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THE EFFECTS OF RIVER SEDIMENT, ENDOSULFAN, AND MODERATE HYPOXIA ON BLUE CRABS (CALLINECTES SAPIDUS) FROM THE TIDAL, FRESHWATER JAMES RIVER

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THE EFFECTS OF RIVER SEDIMENT, ENDOSULFAN, AND MODERATE HYPOXIA ON BLUE CRABS (*CALLINECTES SAPIIDUS*) FROM THE TIDAL, FRESHWATER JAMES RIVER

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

THE EFFECTS OF RIVER SEDIMENT, ENDOSULFAN, AND MODERATE HYPOXIA ON BLUE CRABS (CALLINECTES SAPIIDUS) FROM THE TIDAL, FRESHWATER JAMES RIVER

By Laura Elizabeth Williams, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Studies at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

Major Advisor: Peter L. deFur
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Juvenile male blue crabs move into the tidal, freshwater James River during warmer months to feed and grow by undergoing molting. In crustaceans, growth and molting are hormonally controlled. The physiological effects of a multiple-stressor environment are determined by comparing the blue crab’s oxygen uptake after exposure to pure sand, James River sediment, or endosulfan-spiked sand. The effect of multiple stressors on molting is measured by the activity level of N-acetyl-β-glucosaminidase (NAG), an enzyme in epidermal tissue important to molting. The oxygen uptake was decreased by exposure to James River
sediment but not for exposure to endosulfan for seven days. Exposure to James River sediments over two days caused a similar suppression of epidermal NAG activity as exposure to endosulfan. These results indicate that the blue crab’s exposure to James River sediments and moderate hypoxia has the potential to cause short-term effects on physiology and long-term effects on growth.
Introduction

The blue crab (Callinectes sapidus) is an ecologically and economically important invertebrate in the Chesapeake Bay. It is a key benthic predator that plays an important part in the abundance, diversity and structure of various benthic communities. The blue crab’s habitat spans from the high salinity waters of the Bay to its freshwater tributaries. The blue crab has a sexually dimorphic life history in which the juvenile male blue crab moves into low salinity areas while the female stays in the higher salinity waters at the mouth of the Bay. During warmer months, juvenile males feed and molt among the submerged aquatic vegetation in the Bay’s freshwater tributaries. The tributaries afford important habitat during this vulnerable life stage as they minimize predation while the crab is without a hardened shell for protection (Hines et al. 1987).

Blue Crab Growth and Molting

Crustaceans increase in body size by undergoing molting, or ecdysis. The crab sheds its external shell exposing a new larger exoskeleton which the internal body tissues must fill. The variable characteristics of blue crab molting are the time between molts (intermolt period), and the size increase at molt (molt increment) (Charmantier et al. 1997). Molt increment includes a change in the carapace width (CW) which measures the distance between the lateral spines of the carapace (Figure 1), and the change in mass. The molt increments tend to increase as animal size
increases during the juvenile growth phase (Churchill 1921). The typical increase in carapace width, 25% - 40% for Chesapeake Bay blue crabs (Churchill 1921), has been used as a guideline for blue crabs in Virginia and adjacent states (Van Den Avyle and Fowler 1984). However, a decrease in molt increment has been observed in very high temperatures as well as low salinity water (i.e. salinities < 1 Practical Salinity Units (PSU)) (Tagatz 1968).

Figure 1. Measurement of the carapace width (CW) measured from lateral spine to lateral spine of the blue crab using calipers.

The molt cycle is a continuous preparation for and recovery from the act of molting over the life of the crab. The stages have been previously defined as follows: postmolt (stages A, B, and C1-3), intermolt (stage C4), premolt (stages D0-4) and finally ecdysis (stage E) (Passano 1960) (Table 1). Small crabs molt frequently but the intermolt period increases as crabs grow larger (Passano 1960). The intermolt period can be influenced by multiple environmental factors and may be shorter due to higher temperature, and longer due to lower temperatures (Tagatz 1968) or extreme salinities (Hartnoll 1982). Nutrition, as well as other site-specific factors, can also affect intermolt period (Smith and Chang 2007).
The blue crab is able to live and grow in a range of salinities due to its osmoregulatory ability to regulate ions in its hemolymph (i.e. blood) relative to its environment. At salinities above 27 PSU, the blue crab’s hemolymph is isosmotic with its environment and below 27 PSU, hemolymph is hyperosmotic (Mantel 1967). NaCl is the major osmolyte of blue crab hemolymph, and osmoregulation in freshwater is accomplished through net influx of Na\(^+\) and Cl\(^-\) from the environment (Cameron 1978). During ecdysis, a large influx of water is necessary to build up internal hydrostatic pressure to break open the old cuticle. The elevated uptake of Na\(^+\) in exchange for another monovalent cation creates a gradient across the gill structures of the crab (Towle and Mangum 1985). While the freshwater environment may be a physiological stressor...
that requires maintaining hyperosmotic hemolymph, size at molt increases in freshwater and may reduce the number of molts required to attain a given size (deFur et al. 1988). This may convey an advantage to juvenile male blue crabs to gain a larger size before reaching the Bay to mate with females.

Molting in crustaceans is important for growth and sexual maturation and is hormonally controlled. Ecdysone and other related compounds, or “ecdysteroids,” are major regulators of molting in arthropods (deFur et al. 1999). Ecdysis itself centers on the production and shedding of the exoskeleton. Chitin, a long chain polymer of N-acetylglucosamine, is the primary constituent of all arthropod exoskeletons. The initial event of ecdysis is apolysis, or the separation of the cuticle from the underlying epidermis (Stevenson 1972). The “molting fluid” released during premolt contains the molting hormone ecdysone and several enzymes including chitinase and chitobiase (N-acetyl-β-glucosaminidase, a.k.a. NAG). Ecdysone, which is quickly hydroxylated to 20-hydroxyecdysone, increases as the new cuticle is formed beneath the exoskeleton (deFur et al. 1999). Chitin digestion is accomplished by chitinase, which hydrolyzes the β-(1,4) glycosidic bonds of chitin to dimer and trimer oligomers of NAG. NAG is further hydrolyzed to a monomer by chitobiase (Espie and Roff 1995). The breakdown of the old exoskeleton is integral in the preparation for molting. Chitobiase, or NAG, is also found in the hepatopancreas, as this is the site of digestion and breakdown.

Despite the benefits of freshwater tributaries, the blue crab also experiences stressors in the benthos. The Chesapeake Bay tributaries undergo periods of hypoxia, as well as contaminated sediments. Kuo and Neilson (1987) found that oxygen concentrations of <50% saturation occurred in 75%, 50% and 2% of the surveys for the Rappahannock, York, and James Rivers, respectively. The rare occurrence of hypoxia in the James River is surprising since it
receives the greatest amount of wastewater. Hypoxia, coupled with high contaminant loads, will have a greater effect on benthic organisms when they occur together (see below).

**Hypoxia**

Hypoxia, the depletion of dissolved oxygen in water, is an increasingly common occurrence around the world, especially in shallow coastal and estuarine habitats (Diaz 2001). The number of sites where hypoxia has been reported has increased with an exponential growth rate of 5.54% per year (Vaquer-Sunyer and Duarte 2008). Hypoxia has traditionally been defined as the concentration of oxygen < 2.0 mg/l, and anoxia is the complete absence of oxygen at 0.0 mg/l (Newcombe and Horne 1938, Diaz and Rosenberg 1995). However, the critical oxygen concentration that brings about a lethal or sublethal effect varies among organisms, which have different vulnerabilities at different life stages. It is generally accepted that larval life stages, such as those found in crustaceans, are more sensitive than the adults (Gray 2002).

The two main causes of hypoxia are water stratification, where bottom waters are not mixing with oxygenated surface waters, and decomposition of organic matter. Urbanization of the landscape and higher human population densities result in excess nutrient loading into water bodies, creating eutrophic conditions. The increase in nutrients leads to increased primary production and much of this organic matter reaches the bottom if it exceeds the capacity of the food web to consume it (Diaz 2001). The microbial decomposition of the organic matter increases the oxygen demand on the system, causing hypoxia. Hypoxia is most prevalent during the summer months due to high temperatures and a lack of mixing between the layers within a stratified body of water (Taft 1980). The increase in temperatures world-wide due to global warming will likely increase the occurrence of hypoxia. Higher ambient water temperatures can
adversely affect the respiratory oxygen demand of organisms, water body stratification, and reduce oxygen solubility (Vaquer-Sunyer and Duarte 2008).

Aquatic organisms require oxygen for aerobic respiration and excrete CO$_2$ in the process. CO$_2$ reacts with the aquatic environment as follows: CO$_2$ + H$_2$O $\rightleftharpoons$ H$_2$CO$_3$ $\rightleftharpoons$ H$^+$ + HCO$_3^-$, creating acidic conditions. Within the aquatic ecosystem, photosynthesis removes CO$_2$ and contributes oxygen to the system during the day. Hypoxic and hypercapnic waters occur overnight and in the early morning due to the lack of oxygen input (Burnett 1997), further stressing benthic organisms. The decreased pH becomes a serious secondary stressor during hypercapnia because acidosis of the blue crab hemolymph causes a decrease in the oxygen carrying capacity of most respiratory pigments (Dejours 1981). Hypoxic waters can be considered multiple stressor environments because they often co-occur with high CO$_2$ and decreased pH (Hypes 1999).

Effects of Hypoxia

Hypoxia adversely affects benthic species, abundance, and biomass. Hypoxic waters cause reduced benthic communities, a loss of biodiversity, and are dominated primarily by hypoxia-tolerant species and are re-colonized by opportunistic species (Diaz and Rosenberg 1995). In a review of the lethal effects of hypoxia across aquatic taxa, Vaquer-Sunyer and Duarte (2008) found crustaceans were the organisms most sensitive to hypoxia. Of the large variability in oxygen thresholds among benthic organisms, crustaceans exhibited the highest oxygen concentrations at which death occurred (LC$_{50}$ mean: 2.45 ± 0.14 mg/l; 90$^{th}$ percentile: 5.72 mg/l) and the shortest time till death on exposure to hypoxia (mean: 55.5 ± 12.4 hours; 90$^{th}$ percentile: 1.0 hour). Mobile crustaceans can compensate for their oxygen sensitivity
behaviorally by swimming out of hypoxic waters (Das and Stickle 1993, Pihl et al 1991, Vaquer-Sunyer and Duarte 2008).

Hypoxia can also have sublethal effects on benthic organisms and alter their behavior and physiology. Sublethal stresses include decreased feeding (Diaz and Rosenberg 1995), decreased growth and reproduction, forced migration, less suitable habitat, increased predation, and disruption of life cycles (Vaquer-Sunyer and Duarte 2008). The crustaceans *Penaeus vannamei* and *P. monodon* experienced a significant reduction in growth and in the instantaneous growth rate (k) at the lowest experimental dissolved oxygen of 1ppm after a 16 day exposure (Seidman and Lawrence 1985). Blue crab feeding is affected at 2 – 3 mg O₂/l and the presence of hypoxia inhibits foraging (Gray 2002). The physiological tolerance of blue crabs to low oxygen conditions is limited and varies based on life cycle stage. The utilization of different habitats over the life of a blue crab accounts for the varied encounters with hypoxia (Tankersley and Wieber 2000). Blue crabs are classified as oxyregulators and can maintain their oxygen uptake regardless of ambient oxygen up to a critical oxygen tension. Physiologically, blue crabs can increase ventilation across their gill structures to increase extraction of oxygen by increased scaphognathite beat frequency (Cameron 1989). Prolonged exposure to hypoxia of at least 7 days includes changes to hemocyanin, the oxygen carrying pigment, in the hemolymph of blue crabs. Hemocyanin concentrations increase and the molecule changes structurally to increase oxygen affinity (deFur et al. 1990). Exposure to severe hypoxia also increased lactate concentrations in the hemolymph, indicating a shift to anaerobic metabolism. The increases in hemolymph CO₂ and lactate caused by hypoxia trigger changes in hemocyanin which increase its oxygen affinity (Mangum 1994). Adults are more tolerant than juveniles, but neither is able to survive severe hypoxia for even a short time (deFur et al 1990). Megalopae can maintain a
greater degree of oxyregulation at high oxygen tensions than juveniles (Tankersley and Wieber 2000).

Endocrine Disrupting Compounds

Xenobiotics continue to be a problem in aquatic environments due to anthropogenic point and non-point sources such as agriculture, stormwater runoff, and wastewater treatment discharges. Contaminant concentrations such as metals and organics, in both sediment and tissues, correlate with human population density (Kimbrough 2008). Concern has increased over a particular group of emerging contaminants that have endocrine disrupting capabilities. Soto et al. (1991) found that chemicals leaching from plastic culture dishes exhibited estrogenic properties in the MCF7 breast cancer cell line that is responsive only to estrogen. They then screened other commonly used chemicals and found many exhibited estrogenic activity. Based on the in vitro assay, a number of commonly used chemicals have the potential to activate a hormone pathway known to be a cancer risk factor. The significance of these results is their applicability to other hormones. A broader perspective and more complete picture of endocrine disruption resulted from a focused workshop comparing their effects across vertebrate species (including humans), physiological systems and animal models (workshop proceedings published by Colborn and Clement 1992). It is clear from the results presented and reviewed in Colborn and Clement (1992) that basic physiological mechanisms could be affected by anthropogenic chemicals. The physiological systems of greatest interest in these reviews were endocrine and developmental. During an EPA workshop held in 1995, the EPA defined an endocrine disrupting chemical (EDC) as “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible
for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock et al. 1996).

The crustacean endocrine system, as other endocrine systems, typically functions via cascades that are initiated by environmental or physiological cues and result in a terminal hormone and its action on a target organ. The steroid molting hormones, ecdysteroids, are the secretion product of the Y-organ/molting gland (Figure 2). The Y-organ’s production of ecdysteroids increases dramatically during early to mid premolt stages (Covi et al 2009), initiating the events that lead to molting. The Y-organ is under the regulatory control of the X-organ/sinus gland complex via the secretion of a molt-inhibiting hormone (MIH), a neuropeptide, as well as other circulating signals. The crustacean hyperglycemic hormone (CHH) is a peptide hormone that can independently modulate ecdysteroid activity (Covi et al. 2009). The X-organ’s regulatory role in molting was first indicated by studies that removed the eyestalks of the crab, which caused a shortening of the molt cycle interval (Zeleny 1905). These studies showed that some molt-inhibiting organ was present in the eyestalks. The X-organ also secretes a mandibular organ-inhibiting factor that inhibits the mandibular organ from secreting methyl farnesoate. Methyl farnesoate acts on the Y-organ to stimulate production of ecdysone. Methyl farnesoate is analogous to the Juvenile hormone in insects and may also act directly on tissues via the mandibular organ (deFur et al 1999). Because pesticides are specifically designed to interact with insect molting hormones, crustaceans are then also potentially affected if exposed to these pesticides in the aquatic environment.
Although the number of studies on EDCs and their effects on organisms are growing in number, studies on the specific mechanisms by which EDCs act on the endocrine system are less numerous (Rodriguez et al. 2007). EDCs have the potential to act in several ways. EDCs can act on the estrogen receptor as either an antagonist by blocking the receptor and stopping the endogenous hormone from binding; or as an agonist by binding to the receptor and stimulating the receptor without the presence of the endogenous hormone. EDCs may also increase or decrease the levels of endogenous hormone without interacting with its respective hormone receptor (deFur et al 1999) by increasing or decreasing their excretion rate or biotransformation in various organs (Rodriguez et al 2007).

**Effects of Endocrine Disrupting Compounds**

Several contaminants have been found to have endocrine disrupting effects and include pesticides as well as other chemicals. A critically important result in the early investigation of endocrine disruption was the discovery that male fish in waters downstream from sewage
treatment facilities produced egg protein, vitellogenin, in response to estrogenic chemicals in the discharges (Sumpter and Jobling 1995). Another incidence of altered sexual development in organisms involved tributyltin (TBT), a paint used on the hulls of boats, which gave rise to imposex gastropods due to masculinization of females (Matthiessen and Gibbs 1998). These results demonstrated that external exposure to endocrine disruptors could effectively initiate sub-lethal responses. The significance for crustaceans is that many are aquatic and all are oviparous, as are fish, raising the possibility that aquatic crustaceans may be susceptible to similar exposures and effects. Due to the increasing awareness of these contaminants, the US Geological Survey (USGS) initiated a more comprehensive survey of contaminants in surface waters of the US to include chemicals that had not been previously assessed. USGS termed these chemicals "emerging contaminants" and published the results of a survey of 139 locations across the continental US (Kolpin et al. 2002). The results indicated the widespread occurrence of a range of chemicals in rivers, streams, lakes and ponds. The chemicals include pharmaceuticals (ethinyl estradiol) and industrial chemicals (polychlorinated biphenyls) known to be hormonally active in cell-based or whole animal bioassays (Kolpin et al. 2002). The significance of these results for crustaceans is that aquatic crustaceans in all types of habitats are likely exposed to some range of chemicals known to be hormonally active in animal systems.

Due to the blue crab’s habitat utilization, the juvenile molting crab may be the life stage that is most exposed to chemicals found in tributary sediments. While the larval stages are planktonic and rely on currents within the water column for movement, the juvenile stage begins the blue crab’s settlement into the benthos (Reynes 2004). The juvenile must undergo over a dozen molts before reaching sexual maturity, a process that can be highly influenced by environmental factors and overall health of the individual. During winter, the juveniles must
move into deeper channels to avoid the stress of severely cold surface waters, leaving them in close contact with sediments. Blue crabs in the James River near Richmond have been impacted by a sediment contaminant, chlordecone (Kepone), since contamination in the 1970s. In a study where blue crabs were fed oysters containing Kepone in concentrations similar to those found in oysters from the James River, they either died or molted less frequently than crabs fed Kepone-free oysters (Schimmel et al. 1979).

**Endosulfan**

Endosulfan is a neurotoxic organochlorine insecticide of the cyclodiene family of pesticides. It has been commonly used worldwide on fruits, vegetables, coffee, cotton and tea since the 1950’s. It is poorly soluble in water (0.33 mg/l, 25°C) but readily soluble in organic solvents. Endosulfan is composed of two isomers, alpha and beta, in a ratio of 2:1. The alpha isomer is more volatile and dissipates more readily than the beta isomer, which is more adsorptive and persistent. Under acidic conditions, at 25°C, the half life of both isomers is considerably longer. At neutral pH, the half life of alpha and beta endosulfan was 11 and 19 days, respectively, but at a pH of 5, the half life was more than 200 days for each isomer (Silva and Beauvis 2010).

The different qualities of the isomers allow endosulfan to travel long distances as a vapor and to persist in the environment. It can be transported via runoff or dust dispersion, but subsurface leaching is believed to be negligible. For these reasons, endosulfan can be found almost anywhere in the world, in almost any media (Silva and Beauvis 2010). Endosulfan is primarily degraded by oxidation and hydrolysis, but the effects of photolysis are negligible. Biotic and abiotic processes under aerobic and anaerobic conditions can play a part in the
degradation of endosulfan. Specifically, oxidation of either isomer of endosulfan can result in the formation of endosulfan sulfate, which has a similar level of toxicity but is potentially two times more persistent. The half lives for alpha and beta endosulfan in soils under various environmental conditions ranged from 19-124 and 42-265 days, respectively. The combined half life of alpha and beta isomers and endosulfan sulfate ranged from 9 months to 6 years. Under neutral to alkaline conditions, hydrolysis of endosulfan is favored, producing endosulfan diol. Endosulfan diol is less toxic and more hydrophilic than the parent compound endosulfan (Silva and Beauvis 2010).

Endosulfan is a neurotoxin and a known endocrine disruptor. It is a GABA inhibitor and binds and blocks the Cl- channel linked to the GABA receptor. It prevents Cl- from entering neurons and results in uncontrolled excitation (Silva and Beauvis 2010). Because organochlorines are generally highly lipophilic, endosulfan is readily accumulated in fatty tissues, notably the hepatopancreas of numerous crustaceans (Menone et al. 2000, Blais et al 2003). Zou and Fingerman (1997) found that endosulfan does not affect male differentiation in Daphnia magna but does delay molting. Endosulfan decreases epidermal chitobiase activity in Uca pugilator after a seven-day exposure (Zou and Fingerman 1999).

Cumulative Risk

Often in the analysis of environments and their physiological effects on organisms, one stressor is examined at a time. This approach may provide insight into the cause and effect of an environmental stressor, but it does not replicate the organism’s environment and the multiple stressors it experiences. Stressors may include chemical as well as non-chemical components of an organism’s environment. Combinations of stressors may have synergistic effects, where the
combined effect is more than additive; or an antagonistic effect, where one stressor’s effect is decreased by the presence of the other stressor. The assessment of these multiple stressors is called cumulative risk assessment (CRA) and, as defined by the US EPA in the Framework for Cumulative Risk Assessment, CRA involves “an analysis, characterization, and possible quantification of the combined risks to health or the environment from multiple agents or stressors” (US EPA 2003). Although still not common, field and laboratory analysis of the impacts of multiple stressors on animal physiology are growing. In particular, ongoing anthropogenic activities have impacted aquatic organisms and have introduced additional stressors to the benthic communities. Most notable are the increasing occurrence of hypoxia, and the introduction of xenobiotics with endocrine disrupting activity.
Research Objectives

The blue crab’s life cycle requires adaptation to a range of environmental factors including higher salinity waters of the Chesapeake Bay and the lower salinities of its freshwater tributaries, diverse water and sediment-bound contaminants, and habitat and predatory pressures. Despite these multiple stressors, the blue crab is still able to grow via molting, a physiological phenomenon that leaves them especially vulnerable to these stressors.

The objectives of the present research were: 1) to determine the growth and molting morphometrics of juvenile blue crabs collected from the tidal freshwater James River; 2) to determine if James River sediment and endosulfan have an effect on oxygen uptake in juvenile blue crabs; 3) to determine the effect of a multiple stressor environment (James River sediment and hypoxia or Endosulfan-spiked sediment and hypoxia) on the levels of chitobiase, an enzyme important in growth and molting, in epidermal tissues of juvenile blue crabs.
Methods

Animal Collection

Juvenile blue crabs of less than 90 mm carapace width (CW) were collected from the tidal freshwater James River at the VCU Rice Center (Charles City County, VA) (Figure 3) by beach seine and by crab pots specially designed to catch juveniles. Blue crabs were collected from September to November 2010 and from July to November 2011. For the 2010 collection, the blue crabs were sexed, weighed, and their carapace width (CW) measured. They were brought back to the VCU Life Sciences aquatics facility and maintained in a 265 liter recirculating, filtered aquaria. Blue crabs that molted were weighed and their carapace width measured and returned to the aquarium. For the 2011 collection, blue crabs were housed in individual BPA-free containers (9”L x 6”W x 6.5”H; approx. 2.5 L) at 22 °C (+/- 1 °C). Both 2010 and 2011 collections had normoxic conditions maintained with continuous aeration by air stone. They were housed in freshwater with a pH of > 7.5, salinity < 0.5 ‰ and supplemented with calcium at 5 ml per 38 liters. Crabs were fed frozen fish every three days, at 10% of the crab’s body weight, and water was changed after feeding. Crabs were acclimated to the lab for at least a week prior to experimentation, and feeding was stopped at least 12 hours prior to any experiments.
**Figure 3.** Blue crab collection sites at the VCU Rice Center on the tidal, freshwater James River

*Oxygen Uptake: Respirometry*

To determine the effects on crab oxygen uptake of a multiple contaminant stressor environment (James River sediment) and a single contaminant stressor (endosulfan-spiked sand), the following table outlines the treatment groups.

**Table 2.** The purpose for each oxygen uptake treatment group

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>No substrate</th>
<th>James River sediment</th>
<th>Endosulfan spiked sand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control: Pure Sand</strong></td>
<td>Effect of pure sand versus no substrate</td>
<td>Effect of multiple contaminants from a river system</td>
<td>Effect of endosulfan-spiked pure sand at .33 ppm</td>
</tr>
<tr>
<td>(baseline treatment for each James River and Endosulfan treatment group)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

While in C₄ intermolt, each crab’s baseline oxygen uptake was determined by closed respirometry as controls. Each crab was placed in a plexiglass container fitted with a screw-down lid (175cm x 120cm x 90cm; 1.86 L) with 22°C (± 0.5) water. The respirometer housed a
specially designed “cage” that maintained the crab in a pyrex petri dish of 50 g pure, lab-grade sand (40-100 mesh), which limited their movement during the test. The crab was acclimated to the respirometer overnight by placing the crab in the aerated container and covering the container with a towel to block out any light. The next morning, while the crab was still in the container, the respirometer was flushed with fresh water 1 x the volume of the container. Any sand that had been dislodged from the petri dish overnight was maintained in the container. A stir bar set at approximately 240 rpm was used to avoid an oxygen gradient in the closed respirometer. The respirometer was then closed and PO$_2$ was measured with a fiber optic oxygen transmitter and oxygen dipping probe (PreSens Fibox 3) every 5 minutes starting at 140 Torr. The test was run for approximately 5.5 hours, or an oxygen pressure of approximately 20 torr, whichever came first.

The crab was placed in one of the following exposures for seven days under normoxic conditions: endosulfan-spiked pure artificial sand (n = 6), James River sediment (n = 6), or pure artificial sand (n = 3) (Table 3). The containers were filled with 300 g of the respective sediments: endosulfan exposure: 300 g pure sand; James River exposure: 100 g sediment to 200 g pure sand. The crabs in the pure, artificial sand treatment were run with no substrate present for the basal oxygen uptake. Normoxia was maintained by constant aeration by air stone. Endosulfan-spiked artificial sediments were dissolved in a carrying solvent of ethanol at a concentration of 0.33 ppm. James River sediment was collected by Eckman dredge at the Rice Center at a depth of approximately 2 meters in a shallow channel that runs parallel to the shoreline on September 13, 2011. This sediment collection followed heavy rain events associated with Tropical Storm Lee in early September and the larger Hurricane Irene in late August. The sediment sample was immediately placed in glass jars with screw caps, kept
covered from light, and placed in a refrigerator at 4°C. The James River sediment was mixed with artificial sand at a ratio of 0.33 (James River sediment: artificial sediment). Both the pure, artificial sediment treatment and the James River sediment treatment contained ethanol in the same amount used to spike the endosulfan sediments.

**Table 3.** Seven day exposure control and treatment groups

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Pure artificial sand (n = 3)</th>
<th>Endosulfan-spiked sand (n = 6)</th>
<th>James River Sediment (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No substrate</td>
<td>Pure, artificial sand</td>
<td>Pure, artificial sand</td>
</tr>
<tr>
<td>Treatment</td>
<td>Pure, artificial sand (lab grade, 40-100 mesh)</td>
<td>Endosulfan-spiked sediment at .33 ppm</td>
<td>James River sediment and artificial sand mix (ratio .33)</td>
</tr>
</tbody>
</table>

n = number of blue crabs

Following the seven day exposure, the crab was then placed in the respirometer to acclimate overnight (as described above for basal oxygen uptake) in preparation for the exposure oxygen uptake. The exposure treatment was continued by transferring 75 grams of the exposure sediment from the crab’s holding container into the respirometry container. Exposure oxygen uptake was measured as described above for baseline oxygen uptake.

Additional juvenile blue crabs collected from the tidal freshwater James River, the following exposures were conducted for two days, after which the epidermal and hepatopancreas tissues were collected for the enzyme assay, as described below. Normoxia was maintained by continuous aeration and was measured at 95% saturation or better. Moderate hypoxia was achieved by consumption of oxygen by the individual crab in its container, with minimal aeration to avoid severe hypoxic conditions over the 48 hours. The PO₂ ranged from approximately 100 - 115 Torr (65% - 75% saturation) the first 24 hours, and to avoid any
mortality, was increased to approximately 115 - 130 Torr (75% - 85% saturation) for the second 24 hour period.

**Table 4.** Two day exposure control and treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Control (Pure, artificial sand, lab grade: 40-100 mesh)</th>
<th>Endosulfan-spiked sand (.33 ppm)</th>
<th>James River Sediment (sediment and artificial sand mix, ratio .33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

n = number of blue crabs

**Enzyme Assay**

The NAG enzyme assay was conducted by snap-freezing the epidermal and hepatopancreas tissues with liquid nitrogen and storing at -80°C until analysis. Following Zou (2009), a 0.5 cm² piece of epidermal tissue was sampled from under the carapace, and 30 – 50 mg pieces of hepatopancreas tissue were sampled from each crab. Tissue amounts varied, but at least two samples of each tissue type were collected from each crab. Tissues were homogenized on ice in 0.15 M pH 5.5 citrate-phosphate buffer supplemented with proteolytic inhibitors including 2 µg/ml leupeptin, 1 µg/ml each of aprotonin, pepstatin A, and E-64, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF) as recommended by Finn et al. (1998). After centrifugation at 10,000g for 3 min., 20 µL of supernatant was incubated with 100 µL of 2 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) at 25°C for 15 minutes. The reaction was stopped by addition of 0.9 mL 0.5 M NaOH. Liberated nitrophenol was quantified at 405 nm with a spectrophotometer. Total protein in each sample was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots were stored at -80 °C until assayed for enzymatic activity, expressed as nmol nitrophenol liberated (µg protein)⁻¹ (15 min)⁻¹.
Statistical Methods

For the analysis of oxygen uptake, the following equation (based on MacDonald 1977) calculates on a weight specific basis as micromoles of oxygen per kilogram body weight per minute ($\mu$mol O2/ kg-min), denoted as $\dot{M}O_2$:

$$\frac{\Delta O_2 \text{ in Torr}}{\Delta 30 \text{ min. interval}} \times 1.7582 \ \mu \text{mol} \ \times \frac{(1.86 \ L) - (\text{crab+sediment L}) \ \times 1}{\text{L-Torr}} \times \frac{1}{\text{crab kg}} \times \frac{1}{\text{kg min.}} = \mu \text{mol O}_2$$

The average was taken across n crabs for each time point for the control and exposure treatment oxygen uptake, respectively. The linear regression was calculated for the control oxygen uptake and the exposure treatment oxygen uptake and the slopes compared for significance ($p < 0.05$). To determine if the initial and final $\dot{M}O_2$ for the control and endosulfan exposure (respectively) were significantly different, the mean for $n = 6$ measurements for each treatment were compared using a paired t-test. Finally, a comparison of differences was performed: (average exposure treatment $\dot{M}O_2$ for each time point) – (average respective control $\dot{M}O_2$ for each time point). A linear regression of these was performed as well, and the slopes compared for significance ($p < 0.05$).

For the NAG enzyme analysis, a one-way analysis of variance (ANOVA) and Tukeys (post-test) was used to test the significance of the difference between NAG activities of the blue crabs under different exposure treatments (Zou 2009). A probability value of less than 0.05 was deemed as significant.
Results

2010 Collection

A total of 36 blue crabs were collected, but none were successfully collected on November 20, 2010 when the water temperature had dropped to 11°C (Figure 4).

![Collection Date Temperature and Catch](image)

**Figure 4.** The temperature and catch for each collection date. As temperatures dropped, catch dropped until November 20, 2010 when no crabs were collected.

Despite the blue crab’s sexually dimorphic life history, four females were collected in the tidal freshwater reach of the James River (Table 5).

**Table 5.** Blue crab characteristics and growth morphometrics

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Weight (g)</th>
<th>Carapace Width (mm)</th>
<th>Ave. Days till Molt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
<td>Average  Range</td>
</tr>
<tr>
<td>36</td>
<td>32</td>
<td>4</td>
<td>8.59 0.40-34.44</td>
<td>47 17-88</td>
</tr>
</tbody>
</table>
A linear regression of the percent weight increase per molt event data showed no discernible trend \( (r^2 = 0.0001) \); the average percent weight increase was almost 40\% (Figure 5). There was an increasing trend \( (r^2 = .1136) \) in percent carapace width increase per molt, ranging from a 5\% to almost a 30\% increase per molt.

**Figure 5.** A linear regression of the data shows an almost 40\% weight increase per molt.

**Figure 6.** A linear regression of the data indicates an increasing trend of approximately 10-15\% carapace width increase per molt.
Blue crab collection characteristics are given in Table 6 for the oxygen uptake experiment and Table 7 for the NAG analysis.

**Table 6.** Blue crab characteristics collected for the respirometry experiment

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Weight (g)</th>
<th>Carapace Width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.** Blue crab characteristics collected for the enzyme analysis

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Weight (g)</th>
<th>Carapace Width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>20</td>
<td>16</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Oxygen Uptake**

Oxygen uptake was measured for at least 5.5 hours prior to the seven day exposure and used as the control oxygen uptake. There were no mortalities throughout the experiments. The crab weights and carapace widths were not significantly different across treatment groups. The various treatment substrates were run alone for oxygen uptake, but only the James River sediment had a measurable $\dot{MO}_2$, which was then subtracted from the total (crab + sediment) $\dot{MO}_2$.

There was a non-significant difference in oxygen uptake ($\dot{MO}_2$) between no substrate present and pure sand (Figure 7). Both endosulfan and James River exposures show a lower oxygen uptake relative to their respective controls (Figures 8 and 10), but only the James River exposure is statistically significant relative to its control. However, the paired t-test of initial versus final oxygen uptake was significant for both the control and the endosulfan exposure.
treatment (Figure 9). The \( \text{MO}_2 \) did not change with time during the respirometry experiment for the substrate vs. sand exposure and the James River exposure. In a comparison of differences between the treatments (Figure 11), the apparent suppression of oxygen uptake is apparent for the James River and endosulfan treatments.

**Figure 7.** A linear regression of the average oxygen uptake at each time point after exposure to No Substrate and Pure Sand are not significantly different \((p = 0.65; F = 0.21)\); the intercepts are significantly different \((p < 0.0001; F = 353.77)\).
Figure 8. A linear regression of the average oxygen uptake at each time point after exposure to endosulfan was not significantly different from the control (p = 0.13; F = 2.51); the intercepts are significantly different (p < 0.0001; F = 72.23).

Figure 9. In a comparison of initial versus final oxygen uptake values for both the control and endosulfan, both were found to be significantly different (p < 0.05).
Figure 10. A linear regression of the average oxygen uptake at each time point after exposure to James River sediment was significantly different from the control (p < 0.0001; F = 40.63).

Figure 11. The slopes of the differences (exposure – control) for each treatment were not significantly different (p = 0.056; F = 3.14); the intercepts were significantly different (p < 0.0001; F = 3273.55).
Enzyme Activity

NAG activity was determined in the epidermal and hepatopancreas tissues of crabs exposed to one of six treatments. There were no mortalities throughout the experiments. Epidermal NAG activity was significantly suppressed in the treatment groups (NorJR, HypJR, NorE, HypE) relative to the crabs in hypoxia alone, HypC (* p < 0.001); and significantly suppressed in HypJR and NorE relative to NorC (# p < 0.05) (Figure 12). Unlike epidermal NAG activity, a pattern for hepatopancreas NAG activity is less clear (Figure 13). NAG activity for NorC was significantly different from NorJr (p < 0.001), NorE (p < 0.001), and HypJR (p < 0.01). NAG activity for HypE was significantly different from NorJR (p < 0.05) and NorE (p < 0.001). Additionally, the blue crabs exhibited a non-settling behavior in the hypoxia/endosulfan exposure, followed by a stiffness of the appendages (Figures 14).

**Figure 12.** Epidermal NAG activity was significantly different in the treatment groups (NorJR, HypJR, NorE, HypE) relative to HypC (* p < 0.001); and significantly different in HypJR and NorE relative to NorC (# p < 0.05). NAG activity is expressed as nmol nitrophenol liberated (µg protein)⁻¹ (15 min)⁻¹. Error bars indicate standard error of the mean; F = 10.36. NorC: normoxia and pure sand; HypC: moderate hypoxia and pure sand; NorJR: normoxia and James
River sediment; HypJR: moderate hypoxia and James River sediment; NorE: normoxia and endosulfan; HypE: moderate hypoxia and endosulfan.

**Figure 13.** NAG activity for NorC was significantly different from NorJR and NorE at $p < 0.001$, and HypJR at $p < 0.01$. NAG activity for HypE was significantly different from NorJR ($p < 0.05$) and NorE ($p < 0.001$). NAG activity is expressed as nmol nitrophenol liberated (µg protein)$^{-1}$ (15 min)$^{-1}$. Error bars indicate standard error of the mean; $F = 10.24$. NorC: normoxia and pure sand; HypC: moderate hypoxia and pure sand; NorJR: normoxia and James River sediment; HypJR: moderate hypoxia and James River sediment; NorE: normoxia and endosulfan; HypE: moderate hypoxia and endosulfan.

**Figure 14.** Juvenile blue crab exhibiting non-settling behavior and stiffness of the appendages due to endosulfan exposure.
Discussion

Blue Crab Growth Morphometrics

The results of the molting morphometrics demonstrated that, under conditions maintained in the lab, percent increase in weight and carapace width per molt event were both below that found in the literature. The percent weight increase per molt event was almost 40% (Figure 5), which is considerably lower than that recorded for the freshwater Potomac by deFur et al. (1988) at almost 100% weight increase per molt. There was a positive trend in percent carapace width increase per molt with increasing size, which agrees with the greater size achieved at later molts. However, a 10-15% increase is about half that of the typical increase (Churchill 1921, Newcombe et al. 1949). These lower rates of growth may be an artifact of captivity (Smith 1997), but it may also be due to earlier exposures to environmental contaminants in the Chesapeake Bay and the James River.

Oxygen Uptake

The oxygen uptake values fall within the range found in other studies for juveniles at low salinities and similar temperature. However, results in the literature show an increase in O₂ consumption at lower salinities compared to higher salinities. In King’s study (1965), the juvenile blue crab’s oxygen uptake in low salinity is 33% greater than in pure sea water. This may indicate that higher O₂ is a measure of increased stress during the respirometry experiment.
Also, that there may be a tradeoff between an ion gradient that supports greater molt increment (deFur et al 1988) and greater expenditure of energy in lower salinity waters.

Blue crabs are oxyregulators, capable of maintaining their oxygen consumption rate at a nearly steady state before reaching a critical oxygen concentration ($P_{\text{crit}}$) when the crab becomes an oxyconformer (Randall et al 2002). The non significant increase in oxygen uptake in the presence of a substrate (pure sand) most likely indicates the sand is a tactile stimulus for the crab, which accounts for the higher oxygen consumption. In the case of the endosulfan experiment, the blue crabs may have reached $P_{\text{crit}}$ faster, as indicated by the precipitous drop and the significant difference between initial vs. final. The average consumption after exposure to James River sediment was much lower compared to controls, but was stable across time, which may have indicated the crabs were still oxyregulating. In future studies, analysis of hemolymph lactate would indicate whether there had been a shift to compensatory anaerobic metabolism.

Suppression of oxygen uptake by endosulfan in this study agrees with the literature (Sarojini et al 1989, Venkateshwarlu 2005, Montagna and Collins 2008) but the exact mechanism of action is unknown. For some chemicals, an initial increase in oxygen consumed is required to support the enhanced physiological activities in metabolizing and eliminating pollutants, causing metabolic stress. Other chemicals cause a decrease in oxygen consumed due to the failure of the crab to compensate for the new state of metabolism due to the stress caused by the contaminant (Sarojini et al 1989). Naphthalene, a PAH, has been shown to significantly impair the oxyregulating capacity of the brown shrimp subjected to hypoxia (Zou and Stueben 2006). The multi-step process needed to biotransform naphthalene is well understood in vertebrates, but not in crustaceans. Nevertheless, the oxygen-consumption required by naphthalene in its metabolism may partly explain the reduction in the shrimp’s ability to
oxyregulate (Zou and Stueben 2006). Also, cellular damage or changes to gill structures may contribute to the suppression of oxygen uptake (Lloyd 1960, Bhaven and Geraldine 2000). Bhaven and Geraldine (2000) found extensive damage to the gills and hepatopancreas of freshwater prawns exposed to sublethal doses of endosulfan. The prawns exhibited swelling and fusion of the gill lamellae, as well as thickened basal laminae with hemocytes blocking the tubules of the hepatopancreas. Possible impairment of hemocyanin oxygen affinity, as has been found in cases of prolonged hypoxia, could contribute to changes in oxygen uptake. Pesticides that are cholinesterase inhibitors may inhibit certain enzymatic pathways. Finally, endosulfan is a neurotoxin and GABA inhibitor that blocks Cl- channels. This channel blocking causes uncontrolled excitation which can cause paralysis of muscles and affect the beating frequency of gill structures, resulting in a suppression of oxygen uptake.

James River sediments contain metals, chlordecone (i.e. Kepone), polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), tributyltin (TBT), DDT, and other organophosphates and organochlorines, some at concentrations that exceed sediment quality guidelines set by the National Oceanic and Atmospheric Administration (NOAA) (VA DEQ 2005). Although biota sediment toxicity tests conducted with James River sediment do not show high mortality (VA DEQ 2005), these tests are not run under hypoxic or high temperature scenarios. Natural storm events, like those that occurred in 2011, can greatly increase runoff and produce a large influx of contaminated sediments into the benthic environment (Hose et al. 2002). The influx of contaminants can add up to a combined exposure that may potentially work on the same endpoint, no matter their mechanism of action.
**Enzyme Activity**

This study’s findings are similar to those of Zou (2009) showing a reduction in epidermal NAG activity for brown shrimp (*Penaeus aztecus*) due to hypoxia and a sediment contaminant, naphthalene. Naphthalene was sediment bound at 40 ppm, and dissolved oxygen ranged from 1.0 to 2.6 mg/L, severe hypoxia. Zou showed that, alone, each stressor did not significantly affect the NAG activity, but the combination of the two stressors significantly decreased epidermal NAG activity. This result indicates the possibility for hypoxia to intensify the molt-interfering effects of common sediment contaminants. However, the current experiment utilized only moderate levels of hypoxia (65% - 85% saturation) that are more commonplace than are areas of severe hypoxia (i.e. dead zones). Also, the current study found further significant differences between the contaminant stressors, endosulfan and James River sediment, alone. The most surprising result was the exposure to James River sediment suppressed NAG activity to levels similar to the endosulfan exposure. The concentration of endosulfan used (0.33 ppm) in this study was not an environmentally relevant concentration, but was expected to produce the lower bound for the epidermal NAG activity. However, because the James River sediment results are so similar, these results indicate the molt-interfering capacity of the sediments. Despite the low level concentrations for individual contaminants found in the James River, the presence of several major groups (i.e. metals, PAHs, organochlorines, etc) and their potential to act on a similar endpoint is more relevant in determining toxicity.

The exact mechanisms of several contaminants that interfere with molting have not been determined, but some proposed mechanisms of action have been discussed in the literature. There is evidence that chitinolytic enzymes are the products of ecdysteroid regulated genes in arthropods. In *Uca pugilator*, the levels of chitobiase activity in both the epidermis and the
hepatopancreas change during the molting cycle and correlate with the levels of ecdysteroids in the hemolymph (Hopkins 1983). This correlation is also demonstrated by an increase in the expression of the chitobiase gene in the epidermis and gut of the tobacco hornworm, *Manduca sexta*, after administration of 20-hydroxyecdysone (Zen 1996). Molt-interfering contaminants can attack along any part of the hormonal cascade involved in molting (Zou 2005) (Figure 15). Ecdysteroids regulate gene activities at the transcriptional level, at the ecdysteroid receptor (EcR), which forms a complex with the Retinoid X Receptor (RXR) and binds to DNA. The EcR has been proposed as a possible target for those contaminants that can act as steroid mimics (Wing 1988, Sohi et al 1995). Because the EcR has been identified in both the epidermis and the hepatopancreas, both are possible sites of attack by xenobiotics (Londershausen and Spindler 1985).

![Diagram of hormonal control of molting](image)

**Figure 15.** Hormonal control of molting in decapods crustaceans as depicted by Zou (2005).
Conclusions

The combination of neurotoxic effects most likely decreased the juvenile blue crabs’ ability to oxyregulate while exposed to endosulfan. Exposure to James River sediment also affected oxygen uptake, indicating metabolic stress. To analyze compensatory anaerobic activities, future studies should include hemolymph analysis for calcium, lactate and urate. The suppression of epidermal chitobiase activity in both endosulfan and James River sediment exposures indicates the molt-interfering capacity of both, although the exact mechanisms of action are still unknown. These studies indicate the short-term physiological effects and potential for long-term growth effects of xenobiotics on blue crabs.

Even as endosulfan is currently being phased out across the globe, its mechanism of action on non-target species is still unknown. And as hypoxia spreads due to global warming, studies will need to determine the cumulative physiological effects of the simultaneous exposure to hypoxia, and other stressors, on organisms. A re-evaluation of the definition of hypoxia will need to encompass the more sensitive taxa and life stages. A more relevant value for wildlife will become the study of the sub-lethal effects of hypoxia, as well as the effects of moderate hypoxia. Evaluating multiple stressors in the environment and their physiological effects on organisms remains an area for continued study in the field of toxicology.
Literature Cited


Zou, E. 2009. Effects of hypoxia and sedimentary naphthalene on the activity of N-acetyl-β-


VITA

Laura Elizabeth Williams was born on December 20, 1980 in Virginia and is a US citizen. She graduated from J.R. Tucker High School, Richmond, VA in 1999. Laura received her Bachelor of Science in Biological Sciences from the University of Mary Washington, Fredericksburg, VA in 2003. She studied ornithology and published “Facultative rest-phase hypothermia in free-ranging White-throated Sparrows” in May 2004. Since undergrad, she has worked as a field researcher, professional lab technician, and a collegiate teaching assistant. While enrolled in the Master of Science in Environmental Studies program at Virginia Commonwealth University, Richmond, VA, she has presented her research at multiple conferences and interned at an environmental consulting firm where she now works as a full-time Environmental Scientist.