each species, ploidy and treatment are presented in Table 2, with standard deviation in parentheses. The mean oxygen consumption rate at 12.5 psu and 20°C of triploid *C. ariakensis* was 0.29 mg O_2 hr^{-1} gdw $^{-1}$, and 0.46 mg O_2 hr $^{-1}$ gdw $^{-1}$ for triploid *C. virginica*. The oxygen consumption rate of triploid *C. virginica* was significantly higher than that of triploid *C. ariakensis* (F=14.6, p=0.0087, n=8).

Among diploid oysters, there was no significant interaction among temperature, salinity, and species (F=1.56, p=0.2299, n=36). There were no significant interactions between temperature and species or salinity and species (F=0.058, p=0.812, n=36; F=1.856, p= 0.178, n=36), so the species effect can be examined alone. There was no significant species effect (F=0.0011, p=0.974, n=36). The temperature by salinity interaction was significant (F=7.012, p=0.004, n=36), so these factors were not examined independently. For diploid *C. ariakensis* the Q_{10} value is is 2.37, and for diploid *C. virginica* the Q_{10} value was 2.71.

Discussion

I measured oxygen consumption rates for diploid *C. ariakensis* and *C. virginica* at two temperatures and three salinities in order to determine if there is a difference in the oxygen demand between species that may be causing differential mortality in hypoxia. The average time to gape after reimmersion confirms the

observation made in the anoxic stress experiments that the Asian oyster reopens quickly after being placed in water. The oxygen consumption profiles for the Asian oyster showed that they opened quickly and began consuming oxygen at a stable rate. The native oyster, however, remained closed for much longer and upon reopening consume oxygen at a stable rate. Previous studies showed a period of rapid oxygen consumption upon reopening after valve closure (de Zwaan, 1983; Nichitta and Ellington, 1983). However, this increased post-closure consumption was observed only in three *C. virginica*, most likely because neither species remained closed long enough to acquire a significant oxygen debt.

C. ariakensis remains closed for an average of 11 minutes after reimmersion, significantly less than *C. virginica*, and none of the Asian oysters showed a period of rapid oxygen consumption after reopening. Whether *C. ariakensis* opens because it cannot survive in the absence of oxygen, or whether this is a behavior independent of its oxygen demands is uncertain.

At $25^{o}C$ and a salinity of 25, the oxygen consumption rate of the Eastern oyster was reported to be 9.1 mmol O_2 g ww^{-1} hr^{-1} (Willson and Burnett, 2000), compared to this study's rate of 4.4 O_2 mmol g ww^{-1} hr^{-1} at the same salinity and $20^{o}C$. Results from Shumway and Koehn (1982) give the oxygen consumption rate of *C. virginica* at $10^{o}C$ and 14 psu as 0.233 ml O_2 g^{-1} h^{-1} and 0.461 ml O_2 g^{-1} h^{-1} at $20^{o}C$. The rates we calculated for *C. virginica* are comparable, 0.25 ml O_2 g dw^{-1} h^{-1} and 0.51 ml O_2 g dw^{-1} h^{-1} for $10^{o}C$ and $20^{o}C$ at 15 psu (Table 2).

The Q_{10} value for *C. virginica* at 14 psu for $10^{o}C$ to $20^{o}C$ is 2.05 (Shumway and Koehn, 1982), much lower than the value calculated here, 2.71. This study was

performed in the late winter, and the oysters had been living in water less than 10°C. The rapid acclimation to 20°C during this experiment could have caused a greater increase in oxygen consumption, resulting in a higher Q_{10} value.

With higher oxygen consumption rates, *C. virginica* would be expected to have a shorter time to mortality than *C. ariakensis* under hypoxic conditions. However, this method measures the normoxic oxygen consumption rate, and a difference in the response of the two species to hypoxia could still explain *C. ariakensis'* lower mortality. *C. virginica* triploids may have a higher energy demand than *C. ariakensis*, but may be able to lower or meet this demand during hypoxia by downregulating their metabolism and using alternate energy-producing pathways. This experiment was performed with only 8 oysters and should be repeated with at least 10 triploid oysters of each species to confirm a difference in the two species' oxygen consumption rates.

Given these results, faster mortality in *C. ariakensis* during hypoxia is not due to a higher oxygen consumption demand. If oxygen consumption rates do not explain the differences in mortality, the response of the two species *during* hypoxia may be the reason for this difference. While these results have shown that no significant difference is present in normoxic oxygen consumption rates of the two species, it does not explain how the two species' will respond in hypoxic conditions. The major mechanisms responsible for the survival of *C. virginica* in low dissolved oxygen are the downregulation of metabolism, the switch in pathways from the glycolysis-Krebs Cycle-electron transport chain to a fermentative metabolism, fixation of CO_2, and creation of non-toxic metabolic endproducts and byproducts (Collicutt and

Hochachka, 1977; Hochachka, 1980). Whether these processes are achieved in *C. ariakensis* is the subject of Chapter 4.

A crucial question remaining is how the hypoxic oxygen consumption rate differs between species. The bivalve *Mytilus edulis* decreases its ATP usage by five times during air emersion (de Zwaan and Wijsman, 1979; Ebberink et al., 1979). If *C. virginica* is capable of a similar change during hypoxia, and *C. ariakensis* is not, this could explain their poor survival during anoxia. Furthermore, is *C. ariakensis* able to undergo fermentative metabolism or fix CO_2? If they are capable of these processes, are they able to perform them to the same level as *C. virginica*? In order to investigate the metabolic pathways used by each species in hypoxic conditions, I examined the free amino acid pools of both species before and after exposure to hypoxia.

Chapter 4: Analysis of Free Amino Acid Pools in the Gill Tissue of *Crassostrea ariakensis* and *Crassostrea virginica* under Normoxic and Anoxic conditions

Introduction

Thus far, I have shown that *C. ariakensis* and *C. virginica* show significant differences in survival time during anoxia, with *C. virginica* persisting for substantially longer than its nonnative counterpart. In Chapter 3, I demonstrated that normoxic oxygen consumption rates were not significantly different between the species. Instead, the differential mortality of these two species may be explained by variation in their responses to hypoxia. The ability of bivalve molluscs to survive low dissolved oxygen derives from their ability to downregulate metabolism, undergo fermentation of glycogen and aspartate to produce ATP, create nontoxic endproducts, and generate ATP by synthesizing organic acids (Collicutt and Hochachka, 1977; Fields, 1976; Hochachka, 1980).

In well-oxygenated water, *C. virginica* metabolism is identical to human metabolism: glucose is broken down into two three-carbon molecules, and these pass through the citric acid cycle, generating ATP (the universal energy form) and electron carriers which proceed through the electron transport chain, creating a proton gradient that drives ATP synthase. The final electron acceptor is O_2, and its presence is required to generate approximately 30 ATP per glucose molecule.

When the oyster encounters anoxic stress, both long- and short-term biochemical changes occur in the oyster tissue (Hochachka, 1980; Bayne, 1973; Greenway and Storey, 1999). Marine animals have large free amino acid pools within their cells to regulate the osmotic pressure of the cell by preventing the efflux

of water from the cell by diffusion. During oxygen depletion, the degradation of these amino acids coupled with carbohydrates creates an immediate energy source (Hochachka, 1980; deZwann, 1983; Storey and Storey, 1990). *C. virginica* uses a coupled reaction in which aspartate and glycogen are broken down to alanine and succinate (Storey, 1993; Eberlee et al., 1983). Aspartate fermentation can begin immediately after oxygen depletion begins; there is no lag time (Collicutt and Hochachka, 1977; Ellington, 1981; Ebberink et al., 1979). In the anaerobic pathway, the carbon skeleton of glycogen becomes alanine, while the aspartate skeleton becomes succinate by reversing parts of the citric acid cycle. Other amino acids accumulate as byproducts or endproducts of metabolism, and therefore, levels of free amino acids can be used to determine the pathways utilized by oysters in various metabolic states. As gill tissue is the site of oxygen diffusion, it experiences the first change in free amino acid level and has been used in similar previous studies (Eberlee et al., 1983).

Many enzymes regulate the metabolic pathways in a cell. Though it is important to mention the key active enzymes, the *low* activity of a particular enzyme, lactate dehydrogenase (LDH), allows the bivalve cell to persist for long periods of time in hypoxic conditions. LDH converts pyruvate formed during glycolysis into lactate, whose high K_a is toxic to the cell. Because it is not very active in the oyster cell, pyruvate can become alanine, acetate, or alanopine. Alanopine is created from pyruvate and alanine, catalyzed by alanopine dehydrogenase (ADH) (Fields, 1976; Eberlee et al., 1983). ADH is a small enzyme with almost absolute specificity for pyruvate as a keto acid substrate. Its efficiency for amino acids is 100% on alanine,

and 98% on glycine, so either is commonly used as a substrate (Fields, cited in Hochachka, 1980). Alanine aminotransferase adds an amino group to pyruvate, turning it into alanine; this reaction is coupled with the transamination of glutamate and α-ketoglutarate. In a different reaction, pyruvate can go through two steps to become acetate. Other researchers have measured enzyme activity under various conditions. Rather than measuring the activity of certain enzymes, we are analyzing free amino acid pools before and after exposure to anoxic stress.

The null hypothesis to be tested is: the change in *C. ariakensis* and *C. virginica* free amino acid pools from normoxia to hypoxia is not significantly different. The alternative hypothesis states that the change in free amino acid pools of the two species from a normoxic to a hypoxic state is significantly different. To test this, oysters of both species were exposed to normoxic and hypoxic conditions and the levels of free amino acids present in the gill tissues were measured and compared. This form of metabolic analysis can also be used to indirectly estimate metabolic rates (de Zwaan and Wijsman, 1976; Ebberink et al. 1979). Since we know the ATP equivalents for each end product, based on the amounts of these end products, we can determine ATP production.

Two experiments were performed. In the first, oysters under anoxic treatment were clamped shut for 48 hours and their gill tissue removed. In the second, anoxic-treated oysters were held in water with a dissolved oxygen level of 0.75 mg/L for 48 hours. The first anoxic treatment simulates emersion more than exposure to low dissolved oxygen levels in the water. The second experiment simulates summer conditions in hypoxic areas of the Chesapeake Bay.

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Based on similar studies performed in other bivalves, if alternate energy pathways are used, alanine levels should increase during anoxia with a concomitant drop in aspartate (de Zwaan et al. 1983; Nicchitta and Ellington, 1983). Glycine and alanine may also decrease during hypoxia, however, if alanopine dehydrogenase catalyzes the reaction of alanine and pyruvate to create alanopine and the joining of glycine and pyruvate to form strombine (Fields, 1976). Alanine and glycine are also glucogenic, and can be converted into pyruvate. Glutamate acts as the transaminating shuttle in the aspartate-glycogen pathway, and its levels should be higher in hypoxic cells because of its utility.

In the first experiment, after 2 days of hypoxia, alanine levels were elevated in both species, but were significantly higher in *C. virginica*. Glycine levels dropped in *C. virginica*, but increased in *C. ariakensis*. Aspartate levels were higher both species after anoxic exposure, but were much higher in *C. ariakensis*. Elevated anoxic alanine levels in both species point to coupled fermentation of glycogen and aspartate. That alanine levels are lower and aspartate levels are higher in hypoxic *C. ariakensis* than in hypoxic *C. virginica* indicates that less energy is being created from the non-oxygen dependent metabolic pathways.

Materials and Methods

Experiment 1:

Diploid oysters were taken from the Horn Point Hatchery in Cambridge, MD. Oysters used in the first experiment were collected on July 17th, 2006. The oysters ranged in size from 75-89 mm, with the average length of *C. ariakensis* at 82.6 mm, and the average length of *C. virginica* at 82 mm. The temperature on the collection

day was 20.2°C, and the salinity was 9.4 psu. In both experiments, oysters were maintained at the salinity at which they were collected in order to minimize changes in the FAA pools due to osmoregulation.

C. virginica were harvested from three areas in the Choptank River and held in the oyster hatchery for at least two years. *C. ariakensis* were spawned at the Virginia Institute of Marine Science in 2003 and have been held in the hatchery since 2004. All oysters used in this study were three years old. The hatchery uses a flow-through water system that pulls water from the Choptank River. Temperature and salinity conditions are identical to those found in the Choptank River. Until this experiment, *C. virginica* were held in one tank and *C. ariakensis* in another tank, but the tanks were fed from the same water source.

In the first experiment, oysters for the normoxic treatment were pulled directly out of tanks at the Horn Point Laboratory, shucked, and the gill tissue frozen on dry ice immediately. The remainder of the tissue was weighed, dried for 72 hours at 60°C, and weighed again. Hypoxic-treated oysters were clamped shut and placed on a lab bench for 48 hours at 20°C. After exposure, the oysters were shucked, and the gill tissue removed and placed on dry ice. The rest of the oyster meat was weighed as above. None of the oysters in the hypoxic or normoxic treatments died during the 48 h exposure. FAA levels in the gill tissue were analyzed with the Ez:Faast Kit (Phenomenex), as described below.

Experiment 2:

Diploid oysters used in this experiment were from the same tanks in the Horn Point Laboratory as the oysters in experiment 1 in November, 2007. Oysters of both

species were removed from the tanks and placed one each in a 1 L container filled with artificial seawater at a salinity of 12.5. These dishes were then placed in a water bath at 20°C. Half of the dishes received aeration via an airstone. The other half were held in water that had been sparged with nitrogen gas to bring the dissolved oxygen level down to 1.0 mg/L. These oysters were then sealed inside the container. The oysters were left for 48 hours under these conditions. The experimental conditions were similar to the anoxic stress experiment, except that the oysters were removed and shucked after 48 hours.

After 48 hours, oysters were removed from these conditions, shucked, and a 1.0 g piece of gill tissue was removed. As in the first experiment, none of the oysters died during the treatment period. The tissue was frozen immediately on dry ice to stop metabolic processes that alter levels of free amino acids. The remainder of the tissue was weighed and dried for 72 hrs at 60°C.

A 0.1 g sample of gill tissue was homogenized with 0.2 ml sodium citrate buffer (pH 3.4). Twenty-five ml of homogenate was analyzed using the methods described in the Phenomenex Ez:Faast Total Hydrolysate Amino Acid Analysis Kit. The purified amino acids produced by the kit were run on a Beckman-Coulter GC-MS and compounds were identified based on their mass and retention time using a Phenomenex data library compatible with the Ez:Faast amino acid kit. Levels of amino acids were determined by integration of the corresponding GC peak divided by the dry weight of the gill tissue homogenate. Amino acid levels are measured in mmol/g dry weight of gill tissue. I measured levels of glycine, alanine, glutamate, and aspartate. β-alanine, serine, glutamine, and asparagines were also detected by

this method but were not present in large enough amounts or were not considered important to the metabolism and so were not included in the analysis. This method did not detect succinate and oxaloacetate.

Results were analyzed with JMP using a two-way MANOVA with species and oxygenation as the explanatory factors in a two by two factorial design (2003). The α-level was set at 0.05.

Results

Amino acid levels are reported in Table 3, and differences between anoxic and normoxic amino acid levels are listed in Table 4. There was an overall species effect on the concentrations of amino acids (MANOVA, Wilks' Lambda=0.358, F=3.37, p=0.0204, n=25). There was also an overall oxygenation effect on amino acid levels (MANOVA, Wilks' Lambda=0.28388628, F=4.73, p=0.0047, n=25). Hypoxic levels of alanine were higher than normoxic levels of alanine in the gill tissues of both species, but they increased more during hypoxia in *C. virginica* than *C. ariakensis*. *C. ariakensis* aspartate levels were higher than *C. virginica* levels during normoxia and increase more than *C. virginica* during hypoxia. Hypoxic levels of glutamate were significantly higher than normoxic levels. Levels of glutamate in *C. ariakensis* were two times as high as glutamate levels in *C. virginica*.

In experiment 2, there was an overall species effect on amino acid levels in the gill tissue (MANOVA, Wilks' Lambda=0.1711, F=18.16, p <0.0001, n=40). Oxygenation also had a significant effect on FAA pools (MANOVA, Wilks' Lambda=0.3279, F=7.69, p <0.0001, n=40). Alanine levels increased in both species, but the increase was almost 18 times higher in *C. ariakensis* than in *C. virginica*

(Table 6). In this experiment, aspartate decreased in both species, but showed a greater decrease in *C. virginica* than in *C. ariakensis*. Glutamate increased by 1.6 μmol/gdw in anoxic treated *C. ariakensis* and decreased slightly in *C. virginica*.

Discussion

I held *C. ariakensis* and *C. virginica* in normoxic and anoxic conditions by clamping oysters shut in experiment 1 and by holding oysters in hypoxic water in experiment 2 and then analyzed their free amino acid levels. Changes in free amino acid pools after anoxic exposure are an indirect way of looking at the metabolic pathways used during environmental anaerobiosis. Some studies have indicated that the bivalve response to emersion and its response to hypoxic stress while immersed are different. Air-exposed oysters are thought to metabolize aerobically when emersed, and although these oysters were clamped shut, oxygen uptake from air by *C. virginica* has not been demonstrated (Dwyer and Burnett, 1996). The mechanisms by which oysters can continue to create energy aerobically during emersion are not understood (Willson and Burnett, 2000). Because the oyster is thought to metabolize aerobically, the oyster acidifies as CO_2 levels increase.

Other studies show that during aerial exposure, bivalves use the same mechanisms as those submerged in hypoxic water. The effects of hypoxia and aerial exposure on the blue mussel, *Mytilus edulis*, and oyster free amino acid pools have been studied (Widdows et al., 1979; de Zwaan et al., 1983). Alanine levels in the blue mussel doubled over 24 hours of aerial exposure with a concomitant halving of aspartate levels, while glutamate increased by 1 μmol/g wet weight. Succinate levels

increased approximately 4 mmol/gww (de Zwaan et al., 1983). These results indicate that the exposed mussel is fermenting glycogen and aspartate.

In both the emersion and hypoxia experiments, greater levels of alanine in the low dissolved oxygen treatment for both species suggest that pyruvate was converted to alanine by the co-fermentation of glycogen and aspartate, which indicates the presence of this anaerobic pathway in both species. However, while the pathway does seem to be present in *C. ariakensis*, the higher aspartate levels in this species reveals that this pathway may not be used as much as in *C. virginica*, and may therefore explain why they cannot support anaerobic metabolism as long as the native oyster. In the second experiment, aspartate levels decreased in both species, but decreased more markedly in *C. virginica*.

Using the stoichiometric equation for aspartate and glucose fermentation, for each 2 moles of alanine produced, 4.71 moles of ATP are produced (Gnaiger, 1977). Using this conversion with the amount of alanine produced in the first experiment, 40.5 μmol ATP/gdw were produced in *C. ariakensis* gill tissue, and 67.8 were produced by *C. virginica* during hypoxic exposure. In the second experiment, *C. ariakensis* produced 49.9 μmol ATP/gdw, and *C. virginica* produced 2.8 μmol ATP/gdw. The disparity between the amounts of ATP produced by *C. virginica* could indicate a difference in the energy requirements for air emersion and for hypoxic exposure. They could also point to the disparity between the amount of alanine produced and the amount of aspartate used.

According to the stoichiometry of the glycogen-aspartate anaerobic pathway, for each mole of aspartate consumed, a mole of alanine is created. In neither the first

nor the second experiment did this occur. In the first experiment, aspartate actually increased in both species after hypoxic exposure. This In the second experiment, the activity of ADH could explain why aspartate did not decrease in a 1:1 ratio with the increase in alanine. Alanine and pyruvate may have been converted into alanopine, shrinking the alanine pool.

Glutamate is an intermediate in the aspartate-glycogen fermentation pathway, and by transamination converts to α-ketoglutarate (α-KGA) and back. As such, its levels should increase in the hypoxic treatments if the oyster is using anaerobic pathways, as demonstrated by de Zwaan and colleagues (1983). *C. ariakensis* levels of glutamate during normoxia are 47 µmol/gdw and increase by a factor of 1.7 during hypoxia. *C. virginica* glutamate levels increase from 22 µmol/gdw in normoxia by 1.95.

Future studies should investigate the activities of enzymes isolated from gill tissue, whole oyster tissue, and adductor muscle tissue. The behavioral differences in the gaping of the two species may indicate that *C. ariakensis* has the ability to utilize pathways that create energy quickly, but it may not be able to sustain these levels of energy output. If we know the presence and activity of enzymes like LDH, ADH, and others, we can determine why the Asian oyster is not as successful as the native oyster in surviving hypoxia.

Chapter 5: Summary

This study has shown conclusively that physiological and metabolic differences exist between *C. ariakensis* and *C. virginica*. Gaping occurred very quickly in *C. ariakensis*, while *C. virginica* begans to gape after at least a week in hypoxic water and remained completely closed until that time. The cause of this behavioral difference remains unknown, although putative causes include differential tolerances in the adductor muscle, evolutionary adaptations, and the need to expel toxic endproducts.

Related to the gaping behavior is the shorter time to mortality of *C. ariakensis* during anoxic stress exposure. On average, triploid *C. ariakensis* died after four days in anoxic water, while triploid *C. virginica* persisted until fifteen days. Diploid Asian oysters lived thirteen days on average and diploid Eastern oysters lived significantly longer, an average seventeen days. These differences further suggest metabolic differences between the species. Dissimilarities in the species' energy demand, anaerobic energy pathways, or toxicity of anaerobic endproducts could explain the reduced tolerance of *C. ariakensis* to anoxia.

When we investigated oxygen consumption rates of diploid oysters of both species during normoxic conditions, we found no significant difference at 10 and 20°C or salinities of 5, 15, or 25. This suggests that the energy demand under oxygenated conditions is not different. The experiment should be repeated under hypoxic conditions for both species and should also be repeated with more triploid oysters. Calorespirometry would help determine if the energy demands of the two

species are similar under hypoxic conditions and would also tell us if aerobic or anaerobic metabolism is meeting the demand.

To investigate the pathways used to create energy during anoxia, we analyzed the free amino acid pools in the gill tissue of oysters in normoxic water and oysters in hypoxic water. We found significantly higher levels of alanine in emersed *C. ariakensis* and emersed *C. virginica*. Aspartate levels in emersed *C. ariakensis* were significantly higher than in emersed *C. virginica*, as were glutamate levels. Aspartate consumption in hypoxic water-treated *C. virginica* was higher than in *C. ariakensis* held in hypoxic water. Alanine production and aspartate consumption point towards the usage of the glycogen-aspartate fermentation pathway, although the disparity in the amounts of these amino acids indicates that *C. virginica* may be using this pathway more than *C. ariakensis*. The difference in FAA pools between the emersed and hypoxic water-treated oysters suggests that the metabolic pathways used under these two conditions were not the same.

The disparity in time to mortality indicates a limitation on the survival of *C. ariakensis* in areas of the Chesapeake Bay suffering from prolonged hypoxia. If the Asian oyster is introduced into the Bay, its introduction should be constrained to shallow, well-flushed areas that maintain high dissolved oxygen levels year-round.
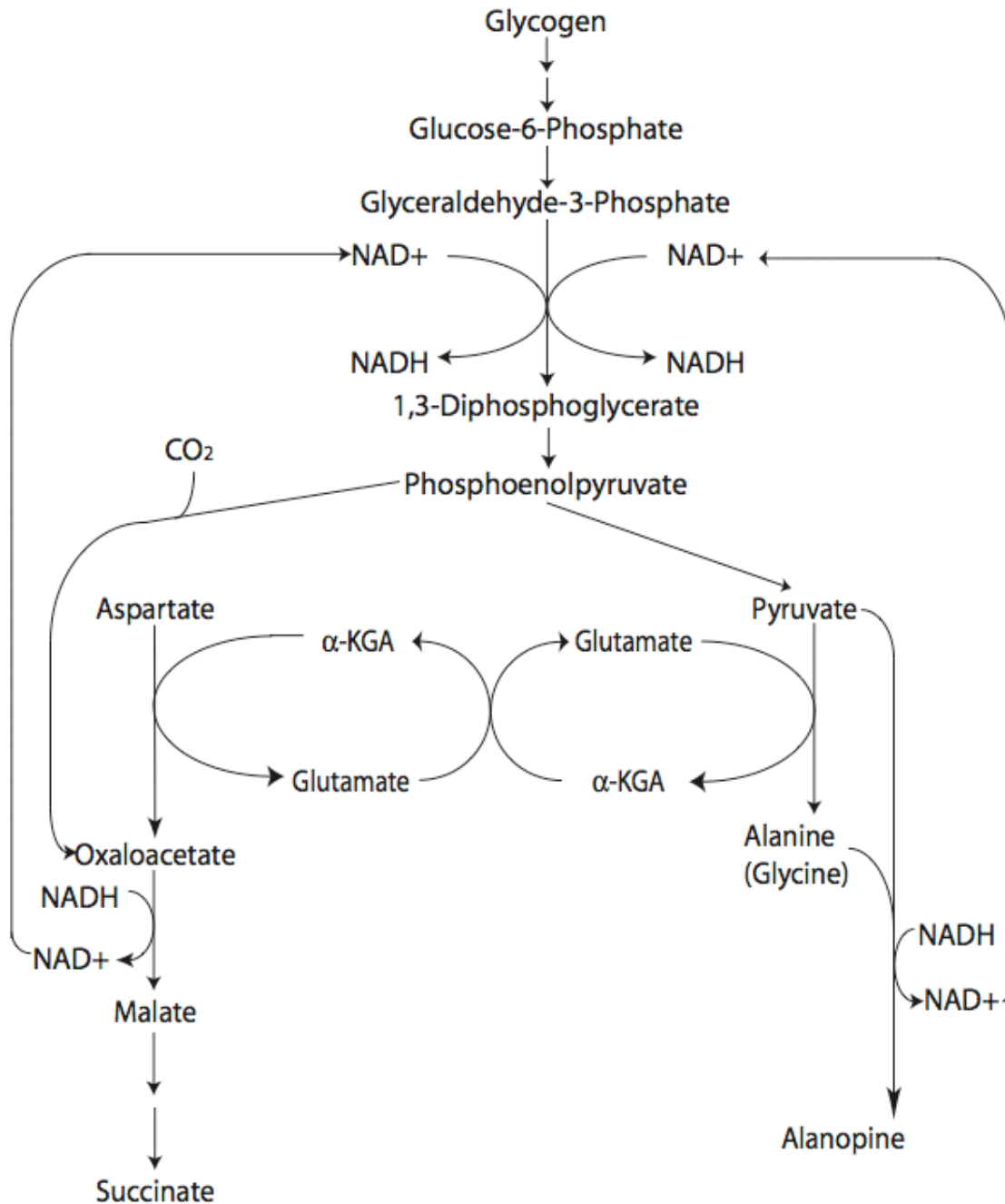
Figure 1. Pathways utilized by *C. virginica* during anoxic exposure. Aspartate and pyruvate are fermented to alanine and succinate through the coupling of a-ketoglutarate (a-KGA) and glutamate cycling. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the fixation of CO_2 with PEP to form oxaloacetate. (After Hochachka, 1980.)
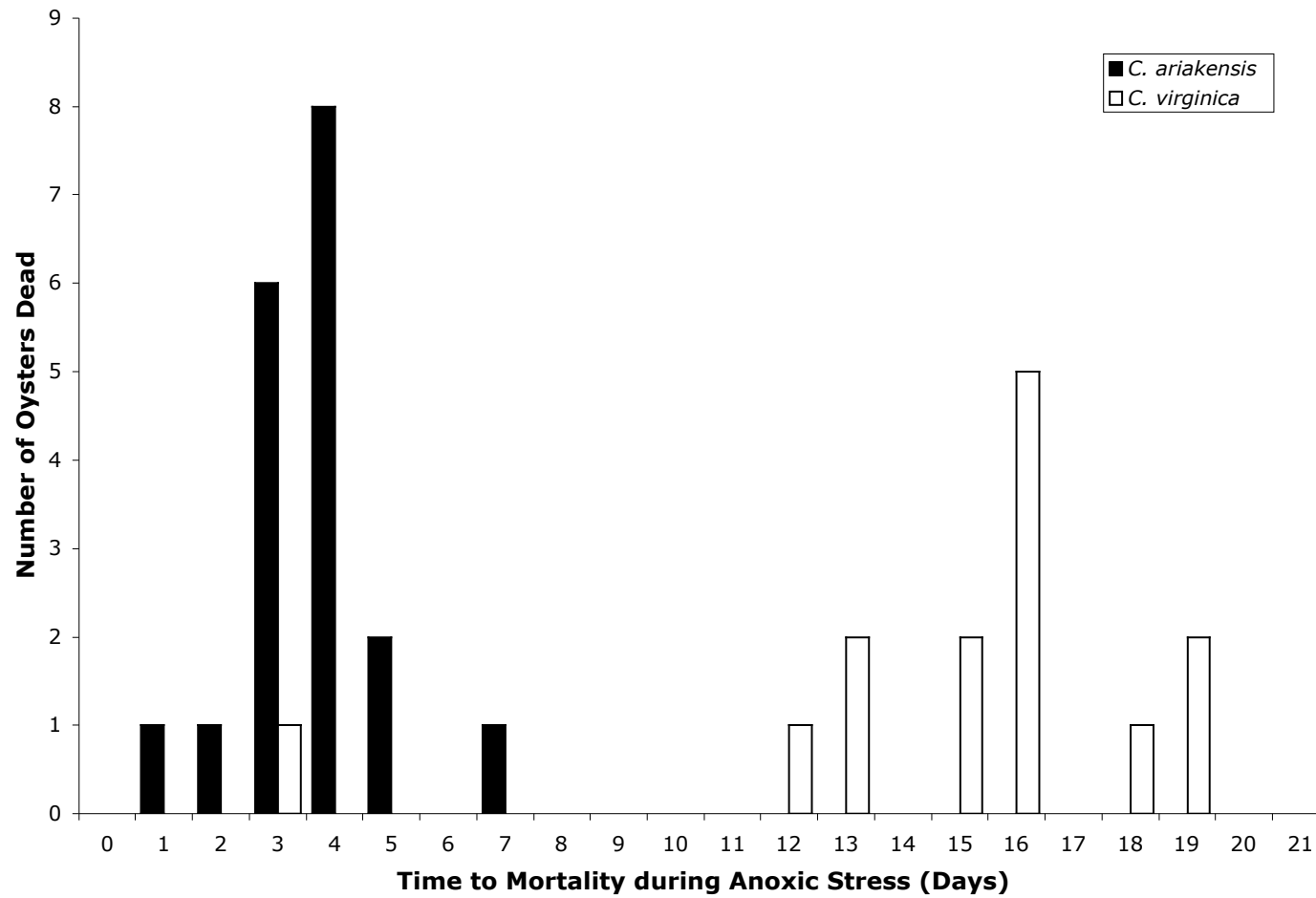
Figure 2. Time to mortality for triploid *C. ariakensis* and *C. virginica* during exposure to anoxic stress. Mortality times were significantly different (ANOVA, F=130.35, p <0.0001, n=33).
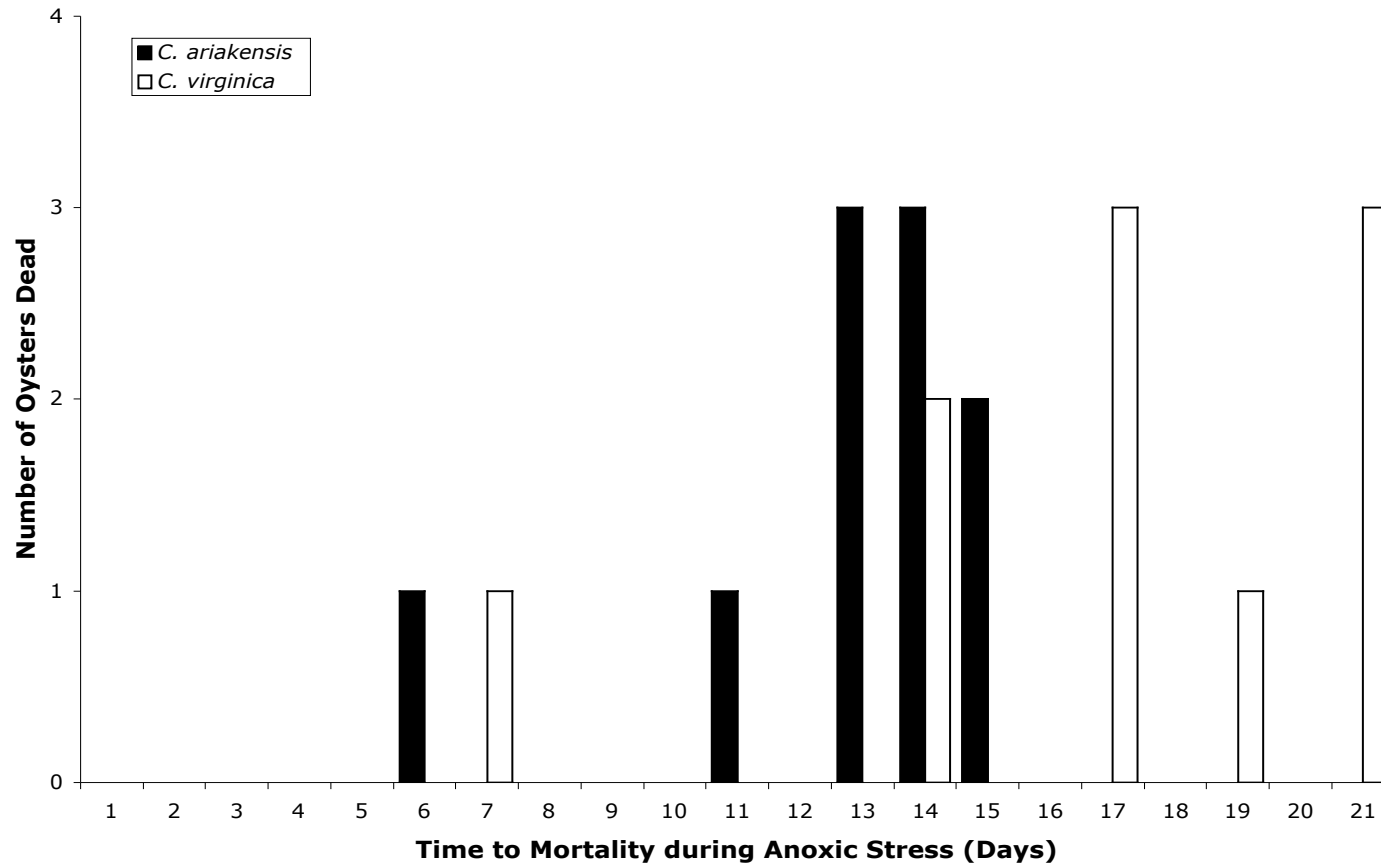
Figure 3. Time to mortality for diploid *C. ariakensis* and *C. virginica* exposed to anoxic stress. Only the hypoxic-treated oysters are shown here, as 80% of the normoxic treated oysters lived the entire 21 days. There was no significant interaction between oxygenation treatment and species (ANOVA, F=0.2654, p=0.6096, n=40). There was a significant species effect on mortality (ANOVA, F=10.96, p=0.0021, n=40) as well as a significant oxygenation effect on mortality (ANOVA, F=7.02, p=0.0119, n=40).
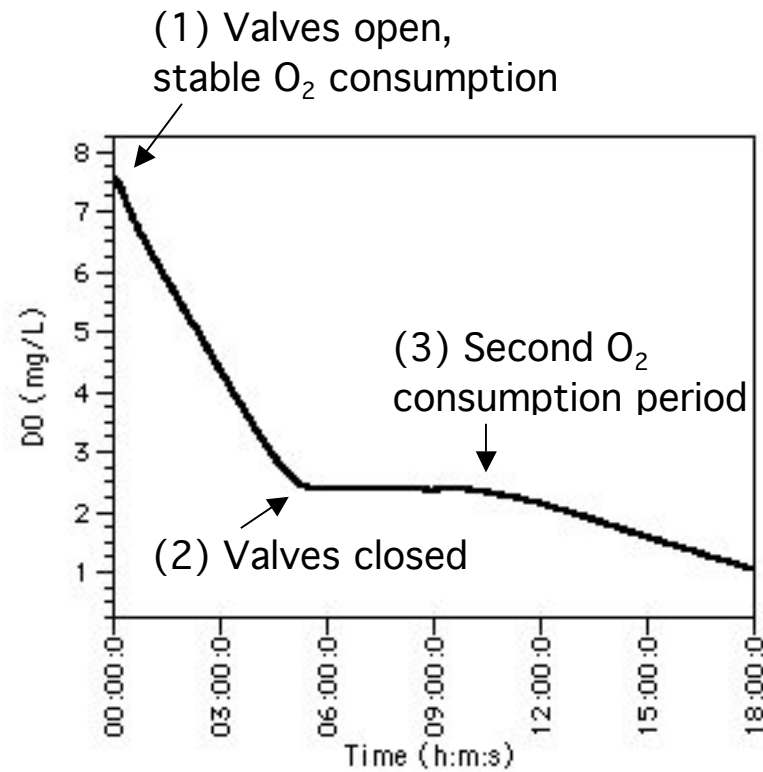
Figure 4. Typical oxygen consumption profile of *C. ariakensis* over an 18 hour period. Note that *C. ariakensis* gaped immediately after reimmersion in water and began consuming oxygen at a stable rate (1). After approximately five hours, the oyster closed its valves (2) and reopened them after another five hours (3).
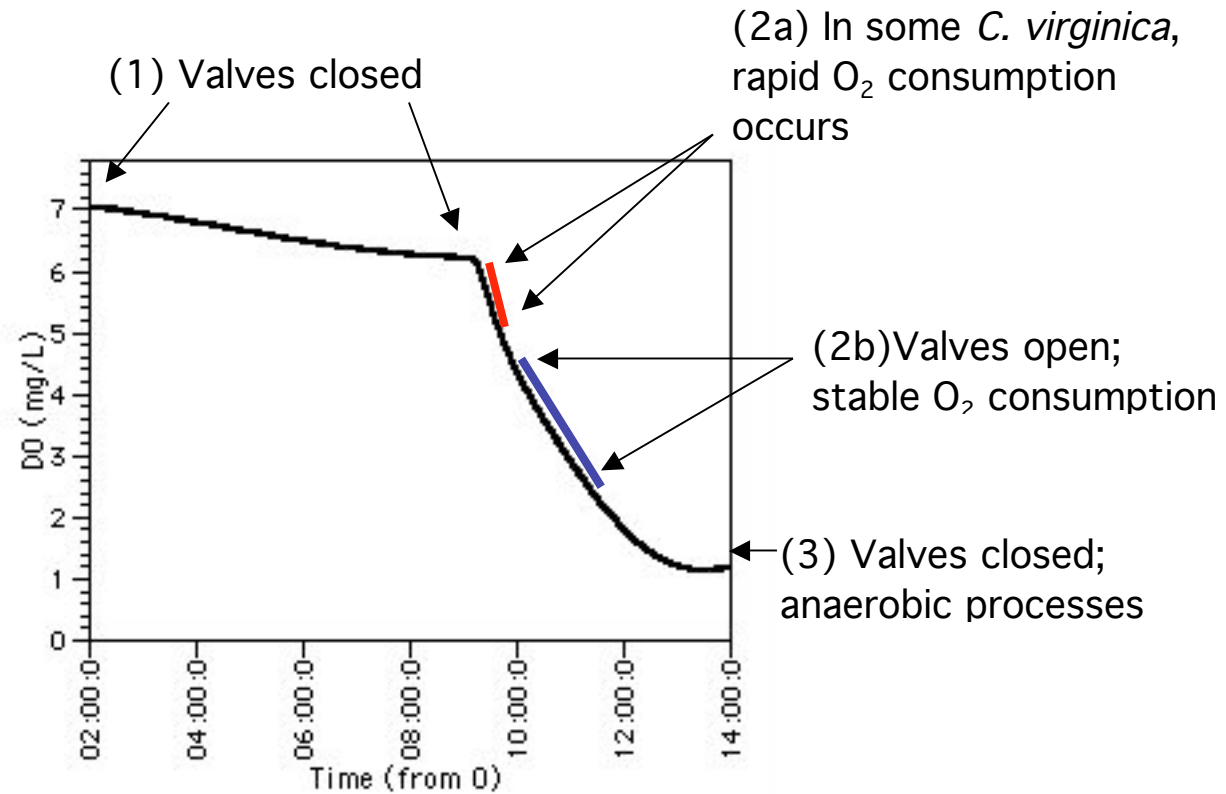
Figure 5. Typical oxygen consumption profile of *C. virginica* over an 18 hour period. *C. virginica* closed for an average of 2 hours after reimmersion in water (1), during which it acquired an oxygen debt; some *C. virginica* consumed oxygen rapidly to "repay" this debt (2a). The oyster then consumed oxygen at a stable rate (2b) until the water was hypoxic, at which point it closed (3). In most cases, oysters in this experiment began stable oxygen consumption after opening.

Table 1. Average time to mortality of diploid and triploid *C. ariakensis* and *C. virginica* at 20°C. Errors represent standard deviation.

Diploid		Triploid	
C. ariakensis	*C. virginica*	*C. ariakensis*	*C. virginica*
(Days)		(Days)	
13 (+/- 2.4)^z	17 (+/- 4.6)^y	4 (+/- 1.3)^x	15 (+/- 4.0)^w

$^{z, y, x, w}$ Days to mortality with the same superscript were not significantly different at the 0.05 level; there was a significant species by ploidy interaction on mortality time (ANOVA, $F = 16.97$, $p < 0.0001$, $n = 45$).

Table 2. Average oxygen consumption rates of diploid and triploid *C. ariakensis* and *C. virginica* at different temperatures and salinities.

Salinity/Ploidy	10°C			20°C	
	C. ariakensis	*C. virginica*		*C. ariakensis*	*C. virginica*
	(mg O$_2$ hr^{-1} gdw^{-1})			(mg O$_2$ hr^{-1} gdw^{-1})	
5/DIP	0.36 (0.08)	0.39 (0.02)		0.52 (0.14)	0.57 (0.31)
15/DIP	0.20 (0.03)	0.25 (0.06)		0.61 (0.13)	0.51 (0.14)
25/DIP	0.27 (0.02)	0.15 (0.05)		0.70 (0.16)	0.70 (0.15)
12.5/TRIP				0.29 (0.07)	0.46 (0.03)

There was no significant species by temperature by salinity interaction on diploid oxygen consumption rates (ANOVA, F=1.563, p=0.2299, n=36). There was a significant temperature by salinity interaction (ANOVA, F=7.01, p=0.0040, n=36), but the interactions of species and temperature and species and salinity were non-significant (ANOVA, F=0.0576, p=0.8123, n=36; F=1.856, p=0.1781, n=36). Diploid oxygen consumption rates did not differ significantly with species (ANOVA, F=0.0011, p=0.9737, n=36). Triploid oxygen consumption rates were significantly different at α =0.05 (ANOVA, F=14.64, p=0.0087, n=8).

Table 3. Average free amino acids levels present in the gill tissue of oysters exposed to normoxic and hypoxic conditions from experiment 1. Numbers in parenthesis are standard deviations.

		Amino Acid Concentration in Gill Tissue (μmol/gdw)		
Species				
Oxygenation	Ala	Gly	Asp	Glu
C. ariakensis				
Normoxic	9.5 (3.2)	13.8 (8.9)	26.9 (8.3)	5.9 (2.0)
Hypoxic	18.1 (7.4)	18.9 (12.4)	35.5 (20.4)	10.1 (5.3)
C. virginica				
Normoxic	3.1 (2.5)	4.3 (4.4)	12.2 (7.0)	2.8 (1.2)
Hypoxic	17.5 (3.3)	3.6 (0.8)	14.6 (9.3)	5.4 (1.9)

There was an overall species effect on the concentrations of amino acids (MANOVA, Wilks' Lambda=0.530, F=3.99, p=0.017, n=25). There was also an overall oxygenation effect on amino acid levels (MANOVA, Wilks' Lambda=0.333, F=9.00, p<0.0001, n=25). The species by oxygenation interaction is non-significant (MANOVA, Wilks' Lambda=0.801, F=1.19, p=0.378, n=25).

Table 4. Differences in amino acid concentrations between hypoxic and normoxic gill tissue from experiment 1.

Differences in Amino Acid Concentration in Hypoxic and Normoxic Gill Tissue (μmol/gdw)

(Hypoxic-Normoxic)

Species	Ala	Gly	Asp	Glu
C. ariakensis	*+8.6*	*+5.1*	*+8.7*	*+4.2*
C. virginica	*+14.4*	*-0.7*	*+2.4*	*+2.7*

There were no significant species by oxygenation interactions for any amino acid. There was a significant oxygenation effect on alanine and glutamate levels. There was also a significant species effect on glycine, aspartate, and glutamate.

Table 5. Average free amino acids levels present in the gill tissue of oysters exposed to normoxic and hypoxic conditions from experiment 2. Numbers in parenthesis are standard deviations.

		Amino Acid Concentration in Gill Tissue (µmol/gdw)		
Species				
Oxygenation	Ala	Gly	Asp	Glu
C. ariakensis				
Normoxic	13.0 (3.6)	10.2 (2.2)	20.5 (9.4)	6.2 (1.4)
Hypoxic	23.6 (10.1)	12.8 (6.1)	19.7 (8.8)	7.8 (4.1)
C. virginica				
Normoxic	16.3 (5.1)	2.7 (1.0)	17.8 (6.9)	5.5 (1.0)
Hypoxic	16.9 (8.9)	2.5 (1.2)	10.4 (5.6)	5.3 (1.8)

There was an overall species effect on amino acid levels in gill tissue (MANOVA, Wilks' Lambda=0.19, F=34.7, p<0.0001, n=40). There was no overall effect of oxygenation on FAA pools (MANOVA, Wilks' Lambda=0.772, F=2.43, p=0.067, n=40). The species by interaction term was non-significant (MANOVA, Wilks' Lambda=0.861, F=1.33, p=0.279, n=40).

Table 6. Differences in amino acid concentrations between hypoxic and normoxic gill tissue from experiment 2.

| | Differences in Amino Acid Concentration in Hypoxic and Normoxic Gill Tissue (µmol/gdw) | | | |
| | (Hypoxic-Normoxic) | | | |
Species	Ala	Gly	Asp	Glu
C. ariakensis	+10.6	+2.7	-0.7	+1.6
C. virginica	+0.6	-0.2	-7.4	-0.2

Multiple pairwise comparisons reveal that only alanine had a significant species by oxygenation interaction (ANOVA, F=4.52, p=0.040, n=40). For alanine, oxygenation had a significant effect on amino acid levels (ANOVA, F=, p=, n=). In glycine, aspartate and glutamate, species had a significant effect on amino acid levels.

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