

ABSTRACT

BELL, GEOFFREY WESZELY. The Impact of Episodic Hypoxia on Blue Crabs (*Callinectes sapidus*): from Molecules to Populations. (Under the direction of David B. Eggleston.)

Episodic hypoxia impacts mobile aquatic animals directly when animals die from exposure to low dissolved oxygen (DO) and indirectly when they avoid intrusions of hypoxic water and aggregate in shallow nearshore habitats where increased competition for resources and spatial overlap between predators and prey reduce growth and increase predation/cannibalism. It is difficult to assess the impact of episodic hypoxia on population dynamics because episodic hypoxic events differ in their severity, duration, and hydrodynamics (i.e., current velocity and strength of the DO frontal boundary). Moreover, some individuals within a population can become acclimated to hypoxia, which affects their behavioral responses to and survival of hypoxia. Therefore, a comprehensive approach examining the behavioral and physiological responses of mobile animals to hypoxia can help predict the impact of hypoxia on population dynamics.

I used a series of laboratory studies, coupled with molecular techniques, to test whether two potential molecular biomarkers (structure and concentration of the hemocyanin respiratory protein) would indicate blue crabs' (*Callinectes sapidus*) degree of physiological acclimation to low DO and influence their behavioral responses to and survival of hypoxia. Only hemocyanin (Hcy) structure correlated with blue crab behavior and survival, suggesting that Hcy "quality" is more important for survival than Hcy "quantity". Blue crabs with hypoxia-tolerant Hcy structures were acclimated to hypoxia, survived longer, and were more active under chronic hypoxic conditions than conspecifics with hypoxia-sensitive Hcy structures

Laboratory flume studies also identified the specific hydrodynamic and hydrographic cues blue crabs use to avoid hypoxia and how their physiology influences these behavioral avoidance responses. Drops in DO stimulated increased movement rates, regardless of whether the change resulted in hypoxia, suggesting that blue crabs may anticipate the onset of episodic hypoxic events. Moreover, faster rates of declining DO stimulated faster movement rates under hypoxic conditions, indicating that the hydrodynamic conditions under which crabs are exposed to hypoxia are important for structuring their behavioral responses. Blue crabs also tended to move down-current with declining DO, suggesting they may use current direction to orient away from hypoxia. Lastly, blue crabs with hypoxia-tolerant Hcy structures were less active under hypoxic conditions than conspecifics with hypoxia-sensitive Hcy structures. Therefore, physiological state affects blue crab survival and behavioral responses to hypoxia.

The final study used the functional relationships between physiology, behavior, and survival generated from the laboratory studies to develop a spatially-explicit, individual-based, population simulation model. This initial model: (i) identified which hydrodynamic factors exert the greatest effect on blue crab population escape responses and mortality rates during episodic hypoxic events, (ii) tested whether physiological acclimation (i.e., hypoxia-tolerant vs. -sensitive populations) can alter these population responses, and (iii) provided a preliminary assessment of the consequences of hypoxia-induced mortality to blue crab population dynamics and the blue crab fishery. The model predicts that: (i) direct mortality from hypoxia is low for blue crabs, (ii) blue crab distribution and abundance patterns are most strongly influenced by the duration of episodic hypoxic events and the strength of the frontal DO gradient, (iii) episodic hypoxic events do not have a substantial impact on blue

crab population dynamics, and (iv) the economic loss to the fishery is minor. Lastly, mortality rates during hypoxic events for blue crab populations with hypoxia-tolerant individuals were 35% lower than for populations with hypoxia sensitive crabs; therefore the physiological state of individuals in a population may influence population-level responses to stressors.

This is the first study documenting a physiological mechanism underlying individual-based differences in the behavioral responses to and survival of an invertebrate hypoxia. Our findings highlight the importance of understanding how physiological stress responses influence behavior and survival because these responses have implications at the population level. Moreover, quantifying the functional relationships between physiology, behavior, and survival is critical for developing mechanistic models that can predict how changes in the severity, duration, and frequency of disturbances over time in coastal ecosystems will impact ecological processes, particularly in the context of global climate change.

The Impact of Episodic Hypoxia on Blue Crabs (*callinectes sapidus*): from Molecules to
Populations

by
Geoffrey Weszely Bell

A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

Marine, Earth, and Atmospheric Sciences

Raleigh, North Carolina

2008

APPROVED BY:

David B. Eggleston
(Chair of Advisory Committee)

Edward J. Noga

James A. Rice

Thomas G. Wolcott

DEDICATION

This dissertation is dedicated to my family, especially my mother, brother, and sister who provided the essential support, guidance, and encouragement needed for its successful completion. Each of them has inspired me in a unique way. To my mother, Johanna Weszely, whose emphasis on education and excellence instilled in me the work ethic necessary to achieve my academic and professional goals. To my brother, Maj. Jeremy Bell, a dedicated brother, father, husband, and soldier who was my role model for the level of commitment required to complete my doctorate degree. To my sister, Kathy Bell, who provided key insights and wisdom that gave me the strength to persevere during the difficult times. Thank you.

BIOGRAPHY

Geoffrey Weszely Bell was born on July 14, 1973 and raised by his mother in Berkshire Valley, NJ. He spent much of his summers near the water, either at Lake Ariel, PA fishing, swimming, and boating or at the Jersey Shore. Geoffrey graduated from high school in May of 1991 and entered college the following fall without a major, but soon realized that he was particularly interested in marine science. He accepted a summer internship at the Rutgers University Marine Field Station (RUMFS) in Tuckerton, NJ where he participated in fish ecology fieldwork and developed an independent research project. This project later became an undergraduate honors thesis and marked the beginning of his career in marine science. Finally, after three different schools and six short years of college education Geoffrey finally graduated with a BS degree from Rutgers University in 1997. Thereafter, he briefly interned for the National Marine Fisheries Service in Sandy Hook, NJ, before returning to RUMFS to work as a technician.

Geoffrey left his home state in 1999 and moved south to Raleigh, NC to enroll in the Masters Degree program in biological oceanography at North Carolina State University. His masters thesis was an exercise in voyeurism as he closely followed blue crab movement and feeding behaviors throughout the Neuse River, patiently waiting for hypoxia. After completing his Masters thesis in 2002, Geoffrey thought: “I’ve been in school for 21 years, what’s another six?”, and enrolled in the PhD program at NC State. He decided to diversify his research skills and incorporate molecular techniques and modeling into his dissertation to gain a broader perspective on how ecological systems work. Geoffrey was very active in

extra-curricular activities while a graduate student. He developed and participated in several educational outreach programs, volunteered to teach classes, and served as an officer for his department's graduate student association. While in graduate school Geoffrey was fortunate to have met his wonderful girlfriend, Meredith, as well as an amazing group of friends.

ACKNOWLEDGEMENTS

I would like to thank my family who inspired me and helped me through the challenging times. My girlfriend, Meredith, also deserves special thanks for being so supportive and understanding while I made the final push to finish my dissertation. I would also like to thank my advisor Dr. David Eggleston for his encouragement that persuaded me to undertake a PhD and his insight that has made me a better scientist, my committee, Drs. Ed Noga, Tom Wolcott, and Jim Rice who provided resources that were critical for the completion of my research and offered unique perspectives that improved the quality of my research. I am also eternally grateful to all of my friends, colleagues, and lab-mates who helped with experiments, discussed difficult scientific issues over beer, provided expert advice, gave me valuable feedback, and commiserated with me when needed: Jone Coralles, Jeana Stephenson, Anirudh Ullal, Jung Kil Seo, Nathalie Reyns, Steve Searcy, Eric Johnson, Todd Kellison, Darren Parsons (a.k.a. Capucco), Chris Taylor, Felix Deltoro Silva, Gayle Playa, Grant Parkins, Ashton Drew, Cindy Huggett, Brandon Puckett, Ray Mroch, Mary Radlinski, Christina Durham, Carrie Thomas, Michelle Moorman, Sunny Snider, Jun Yoshizaki, Suzanne Snider (a.k.a S-Dog), John Bucci, Marius Brouwer, and Gerald Godette. I would also like to express my appreciation for the funding provided by the EPA STAR Fellowship Program, the NC Beautiful Fellowship Foundation, the National Science Foundation, the NC Blue Crab Research Program, the National Sea Grant College Program, the National Oceanic and Atmospheric Administration Marine Biotechnology Program, and the USDA - NRI Competitive Grant Program.

TABLE OF CONTENTS

	Page
LIST OF TABLES	ix
LIST OF FIGURES	x
CHAPTER 1	1
ABSTRACT	1
MATERIALS AND METHODS	6
RESULTS.....	11
<i>Survival</i>	11
<i>Physiology</i>	12
<i>Behavior</i>	14
DISCUSSION	15
ACKNOWLEDGEMENTS	20
LITERATURE CITED	21
APPENDIX	34
APPENDIX 1A.....	35
APPENDIX 1B	35
APPENDIX 1B	36
CHAPTER 2	37
ABSTRACT	37
MATERIALS AND METHODS	43

<i>Capture & Maintenance</i>	43
<i>Experimental Procedures</i>	43
<i>Hemocyanin Analyses</i>	46
<i>Statistical Analyses</i>	48
RESULTS.....	49
<i>Movement rates</i>	49
<i>Directionality</i>	52
<i>Burial</i>	53
<i>Physiology</i>	53
DISCUSSION	54
ACKNOWLEDGEMENTS	60
LITERATURE CITED	61
CHAPTER 3	73
ABSTRACT	73
METHODS.....	79
<i>Model description</i>	79
<i>Model Parameterization</i>	81
<i>Model Simulations, Hypotheses, & Statistical analyses</i>	90
RESULTS.....	94
<i>Hydrodynamics</i>	94
<i>Physiology</i>	98
<i>Sensitivity</i>	99

DISCUSSION	100
<i>Hydrodynamic and physiological mechanisms</i>	101
<i>Implications for other estuarine species</i>	104
<i>Implications for the blue crab fishery and population dynamics</i>	105
ACKNOWLEDGEMENTS	109
LITERATURE CITED	110
APPENDICES	129
APPENDIX 1A	130
APPENDIX 1B	131
APPENDIX 1C	133
APPENDIX 2A	136
APPENDIX 2B	138
APPENDIX 2C	140
APPENDIX 3A	142

LIST OF TABLES

	Page
CHAPTER 3	
Table 1	AIC results assessing the effects of hydrodynamic factors on the percent of crabs successfully avoiding hypoxic upwelling events.....118
Table 2	AIC results assessing the effects of hydrodynamic factors on the blue crab mortality during hypoxic upwelling events.....119
Table 3	AIC results assessing the relative effects of hydrodynamics and hypoxia tolerance on the percent of crabs successfully avoiding hypoxic upwelling events.....120
Table 4	AIC results assessing the relative effects of hydrodynamics and hypoxia tolerance on blue crab mortality.....121

LIST OF FIGURES

CHAPTER 1	Page
Figure 1 Locations of blue crab collections in coastal North Carolina.....	28
Figure 2 Image of PAGE gel and densitometer scan.....	29
Figure 3 Median Time to Death estimates.....	30
Figure 4 Hcy structure comparison.....	31
Figure 5 Functional relationship between Hcy subunits and Median time-to-death.....	32
Figure 6 Movement and burial behavior during hypoxic exposure.....	33
CHAPTER 2	
Figure 1 Effect of declining DO on movement rate.	68
Figure 2 Effect of declining DO on percent of time spent in the downstream end of the flume.....	69
Figure 3 Effect of declining DO on the percent of time spent buried.....	70
Figure 4 Comparison of Hcy subunit composition for fast- and slow-moving blue crabs.	71
Figure 5 Functional relationship between Hcy subunit proportion and movement rate.....	72
CHAPTER 3	
Figure 1 Conceptual diagram of model inputs, functions, and outputs.	122
Figure 2 Spatial distribution of blue crabs during chronic hypoxia and episodic hypoxic events.	123

Figure 3	Hydrodynamic factors influencing crab escape responses	124
Figure 4	Hydrodynamic factors influencing crab mortality	125
Figure 5	Effect of hypoxia tolerance on blue crab escape responses	126
Figure 6	Effect of hypoxia tolerance on blue crab mortality	127
Figure 7	Sensitivity analyses of blue crab movement parameter estimates.....	128

CHAPTER 1

MOLECULAR KEYS UNLOCK THE MYSTERIES OF VARIABLE SURVIVAL RESPONSES OF BLUE CRABS EXPOSED TO HYPOXIA

ABSTRACT

Hypoxia is a major stressor in coastal ecosystems, yet predicting its impacts on fish and shellfish populations across hypoxic events is difficult due to individual variability in behavioral responses and survival. Although aquatic animals have evolved diverse physiological responses to cope with hypoxia, we know little about the physiological basis for survival and behavioral decisions that modify survival under hypoxic conditions. We used a laboratory study coupled with molecular techniques to determine how extrinsic factors (e.g. collection site and temperature) and respiratory physiology (hemocyanin concentration and structure) affect survival and behavior of adult blue crabs (*Callinectes sapidus*) exposed to different levels of chronic hypoxia. Nearly 100% of crabs survived 30 hr at DO concentrations greater than $1.0 \text{ mg DO} \cdot \text{l}^{-1}$, suggesting that adult blue crabs are tolerant of severe hypoxia. Below $1.0 \text{ mg DO} \cdot \text{l}^{-1}$, median time-to-death decreased at lower DO and higher temperatures. Crabs collected from the hypoxic Neuse River Estuary, NC, USA survived longer and were more active under hypoxic conditions than crabs from normoxic Bogue and Back Sounds, NC indicating that blue crab populations in hypoxic estuaries can acclimate to hypoxia which enhances their tolerance of hypoxia. Hemocyanin (Hcy) concentration did not explain these geographic differences, but hypoxia-tolerant crabs had

Hcy structures indicative of acclimation (i.e, high O₂ affinity) suggesting Hcy “quality” (i.e. structure) may be more important than Hcy “quantity” (i.e. concentration) for surviving hypoxia. This is one of the first studies to document a physiological mechanism to explain differences among individuals in their survival and behavioral responses to a stressor. The geographic differences in survival we observed also highlight the importance of carefully selecting experimental animals when planning to extrapolate laboratory results to the population level.

INTRODUCTION

Oxygen depletion (i.e., hypoxia and anoxia) is among the most widespread and important environmental stressors facing coastal ecosystems today. The frequency, duration, and spatial extent of low dissolved oxygen (DO) in estuaries worldwide have increased over the past several decades due largely to increased nutrient loading from coastal development (Diaz and Rosenberg 1995). The impacts of hypoxia on populations of mobile animals are well-documented and include increased mortality rates from direct exposure (Breitburg 1992; Paerl et al. 1999; Farwell et al. 2007), habitat compression resulting from individuals avoiding hypoxia and aggregating in normoxic refuge areas (Pihl et al. 1991; Eby and Crowder 2002), and altered trophic dynamics (Pihl et al. 1992; Eggleston et al. 2005). Predicting the magnitude of these impacts for a given hypoxic event is difficult, however, since hypoxic bottom water is spatiotemporally dynamic (Breitburg 1990; Luettich et al. 2000; Reynolds-Fleming and Luettich 2004) and individuals within populations vary in their behavioral and survival responses to hypoxia (Breitburg 1992; van Raaij et al. 1996; Bell et al. 2003).

During some hypoxic upwelling events, thousands of mobile animals successfully avoid hypoxia, thereby forming dense aggregations along shorelines, termed “Jubilees” (Loesch 1960). At the other extreme, hundreds of thousands of fish can die during “fish kills” when they are trapped in upwelled hypoxic water (Breitburg 1992; Paerl et al. 1998). For moderate hypoxic upwelling events that occur almost daily in estuaries (Bell et al. 2003; Reynolds-Fleming and Luettich 2004), some individuals of a given species successfully avoid hypoxia while others are trapped in low DO for several hours and may suffer substantial mortality in

some cases (Breitburg 1992; Bell et al. 2003; Bell and Eggleston 2005). It is unknown why mobile animals exhibit such large variation in their behavioral and survival responses to dynamic hypoxia. Discovering the physiological mechanisms for this variability may be key to predicting when hypoxia alters distribution and abundance patterns of mobile species, as well as its associated direct and indirect effects (e.g. increased mortality rates, altered trophic dynamics, and depressed growth rates).

Risk of mortality from hypoxia exposure may be mitigated by the physiological state of an individual if prior sublethal hypoxia encounters induce physiological changes that allow individuals to acclimate to low DO concentrations (Rees et al. 2001). Studying hypoxia-induced changes in the oxygen-binding proteins of aquatic animals, in particular hemoglobins (Hgb) for fish and hemocyanins (Hcy) for crustaceans, is perhaps the best way to understand the significance of physiology for acclimation to stressors because these molecules respond directly to DO (Powers 1980). Increased respiratory protein concentration is one of the most common responses to hypoxic exposure for both fish and invertebrates because it raises the oxygen carrying capacity of the blood/hemolymph (Mangum 1997; Pirow et al. 2001; Timmerman and Chapman 2004). Unlike vertebrate Hbs, however, crustacean Hcys are oligomers composed of a dozen or more different monomer subunits. During exposure to low DO, some crustaceans synthesize new Hcy molecules using a portion of the subunit types; the new Hcy molecules have a higher oxygen affinity than Hcys synthesized under normoxic conditions (DeFur et al. 1990; Mangum 1997). After several days of exposure to low DO, the relative proportion of the subunit types in the hemolymph will change as specific subunits are preferentially used to synthesize new Hcys.

Although acclimation to hypoxia can enhance survival, very few studies have taken the next step to bridge the gap between physiology and ecology (Brouwer et al. 2007) by testing which physiological acclimation mechanisms are most important for enhancing survival under hypoxic conditions. Identifying these mechanisms and quantifying their relationship with survival is important for predicting if inter-individual variability in physiological state affects population dynamics.

In this study, we examine the interactive effects of hypoxia, temperature, and physiological state on survival of a euryhaline crustacean, the blue crab (*Callinectes sapidus*). Blue crabs are ecologically and economically important and are one of the most common mobile animals in estuaries prone to hypoxia (Pihl et al. 1991; Bell and Eggleston 2005). Blue crabs have many physiological responses to hypoxia (Mangum 1997) but only two, Hcy concentration and Hcy structure, have been shown to vary among individuals in wild populations (Engel et al. 1993; Mangum 1994). Exposure of blue crabs to low DO increases their Hcy concentration (Mangum 1997; Brouwer et al. 2004), which raises the oxygen carrying capacity of the hemolymph. Blue crab Hcy is an oligomer protein containing six monomer subunits and when crabs are exposed to moderate hypoxia they synthesize Hcys with three of the six subunit types (DeFur et al. 1990; Mangum 1997). After approximately one week of exposure to low DO the relative proportions of the subunit types in the hemolymph change. Hcys with only three of the subunit types have higher O₂ affinity than Hcys with the full complement of all six subunits (DeFur et al. 1990); therefore, blue crabs may acclimate to hypoxia by increasing the number of Hcy molecules in their hemolymph (i.e., Hcy concentration) or by synthesizing Hcys with three of the six subunit

types (i.e., Hcy structure). These acclimation responses are assumed to be adaptive for survival in hypoxia (Mangum and Towle 1977) but the relative importance of Hcy concentration and structure for survival has not been tested.

We used mortality experiments analyzed with time-to-event models to examine how individual-level characteristics (e.g. sex, size, collection site, etc.) and molecular-level differences (Hcy concentration and structure) influence survival. Time-to-event models (Dixon and Newman 1991) are preferable to more traditional LC_{50} methods because they generate functional relationships between any number of independent variables and ecologically meaningful estimates of survival (e.g., median time-to-death). Use of time-to-event models can establish links between animal physiology and ecology to help understand the adaptive significance of physiological acclimation and provide a mechanistic understanding of how environmental stressors affect wild populations.

MATERIALS AND METHODS

Adult blue crabs (carapace width [CW] > 100 mm) were collected during summer of 2004 from two general locations in coastal North Carolina: (i) Bogue and Back Sounds, and (ii) the Neuse River Estuary (NRE, Fig. 1). Although average summer temperatures are similar at the two sites ($\sim 28^{\circ}\text{C}$), Bogue and Back Sounds have higher summer salinity ranges (32 – 35 psu) than the NRE (12 – 17 psu). The frequency, duration, and spatial extent of hypoxia also differ between the sites. Hypoxia is either rare or nonexistent in Bogue and Back sounds but the NRE experiences seasonal hypoxia and anoxia annually during summer months (Paerl et al. 1998; Luetlich et al. 2000). Hypoxic bottom water in the NRE is

spatiotemporally dynamic, lasts from weeks to months, and can cover as much as 100 km² of bottom habitat (Luettich et al. 2000).

We used crab pots, gill nets, and various trawls to collect crabs. Crabs were transported in coolers to the lab and placed in holding tanks (1 m x 2 m) that received flow-through, filtered seawater from Bogue Sound. Temperature and salinity in the holding tanks ranged from 25.1 – 29.3°C and 26.9 – 33.6 psu, respectively. Crabs were fed *ad libitum* daily with dead fish. Crabs were held for no more than one week because it takes roughly seven days for blue crabs to significantly alter their Hcy structure in response to either salinity or hypoxia (Mason et al. 1983; DeFur et al. 1990). Experiments were conducted in eight rectangular 62-l aquaria, which were part of a 1000-l recirculating system that generated three hypoxic treatments and a normoxic control (6.0 mg·l⁻¹) using a series of three columns that sequentially stripped oxygen by bubbling nitrogen gas (Taylor and Miller 2001). Target DO concentrations for the three hypoxia treatments were 0.5 mg·l⁻¹, 1.0 mg·l⁻¹, and 1.5 mg·l⁻¹. The actual DO concentrations achieved during experiments were slightly lower due to DO fluctuations (0.42 ± 0.01 mg·l⁻¹, 0.78 ± 0.02 mg·l⁻¹, and 1.30 ± 0.03 mg·l⁻¹, respectively). Water from the columns fed 19-l buckets that provided constant head pressure and maintained a flow rate of 0.60 l·min⁻¹ through each tank (a complete exchange of water every 1.7 hr). DO treatments were randomly assigned to aquaria and for each trial, one of two temperature treatments (25°C or 30°C) was applied to all aquaria.

Three sides of each aquarium were wrapped in polystyrene insulation to maintain a constant temperature and prevent crabs from reacting to the observer or to crabs in other tanks. The fourth side of each tank was left clear for behavioral observations. To limit the

diffusion of oxygen into experimental tanks, a rigid, opaque plastic sheet with flexible plastic sheets taped to its edges was floated on the surface of the water to seal off the air-water interface. DO and temperature were spatially homogeneous within each of the tanks. A 3-cm thick layer of sandy substrate was placed in each tank so that burial of crabs, which is an important anti-predator and habitat utilization behavior, could be observed.

Only intermolt males and females, as well as mature females past terminal molt but without eggs were used in experiments. Crabs were measured (mm CW), sexed, and bled for hemolymph (see below) before introduction to an aquarium. One crab was randomly assigned to each of the aquaria, which contained the same seawater that supplied the holding tank. After all crabs were placed in the aquaria, they were brought to experimental conditions (salinity: 17 psu and temperature: 25°C or 30°C) over a 3-hr period. Crabs were then given 15 hr to acclimate to these conditions before starting experiments. The acclimation period allowed crabs to recover from handling stress, adjust to the experimental tanks, and provided Bogue Sound crabs with enough time for short-term, salinity-induced physiological responses to subside (e.g., increased O₂ consumption, cardiac output, and urine production, as well as deamination of free amino acids) (Gerard and Gilles 1972; Robinson 1994; McGaw and Reiber 1998). Although the relatively low salinity in our experiment may have caused more osmoregulatory stress for Bogue and Back Sound crabs than for NRE crabs, it is unlikely that salinity substantially influenced the differences in survival between collection sites (see Discussion).

Experiments began when target DO concentrations were reached in each aquarium (~ 3 hr after nitrogen bubbling had started in the columns). Every 3 hr, DO and temperature were

monitored and adjusted as needed, crab behavior (movement, posture, burial) was observed for one minute, and any deaths were recorded. If crabs appeared moribund (e.g. quiescent, no antennule flicking, and reduced responsiveness to physical stimulation) during an observation period they were monitored more frequently to accurately pinpoint time of death. Time of death was recorded when crabs showed no appendage, eye stalk, or antennule movement in response to mechanical stimulation. Experiments lasted 30 hr to correspond with the maximum exposure time of free-ranging crabs to hypoxia (Bell et al. 2003).

Before each crab was placed in an aquarium, a 0.1 ml sample of hemolymph was collected from the base of the swimmerette with a needle and stored at -70 °C. The clotted hemolymph was thawed, homogenized with a microtube pestle for 5 min, and centrifuged at 15,000 rpm and 4 °C for 15 min to collect the supernatant. To measure hemocyanin concentration, duplicate 1:60 dilution samples were prepared by mixing 3 µl of serum with 177 µl of a 50 mM Tris HCl + 10 mM CaCl₂ buffer solution (pH = 8.0). The absorbance of each duplicate sample was measured against a blank of pure Tris-Ca buffer at 334 nm wavelength (maximum absorbance of Hcy) with a spectrophotometer. Hemocyanin concentration (mg·ml⁻¹) was calculated using an extinction coefficient of 2.3 (Johnson et al. 1984).

We used native discontinuous polyacrylamide gel electrophoresis (PAGE) to quantify Hcy structure. We diluted 25 µg of hemocyanin 1:60 (hemolymph volume needed to obtain this amount of Hcy was calculated using each crab's Hcy concentration) with sample buffer (0.05 M Tris-HCl + 0.01 M EDTA + 10% glycerol + 0.002% Bromophenol blue tracking dye). Samples were kept overnight at 4°C to dissociate Hcy into its component subunits. The 0.75-mm thick gels (8 x 7.4 cm) consisted of a 3% stacking gel atop a 12.5% resolving gel (Hames

and Rickwood 1990). The discontinuous buffer system consisted of a Tris-Glycine (0.025 M Tris, 0.20 M glycine; pH 8.1) upper chamber buffer and 0.025 M Tris-HCl (pH 8.9) lower chamber buffer. Gels were run at 4 °C (to minimize heating) and constant current (15 mA for 2 gels) until voltage reached 180 V (~ 7 hr for 2 gels). The resolving gel was stained with Coomassie Blue R-250 for 40 min and then destained overnight with a 40% methanol, 10% acetic acid solution. Gels were scanned with image analysis software to identify and measure the optical density of subunit bands. Subunits were identified as 1-6 based on their relative electrophoretic mobility from prior studies (Fig. 2) (DeFur et al. 1990; Mangum 1994). We calculated the proportion of each subunit band relative to the total optical density of all subunit bands.

Parametric and semi-parametric time-to-event models were used to assess the survival of blue crabs collected from the NRE as well as Bogue and Back Sounds exposed to various DO and temperature combinations while accounting for several covariates including sex, size (i.e. CW), as well as Hcy concentration and structure. The ‘event’ was the time (hr) of death, relative to the start of the experiment. Parametric models compute median time-to-death (MTTD) for specific sets of experimental conditions and individual covariates. These models require that the underlying error distribution be correctly specified (Dixon and Newman 1991; Newman and Dixon 1996), so we used Akaike’s information criterion (AIC) estimates to objectively choose from five commonly used error distributions (Newman and Dixon 1996). The log-logistic distribution was chosen as the underlying error distribution for all models because its AIC value was always smallest and its model probability was never less than 0.71 (i.e. probability of this error distribution being the correct error distribution

among all distributions tested). In contrast to parametric models, semi-parametric Cox proportional hazard models test for differences in survival functions between treatments. Survival functions describe the probability of survival across stressor exposure times given a specific set of experimental conditions and individual covariates. The differences in survival responses to hypoxia among treatment levels were similar for both model types; therefore, we only present results for the parametric time-to-event models (see Appendix 1 for semi-parametric Cox proportional hazard model results). Results are also only presented for DO concentrations less than $1.2 \text{ mg DO}\cdot\text{l}^{-1}$ because survivorship was nearly 85% after 30 hr in the $1.5 \text{ mg DO}\cdot\text{l}^{-1}$ treatment and there was no mortality during the $6.0 \text{ mg DO}\cdot\text{l}^{-1}$ control.

Several ANOVA and MANOVA models tested for patterns in crab behavior (e.g., movement activity and burial), Hcy concentration, and Hcy subunit structure as a function of exposure to low DO. In cases where response variables violated the ANOVA assumptions of normality or homogeneity of variance, nonparametric analyses were used. Wilcoxon tests were used for main effects with only two treatment levels and Kruskal-Wallis tests for main effects with more than two treatment levels (Sokal and Rohlf 1981). Separate Wilcoxon tests were also used for multiple comparisons among treatment levels for significant main effects.

RESULTS

Survival

Although survival of adult blue crabs generally declined with increasing severity of hypoxia (Fig. 3), crabs survived well under severe hypoxia and near-anoxia conditions. After 30 hr, 84.4% of crabs in the $1.5 \text{ mg}\cdot\text{l}^{-1}$ treatment were still alive, whereas 54.8% survived at $1.0 \text{ mg}\cdot\text{l}^{-1}$. Percent survival after 30 hr was low (3.1%) only when crabs were exposed to DO

concentrations below $0.5 \text{ mg}\cdot\text{l}^{-1}$. Median-time-to-death (MTTD) significantly increased with increasing DO ($p < 0.05$) in all cases but depended upon collection site, temperature, and crab size (Fig. 3). Crabs from the Neuse River survived twice as long as crabs from Bogue and Back Sound, irrespective of DO concentration (Fig. 3A). The absolute difference in MTTD between crabs from the two collection sites was more pronounced at higher DO concentrations, increasing from 5.5 hr at near-anoxia to 20 hr at severe hypoxia ($\sim 0.8 \text{ mg DO}\cdot\text{l}^{-1}$). Significant collection site-by-DO ($p = 0.035$, $df = 1$) and collection site-by-size interactions ($p = 0.041$, $df = 1$) precluded interpretation of the collection site effect and required that we develop separate models for each site.

Survival was lower at the higher temperature for crabs from both collection sites. MTTD of Bogue and Back Sound crabs was significantly greater at 25 C than 30 C ($p = 0.001$), with the temperature difference in MTTD less pronounced at the lowest DO concentrations (~ 4 hr at $0.25 \text{ mg}\cdot\text{l}^{-1}$) compared to higher DO concentrations (~ 17 hr at $0.9 \text{ mg}\cdot\text{l}^{-1}$) (Fig. 3B). For NRE crabs, MTTD significantly decreased by 10 - 20 hr between 25°C and 30°C across all hypoxic DO concentrations (compare open vs. closed symbols in Fig. 3C) ($p < 0.001$, $df = 1$). Although body size (CW) had no effect on the survival of crabs from Bogue and Back Sounds, survival time was inversely related to carapace width for NRE crabs (Fig. 3C). MTTD was greater by 3 - 9 hr for small (mean CW = 110 mm) than large (mean CW = 140 mm) NRE crabs given any combination of DO and temperature (Fig. 3C).

Physiology

Based on our findings from the time-to-event models that collection site and NRE crab CW influence survival, we hypothesized that crabs with greater hypoxia tolerance

would have greater Hcy concentrations than crabs that were less tolerant of hypoxia (i.e., NRE vs. Bogue and Back Sound crabs, and small vs. large NRE crabs). Contrary to our original hypothesis, the more tolerant NRE crabs had Hcy concentrations ($\mu = 33.43 \text{ mg}\cdot\text{ml}^{-1} \pm 1.94$) that were nearly half that of the less tolerant Bogue and Back Sound crabs ($\mu = 60.71 \text{ mg}\cdot\text{ml}^{-1} \pm 4.41$) (ANOVA: $p < 0.001$, $df = 1$). There was no significant effect of carapace width on Hcy concentration nor was there a significant interaction between collection site and crab size on Hcy concentrations (ANOVA: $p > 0.05$, $df = 1$).

Although hypoxia-tolerant crabs did not possess higher Hcy concentrations than hypoxia-sensitive crabs they did have different Hcy structures than their less tolerant counterparts. For example, overall Hcy structure significantly differed between NRE and Bogue/Back Sound crabs (MANOVA – Wilkes Lambda statistic: $p < 0.001$, num. $df = 6$, den. $df = 51$; Fig. 4A), as well as small and large NRE crabs (Wilkes Lambda statistic: $p = 0.003$, num. $df = 6$, den. $df = 26$; Fig. 4B). In general, the Hcys of hypoxia-tolerant crabs had a higher proportion of subunit 1 and reduced proportions of all other subunits (Fig. 4). The proportions of subunits 1, 3, 4, and 6 were significantly different between collection sites, whereas the proportions of subunits 1, 2, 4, 5, and 6 were significantly different between large and small Neuse River crabs (MANOVA univariate results: $p < 0.05$, $df = 1$). The consistent differences in Hcy structure between hypoxia-tolerant and hypoxia-sensitive crabs suggests that Hcy structure may be the reason for individual-level variation in blue crab survival.

To quantify the functional relationship between Hcy structure and survival, we used percent composition of subunits 1 & 4 (subunits showing the strongest correlation with

survival) as covariates in parametric time-to-event models to predict MTTD at each DO and temperature treatment combination. Inter-individual variation in percent composition of subunit 1 was greater than any other subunit (range = 1.4% - 33.7%) and was the only subunit positively related to MTTD (Fig. 5A). MTTD increased significantly with increasing percent composition of subunit 1 for all DO and temperature combinations (parametric time-to-event model: $p < 0.001$, $df = 1$). A 30% increase in subunit 1 percent composition increased MTTD by 3.2 hr in the most severe hypoxic exposure ($0.5 \text{ mg} \cdot \text{l}^{-1}$ DO & 30°C) and increased MTTD by 6.5 hr and 9.7 hr in the $0.5 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ & 25°C and $1.0 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ & 30°C treatments, respectively. The 15% increase in subunit 1 percent composition in the least severe hypoxic exposure ($1.0 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ & 25°C) raised MTTD by 6.2 hr. Percent composition of subunit 4 was less variable among crabs (range = 9% - 36%) than subunit 1 but its negative relationship with MTTD was representative of the pattern for most other subunits (Fig. 5B). MTTD significantly decreased with increasing percent composition of subunit 4 for all DO and temperature combinations (Fig. 5B; $p < 0.001$, $df = 1$). Increases in percent composition of subunit 4 decreased MTTD by as little as 8.5 hr and as much as 26.4 hr depending on the DO and temperature treatment combination (Fig. 5B).

Behavior

As expected, blue crab movement and burial behavior were diminished in severe hypoxia. Percent of time spent moving at the $0.5 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ ($6.80\% \pm 1.68$) and $1.0 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ ($9.57\% \pm 2.73$) treatments were significantly less than at the $1.5 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ treatment ($24.39\% \pm 3.74$;) and $6.0 \text{ mg} \text{ DO} \cdot \text{l}^{-1}$ control ($30.82\% \pm 6.74$) (Fig. 6A; multiple comparison Wilcoxon tests: $p < 0.008$ for all comparisons, $df = 1$). There was no significant difference in crab

movement between the 1.5 mg DO·l⁻¹ and 6.0 mg DO·l⁻¹ treatments (Fig. 6A; multiple comparison Wilcoxon test: $p = 0.25$, $df = 1$). NRE crabs, however, were able to sustain greater locomotor activity under moderate to severe chronic hypoxia than were Bogue and Back Sound crabs. NRE crabs spent a significantly greater percent of their time moving than Bogue Sound crabs at the 1.0 and 1.5 mg DO·l⁻¹ treatments (Fig. 6A; multiple comparison Wilcoxon tests: $p < 0.001$ for all comparisons, $df = 1$). The percent of time crabs spent buried in all hypoxic treatments was significantly less than in the 6.0 mg/l control ($p < 0.005$; $df = 1$). Moreover, percent of time spent buried was significantly less at 0.5 and 1.0 mg DO·l⁻¹ than at 1.5 mg·l⁻¹ ($p < 0.007$ for both comparisons, $df = 1$) (Fig. 6B). Therefore, although DO concentrations below 1.5 mg DO·l⁻¹ likely cause blue crabs to emerge from burial, movement activity does not diminish until DO concentrations drop below 1.0 mg DO·l⁻¹. The degree to which movement activity declines depends on the hypoxia tolerance of individual crabs.

DISCUSSION

In general, adult blue crabs in this study were highly tolerant of severe hypoxia and near-anoxia during chronic exposures (1-2 days) compared to other common mobile species in hypoxic estuaries including spot (*Leiostomus xanthurus*), Atlantic menhaden (*Brevoortia tyrannus*), bay anchovy (*Anchoa mitchilli*), and naked goby (*Gobiosoma bosc*) (Breitburg 1994; Shimps et al. 2005). There was, however, considerable variability in survival among individual crabs for any given treatment combination, with some crabs surviving only a few hours and others remaining alive for the entire 30-hr experiment. Much of this variability was attributed to two characteristics: collection site and body size (for NRE crabs only). The

greater survival and higher movement rates of Neuse River crabs under hypoxic conditions compared to Bogue and Back Sound crabs suggests that blue crab populations can acclimate to hypoxia. Furthermore, the higher survival of small versus large NRE crabs suggests that the degree of acclimation to hypoxia and hypoxia-tolerance vary among individuals within a population.

Hcy structure is likely an important molecular mechanism controlling variability in blue crab survival and behavior under chronically hypoxic conditions. The effect of physiological state on survival and behavior of mobile animals is poorly understood, but there are cases where variation in physiological stress responses has correlated with individual differences in survival and behavior in higher vertebrates (Bohus et al. 1987) and a teleost fish (van Raaij et al. 1996). To the best of our knowledge, this is the first study to document such a response in an invertebrate.

In general, crabs with lower proportions of Hcy subunits three through six and increased proportions of subunit one survived longer and were more active in hypoxic water. Although Hcy subunits one and four (the two most variable subunits in our study) were considered invariant in relation to exposure to hypoxia in a previous study (DeFur et al. 1990), that study was one of the first to describe variation in blue crab subunit composition and qualitatively compared differences in gel banding patterns for three crabs from different hypoxia exposure treatments. Our study quantified gel banding patterns for 30 crabs, which may have allowed us to detect greater variability in subunits one and four. The smaller proportions of Hcy subunits three, five, and six for hypoxia-tolerant vs. hypoxia-sensitive crabs, however, were consistent with other studies showing reduced amounts of these three subunits in blue crabs

exposed to moderate hypoxia (Mangum and Rainer 1988; DeFur et al. 1990). Reducing these three subunits increases Hcy O₂ affinity (Mangum and Rainer 1988; DeFur et al. 1990), therefore, our more tolerant crabs had Hcy phenotypes which enabled them to extract more oxygen from the surrounding water. Although High Hcy O₂ affinity enhances the uptake of O₂ at the gills, it also makes it more difficult to offload O₂ at the tissues. Blue crabs, however, increase their activity and ventilation rates during exposure to hypoxia, which reduces pH at the tissues (Mangum and Weiland 1975; Batterton and Cameron 1978) and induces a Bohr shift that lowers the O₂ affinity of Hcy. This lowering of O₂ affinity may be sufficient to counteract the elevated O₂ affinity of Hcy in hypoxia-tolerant blue crabs, promoting survival and allowing for higher movement rates in hypoxic water.

Changes in the proportion of different Hcy monomers are environmentally induced by exposure to hypoxia and, to a lesser extent low salinity, (DeFur et al. 1990; Mangum 1997). Blue crabs encounter low salinity and hypoxia as they migrate up-estuary during ontogeny (Mangum and Towle 1977); therefore, the greater hypoxia tolerance of NRE crabs compared to Bogue and Back Sound crabs is likely due to NRE crabs being acclimated to the lower salinities and hypoxic conditions characteristic of the NRE. The lower total oxygen demand by smaller vs. larger crabs may account for the greater tolerance to hypoxia by small versus large NRE crabs, as has been proposed for fish (Moyle and Cech 2000); however, it is unclear why smaller crabs had the Hcy structure with high oxygen affinity. Presumably, small crabs would have to either encounter hypoxia more frequently than large crabs to stimulate the synthesis of the higher O₂ affinity Hcy or have higher metabolic rates that

would allow for faster turnover of Hcys from the low to high O₂ affinity structures.

Ontogenetic changes in hypoxia exposure and Hcy synthesis have not been investigated.

It was somewhat surprising to observe no effect of Hcy concentration on blue crab survival considering that increased respiratory pigment concentration is a common physiological response to hypoxic exposure in aquatic animals (Powers 1980; Hagerman 1986; Peterson 1990; Timmerman and Chapman 2004). These responses are assumed to be adaptive for animals living in hypoxic systems since elevated respiratory pigment concentrations increase the oxygen carrying capacity of hemolymph/blood (Mangum 1980; Powers 1980). In contrast, our study found an inverse relationship between Hcy concentration and survival, therefore, increases in Hcy concentration may not be an adaptive response to hypoxia for blue crabs.

Hcy concentration in crustaceans is low compared to other aquatic animals because small increases in the number of Hcy molecules cause an exponential rise in the colloidal osmotic pressure of hemolymph which disturbs fluid balance and desiccates tissues (Mangum and Johansen 1975). Increased Hcy concentrations could also raise the viscosity of hemolymph, thereby increasing the energetic cost of circulating hemolymph. Moreover, increases in blue crab Hcy concentration during hypoxia exposure come not from increased protein synthesis, but from reduced protein degradation. Blue crabs down-regulate many genes, including those that code for Hcy, to conserve energy during hypoxic stress (Brown-Peterson et al. 2005). Since there can be significant physiological costs associated with increased Hcy concentration, a positive correlation between Hcy concentration and hypoxic exposure may not be adaptive, but merely the byproduct of another acclimation response--energy

conservation via reduced protein synthesis and degradation. Our results also suggest that changing O₂ affinity (i.e., Hcy “quality” via altered subunit composition) is more adaptive than increasing O₂ carrying capacity (i.e., Hcy “quantity” via increased Hcy concentration).

Hcy structure is likely an important physiological mechanism influencing variability in blue crab survival to hypoxia, even though other physiological mechanisms could have influenced survival. Differences in energy demands for osmoregulation between crabs from the two collection sites may have influenced survival; however, it is unlikely that this is the main reason for differences in survival between collection sites because: 1) NRE crabs were also held at 30 psu for several days prior to experiments, 2) euryhaline blue crabs occur in areas with temporally variable salinity and quickly acclimate to salinity changes (Findley and Stickle 1978), 3) salinity variations between 10 – 30 psu have negligible effects on the metabolism of blue crabs (Laird and Haefner 1976), and 4) short-term (~24 hours), salinity-induced increases in blue crab oxygen consumption (presumptive increased energy demand) result not from osmoregulatory demand but increased locomotor activity (McGaw and Reiber 1998), which did not occur for Bogue Sound crabs (see Fig. 6A). Survival may have been influenced by other physiological responses to hypoxia that were not tested in this study, including increased lactate production during anaerobic metabolism (Lowery and Tate 1986), demineralization of the exoskeleton (DeFur et al. 1990; Mangum 1997), and hyperventilation-induced alkalosis (Batterton and Cameron 1978), however, individual variability in these responses has not been examined.

Identifying physiological mechanisms responsible for variability in survival and behavior among and within populations can help assess the adaptive significance of various

physiological responses to environmental stressors. Given the geographic differences in blue crab survival we observed, researchers should be careful when selecting their experimental animals if they plan to generalize their results. A logical next step for blue crabs would be to determine how variability in physiological state affects population dynamics. Individual-based models are powerful tools for identifying how individual-level characteristics influence populations (DeAngelis et al. 1994). Using models that correlate variation in molecular-level biomarkers, such as Hcy structure, with survival might allow scientists and managers to predict how the severity, duration, and frequency of hypoxia will affect populations (Adams et al. 1989).

ACKNOWLEDGEMENTS

We would like to thank the following people for their valuable help with lab experiments and input on molecular techniques: G. Plaia, C. Huggett, J. Stephenson, J. Corrales, A. Ullal, J-K. Seo, G. Godette, and M. Brouwer. Funding for this research was provided by research grants from the National Science Foundation (OCE-0221099) and the NC Blue Crab Research Program (01-POP-08) to D. B. Eggleston; the NC Blue Crab Research Program (03-BIOL-01), the National Sea Grant College Program, National Oceanic and Atmospheric Administration (Marine Biotechnology Program (Grant BR-01, NA16-RG-2251), and the USDA - NRI Competitive Grant Program (Project #2006-35204-17508) to E.J. Noga, as well as EPA STAR and NC Beautiful fellowships to G. W. Bell.

LITERATURE CITED

- Adams, S. M., K. L. Shepard, M. S. Greeley, Jr., B. D. Jimenez, M. G. Ryon, L. R. Shugart and J. F. McCarthy (1989). "The use of bioindicators for assessing the effects of pollutant stress on fish." Mar Environ Res **28**: 459-464.
- Batterton, C. V. and J. N. Cameron (1978). "Characteristics of resting ventilation and response to hypoxia, hypercapnia, and emersion in the blue crab *Callinectes sapidus* (Rathbun)." J Exp Zool **203**: 403-418.
- Bell, G. W. and D. B. Eggleston (2005). "Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia." Mar Biol **146**: 761-770.
- Bell, G. W., D. B. Eggleston and D. L. Wolcott (2003). "Behavioral responses of free-ranging blue crabs to episodic hypoxia. I. Movement." Mar Ecol Prog Ser **259**: 215-225.
- Bohus, B., R. F. Benus, D. S. Fokkema, J. M. Koolhaas, C. Nyakas, G. A. Van Oortmerssen, A. J. A. Prins, A. J. H. De Ruiter, A. J. W. Scheurink and A. B. Steffens (1987). "Neuroendocrine states and behavioral and physiological stress responses." Prog Brain Res **72**: 57-70.
- Breitburg, D. L. (1990). "Near-shore hypoxia in the Chesapeake Bay: patterns and relationships among physical factors." Est Coast Shelf Sci **30**: 593-609.
- Breitburg, D. L. (1992). "Episodic hypoxia in Chesapeake Bay: interacting effects of recruitment, behavior, and physical disturbance." Ecol Monogr **62**(4): 525-546.
- Breitburg, D. L. (1994). "Behavioral response of fish larvae to low dissolved oxygen concentrations in a stratified water column." Mar Biol **120**: 615-625.

- Brouwer, M., N. J. Brown-Peterson, P. Larkin, P. Patel, N. Denslow, S. Manning and T. H. Brouwer (2007). "Molecular and whole animal responses of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia." J Exp Mar Biol Ecol **341**: 16-31.
- Brouwer, M., P. Larkin, N. J. Brown-Peterson, C. King, S. Manning and N. Denslow (2004). "Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes sapidus*." Mar Environ Res **58**: 787-792.
- Brown-Peterson, N. J., P. Larkin, N. Denslow, C. King, S. Manning and M. Brouwer (2005). "Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*." Mar Ecol Prog Ser **286**: 203-215.
- DeAngelis, D. L., K. A. Rose and M. A. Huston (1994). Individual-oriented approaches to modeling ecological populations and communities. Frontiers in Mathematical Biology. S. A. Levin. Berlin, Springer: 390-410.
- DeFur, P. L., C. P. Mangum and J. E. Reese (1990). "Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia." Biol Bull **178**: 46-54.
- Diaz, R. J. and R. Rosenberg (1995). "Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna." Oceanogr Mar Biol Ann Rev **33**: 245-303.
- Dixon, P. M. and M. C. Newman (1991). Analyzing toxicity data using statistical models for time-to-death: an introduction. Metal Ecotoxicology: Concepts and Applications. M. C. Newman and A. W. McIntosh. Chelsea, MI, Lewis Publishers: 207-242.

- Eby, L. A. and L. B. Crowder (2002). "Hypoxia-based habitat compression in the Neuse River Estuary: context-dependent shifts in behavioral avoidance thresholds." Can J Fish Aquat Sci **59**(6): 952-965.
- Eggleston, D. B., G. W. Bell and A. D. Amavisca (2005). "Interactive effects of episodic hypoxia and cannibalism on juvenile blue crab mortality." J Exp Mar Biol Ecol **325**: 18-26.
- Engel, D. W., M. Brouwer and S. McKenna (1993). "Hemocyanin concentrations in marine crustaceans as a function of environmental conditions." Mar Ecol Prog Ser **93**: 235-244.
- Farwell, M., M. G. Fox, C. D. Moyes and G. Burness (2007). "Can hypoxia tolerance explain differences in distribution of two co-occurring north temperate sunfishes?" Environ Biol Fish **78**: 83-90.
- Findley, A. M. and W. B. Stickle (1978). "Effects of salinity fluctuation on the hemolymph composition of the blue crab *Callinectes sapidus*." Mar Biol **46**: 9-15.
- Gerard, J. F. and R. Gilles (1972). "The free amino-acid pool in *Callinectes sapidus* (Rathbun) tissues and its role in the osmotic intracellular regulation." J Exp Mar Biol Ecol **10**: 125-136.
- Hagerman, L. (1986). "Haemocyanin concentration in the shrimp *Crangon crangon* (L.) after exposure to moderate hypoxia." Comp Biochem Physiol A **85**(4): 721-724.
- Hames, B. D. and D. Rickwood (1990). Gel Electrophoresis of Proteins, a Practical Approach. New York, Oxford University Press.
- Johnson, B. A., C. Bonaventura and J. Bonaventura (1984). "Allosteric modulation of *Callinectes sapidus* hemocyanin by binding of L-Lactate." Biochemistry-US **23**: 872-878.

- Laird, C. E. and P. A. Haefner, Jr. (1976). "Effects of intrinsic and environmental factors on oxygen consumption in the blue crab, *Callinectes sapidus* Rathbun." J Exp Mar Biol Ecol **22**: 171-178.
- Loesch, H. (1960). "Sporadic mass shoreward migrations of demersal fish and crustaceans in Mobile Bay, Alabama." Ecology **41**(2): 292-298.
- Lowery, T. A. and L. G. Tate (1986). "Effect of hypoxia on hemolymph lactate and behavior of the blue crab *Callinectes sapidus* Rathbun in the laboratory and field." Comp Biochem Physiol A **85**(4): 689-692.
- Luettich, R. A., Jr., J. E. McNinch, H. W. Paerl, C. H. Peterson, J. T. Wells, M. Alperin, C. S. Martens and J. L. Pinckney (2000). Neuse River Estuary modeling and monitoring project stage 1: hydrography and circulation, water column nutrients and productivity, sedimentary processes and benthic-pelagic coupling, and benthic ecology, Water Resources Research Institute of the University of North Carolina: 172.
- Mangum, C. P. (1980). "Respiratory function of the hemocyanins." Am Zool **20**: 19-38.
- Mangum, C. P. (1994). "Subunit composition of hemocyanins of *Callinectes sapidus*: phenotypes from naturally hypoxic waters and isolated oligomers." Comp Biochem Physiol B **108**(4): 537-541.
- Mangum, C. P. (1997). "Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*." Am Zool **37**(6): 604-611.
- Mangum, C. P. and K. Johansen (1975). "The colloid osmotic pressures of invertebrate body fluids." J Exp Biol **63**: 661-671.

- Mangum, C. P. and J. S. Rainer (1988). "The relationship between subunit composition and O₂ binding of blue crab hemocyanin." Biol Bull **174**: 77-82.
- Mangum, C. P. and D. W. Towle (1977). "Physiological adaptation to unstable environments." Am Zool **65**: 67-75.
- Mangum, C. P. and A. L. Weiland (1975). "The function of hemocyanin in respiration of the blue crab *Callinectes sapidus*." J Exp Zool **193**: 257-264.
- Mason, R. P., C. P. Mangum and G. Godette (1983). "The influence of inorganic ions and acclimation salinity on hemocyanin-oxygen binding in the blue crab *Callinectes sapidus*." Biol Bull **164**: 104-123.
- McGaw, I. J. and C. L. Reiber (1998). "Circulatory modification in the blue crab *Callinectes sapidus*, during exposure and acclimation to low salinity." Comp Biochem Physiol A **121**: 67-76.
- Moyle, P. and J. Cech (2000). Fishes: An Introduction to Ichthyology. Uppersaddle River, Prentice Hall.
- Newman, M. C. and P. M. Dixon (1996). Ecologically meaningful estimates of lethal effect in individuals. Ecotoxicology: a heirarchical treatment. M. C. Newman and C. H. Jagoe. Boca Raton, FL, Lewis Publishers: 225-253.
- Paerl, H. W., J. L. Pinckney, J. M. Fear and B. L. Peierls (1998). "Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA." Mar Ecol Prog Ser **166**: 17-25.

- Paerl, H. W., J. L. Pinckney, J. M. Fear and B. L. Peierls (1999). "Fish kills and bottom-water hypoxia in the Neuse River and Estuary: reply to Burkholder et al." Mar Ecol Prog Ser **186**: 307-309.
- Peterson, M. S. (1990). "Hypoxia-induced physiological changes in two mangrove swamp fishes: sheepshead minnow, *Cyprinodon variegatus* Lacepede and sailfin molly, *Poecilia latipinna* (Lesueur)." Comp Biochem Physiol A **97**(1): 17-21.
- Pihl, L., S. P. Baden and R. J. Diaz (1991). "Effects of periodic hypoxia on distribution of demersal fish and crustaceans." Mar Biol **108**: 349-360.
- Pihl, L., S. P. Baden, R. J. Diaz and L. C. Schaffner (1992). "Hypoxia-induced structural changes in the diet of bottom-feeding fish and crustacea." Mar Biol **112**: 349-361.
- Pirow, R., C. Baumer and R. J. Paul (2001). "Benefits of haemoglobin in the cladoceran crustacean *Daphnia magna*." J Exp Biol **204**: 3425-3441.
- Powers, D. A. (1980). "Molecular ecology of teleost fish hemoglobins: strategies for adapting to changing environments." Am Zool **20**: 139-162.
- Rees, B. B., F. A. Sudradjat and J. W. Love (2001). "Acclimation to hypoxia increases survival time of zebrafish, *Danio rerio*, during lethal hypoxia." J Exp Zool **289**: 266-272.
- Reynolds-Fleming, J. V. and R. A. Luettich, Jr. (2004). "Wind-driven lateral variability in a partially mixed estuary." Est Coast Shelf Sci **60**: 395-407.
- Robinson, G. D. (1994). "Effects of acclimation salinity on sodium fluxes in the blue crab (*Callinectes sapidus*)." Comp Biochem Physiol A **108**(1): 69-73.
- Shimps, E. L., J. A. Rice and J. A. Osborne (2005). "Hypoxia tolerance in two juvenile estuary-dependent fishes." J Exp Mar Biol Ecol **325**(2): 146-162.

- Sokal, R. R. and F. J. Rohlf (1981). Biometry. New York, W.H. Freeman and Company.
- Taylor, J. C. and J. M. Miller (2001). "Physiological performance of juvenile southern flounder, *Paralichthys lethostigma* (Jordan and Gilbert, 1884), in chronic and episodic hypoxia." J Exp Mar Biol Ecol **258**: 195-214.
- Timmerman, C. M. and L. J. Chapman (2004). "Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*)."
Physiol Biochem Zool **77**(4): 601-610.
- van Raaij, M. T. M., D. S. S. Pit, P. H. M. Balm, A. B. Steffens and G. E. E. J. M. van den Thillart (1996). "Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia." Horm Behav **30**: 85-92.

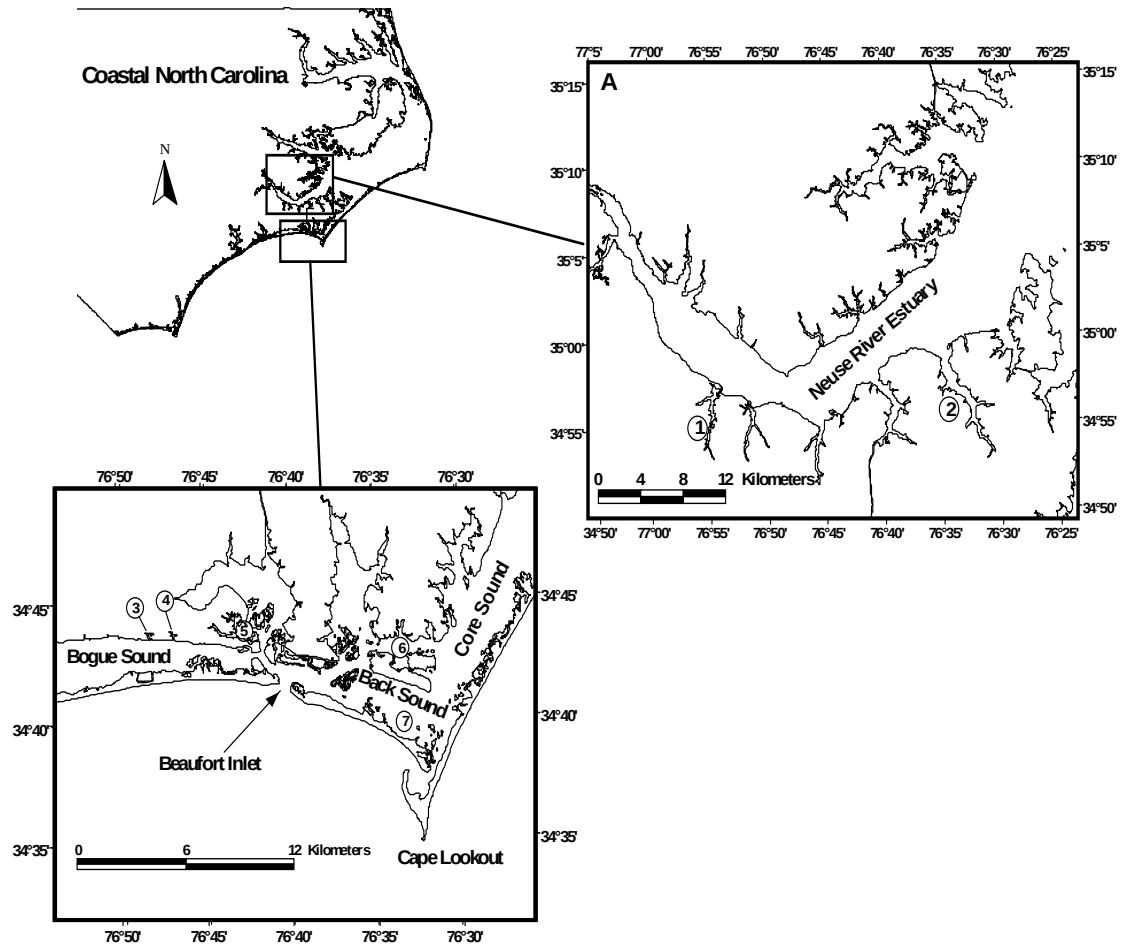


Figure 1 Locations of blue crab collections in coastal North Carolina. (A) Neuse River Estuary: ¶ Hancock Creek and ¶ South River.

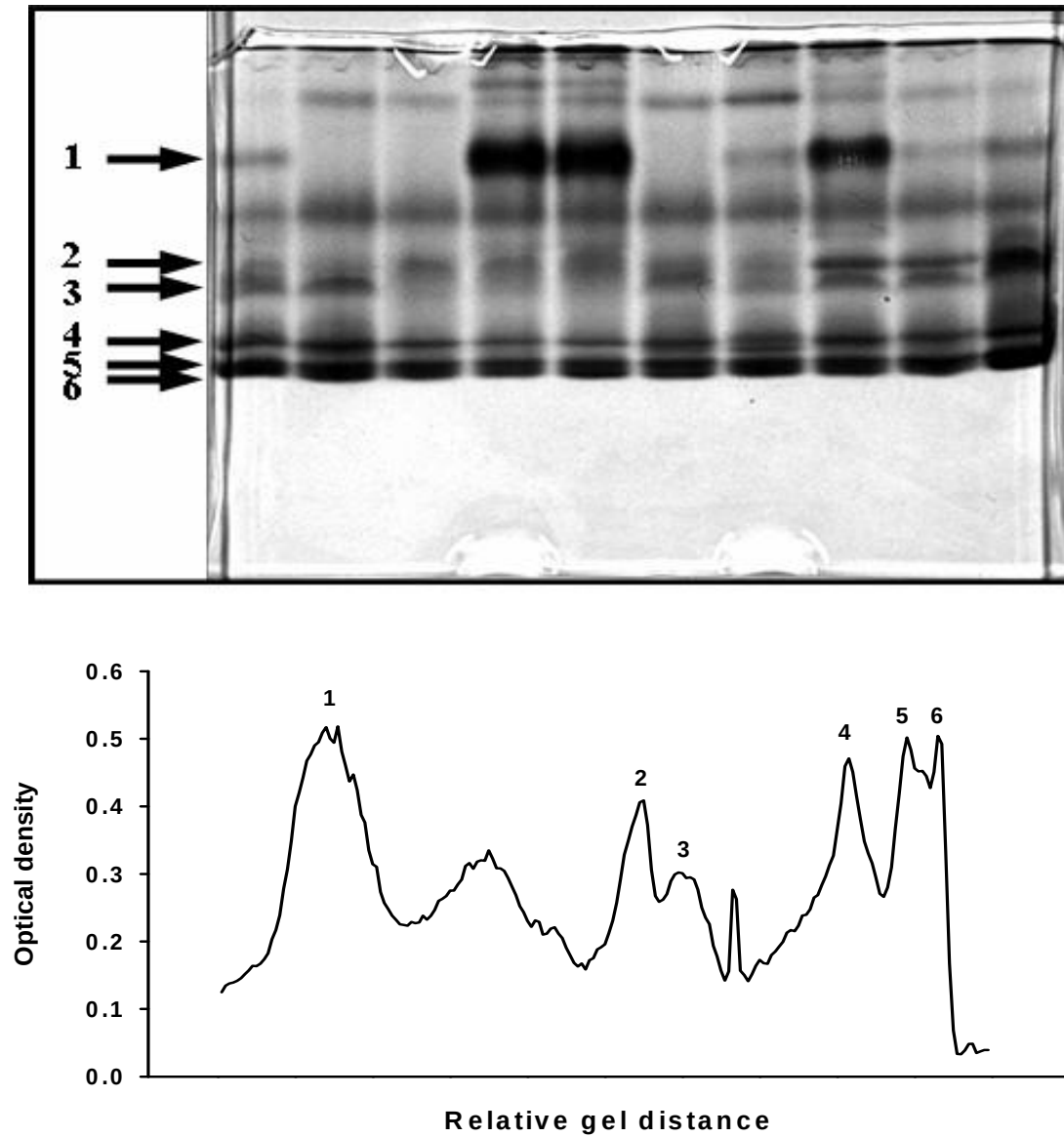


Figure 2 Typical PAGE gel banding patterns of the six dissociated Hcy subunits. Top panel is image of a representative gel that has been stained. Each vertical lane is a single hemolymph sample and each band is labeled with its corresponding Hcy subunit. Bottom panel is a representative densitometer scan of a single vertical lane (left side of X-axis is the top of the gel) where each peak corresponds to a specific Hcy subunit.

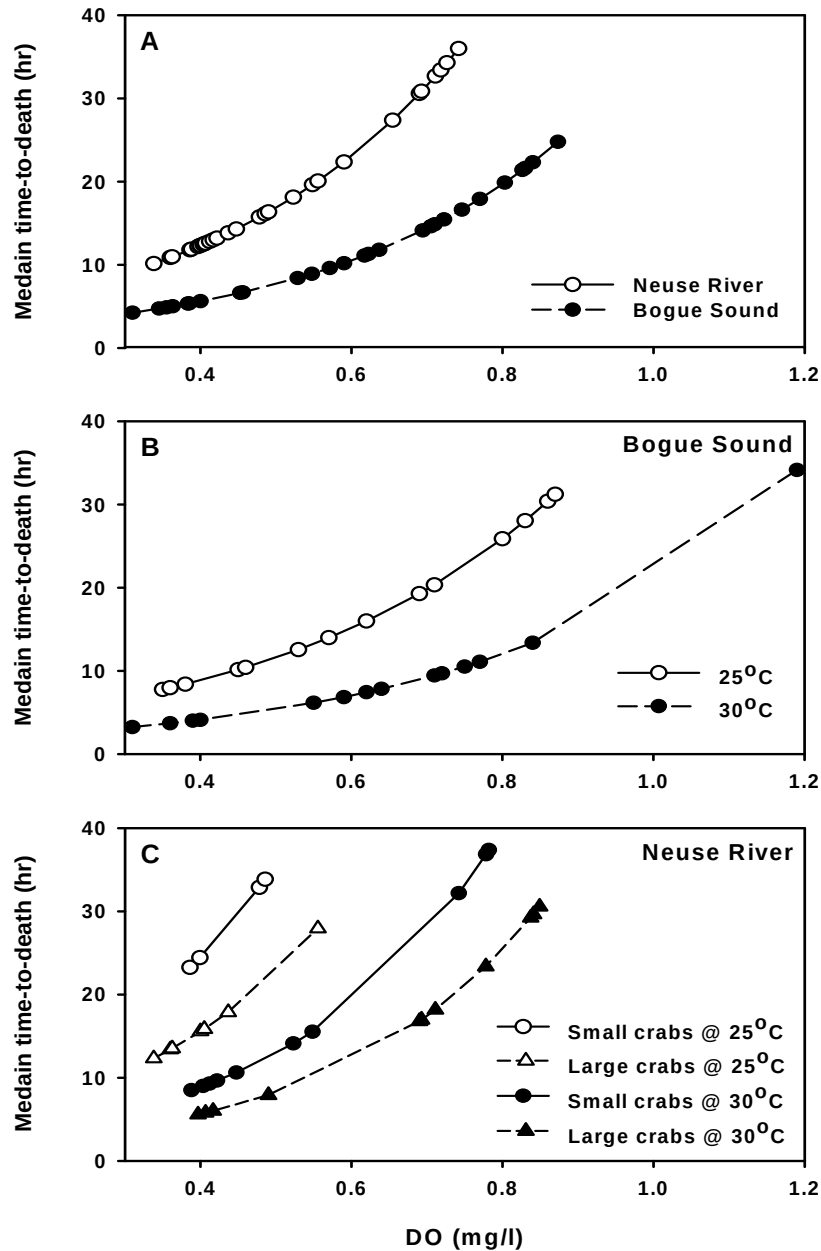


Figure 3 Differences in blue crab median time-to-death estimates as a function of DO concentration from time-to-event model between: (A) collection sites (data pooled across all treatments), (B) temperature (25 C & 30 C) for Bogue Sound crabs (no significant size effect), and (C) averaged size large (CW = 140 mm; triangles) and small (CW = 110 mm; circles) Neuse River crabs at 25 C (open symbols) and 30 C (closed symbols).

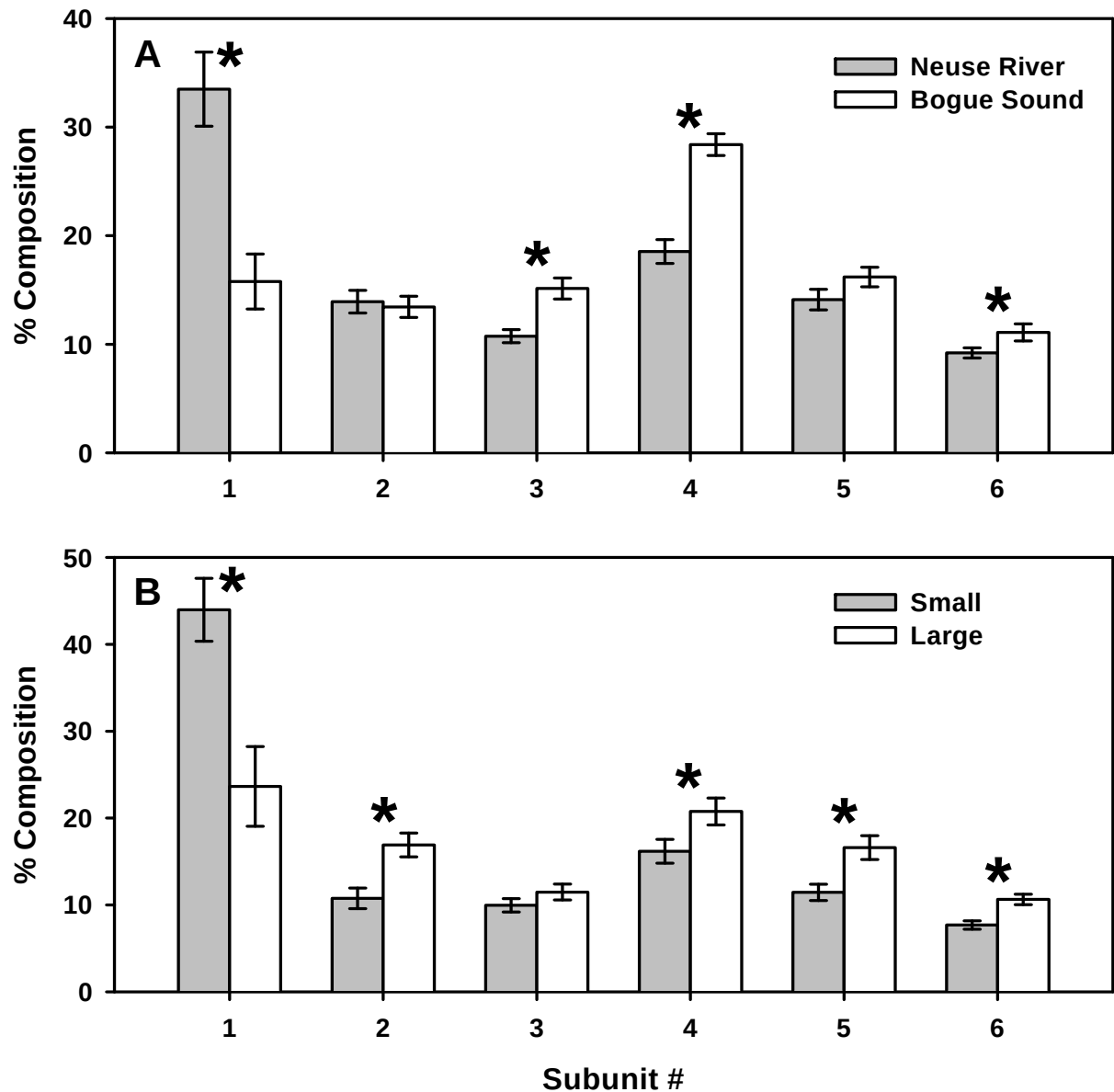


Figure 4 Hcy structure comparison for factors with significant differences in treatment levels from parametric time-to-event models: (A) Neuse River vs Bogue and Back Sounds crabs, and (B) small vs large Neuse River crabs). Asterisks denote which subunits were significantly different between groups.

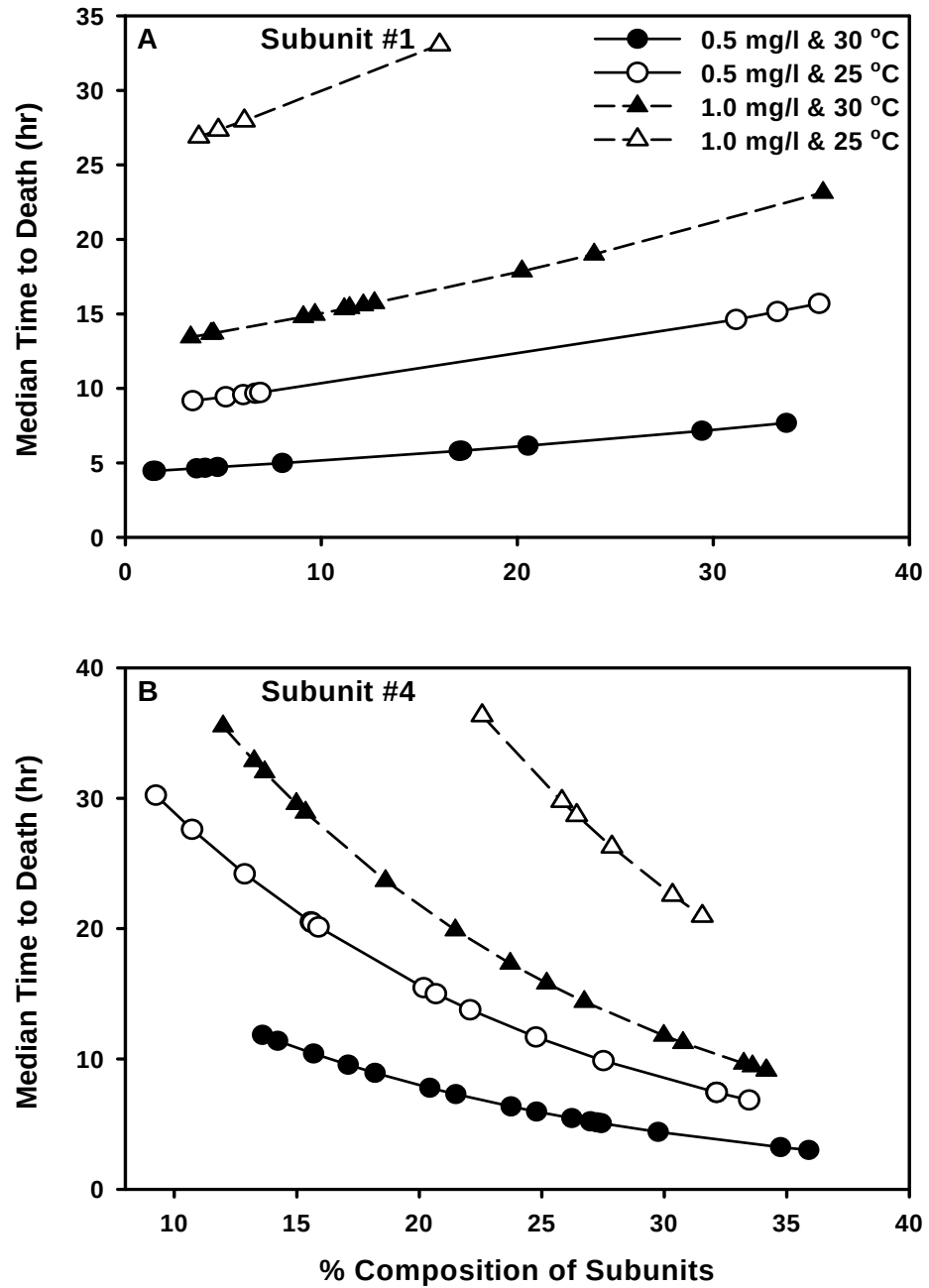


Figure 5 Functional relationship between percent relative composition of two Hcy subunits (A: subunit 1, B: subunit 4) and median time-to-death estimates from time-to-event models. Median time-to-death is plotted for each DO and temperature treatment combination.

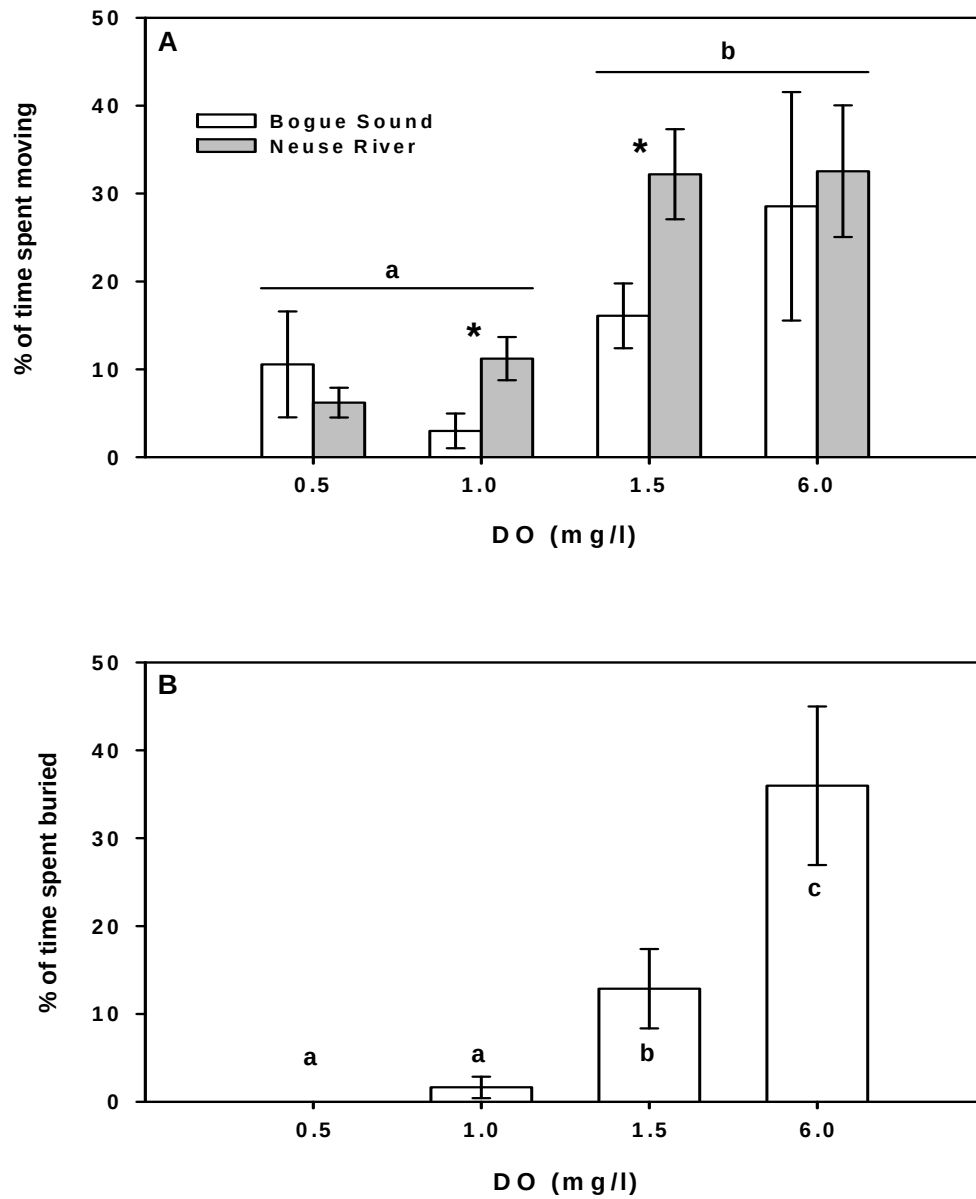
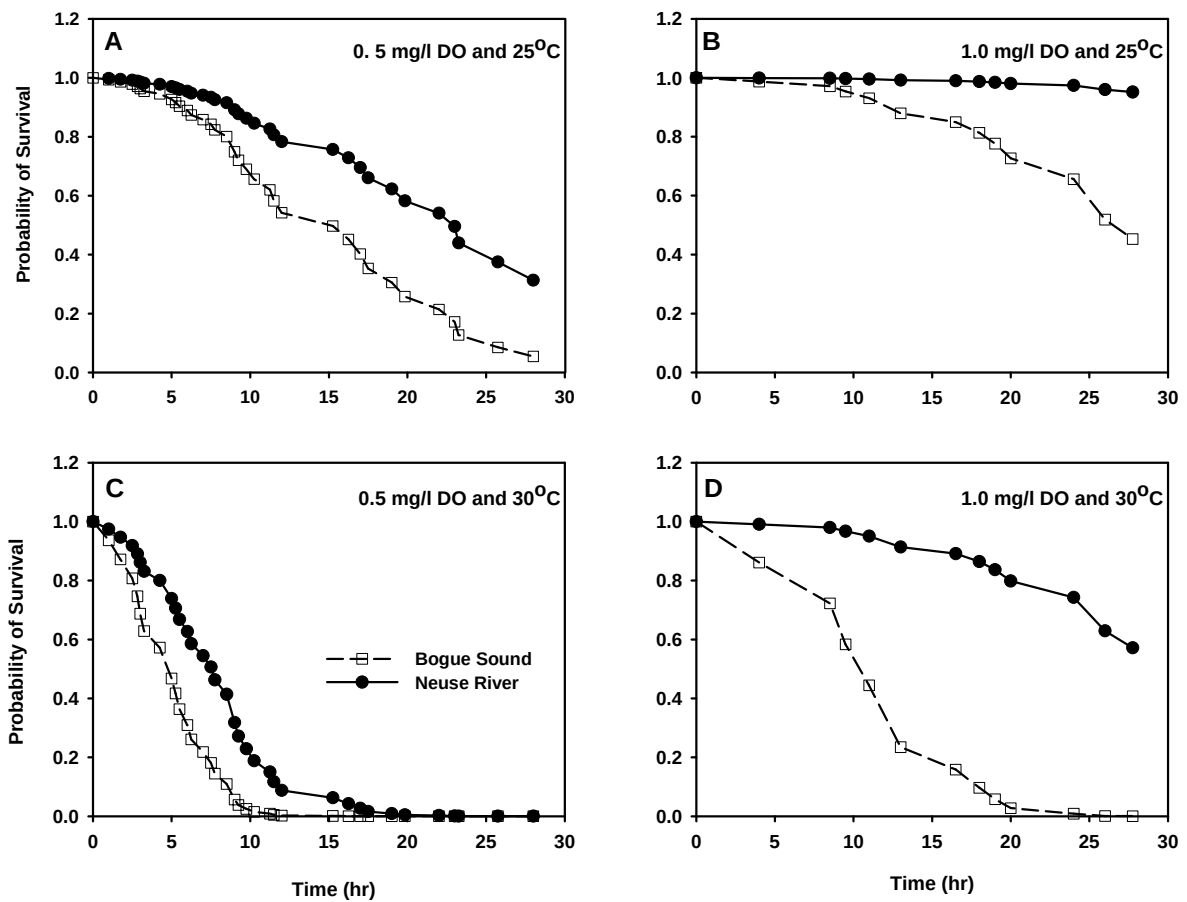


Figure 6 Effects of hypoxia and collection site on mean percent of time blue crabs spent (A) moving and (B) buried (pooled across collection site) ($\pm$ SE) for each hypoxic treatment and the 6.0 mg DO·l⁻¹ control. Lowercase letters denote significant differences in movement and burial behavior among DO concentrations. Asterisks denote significant differences in movement between crabs from the two collection sites within each DO concentration.

APPENDIX

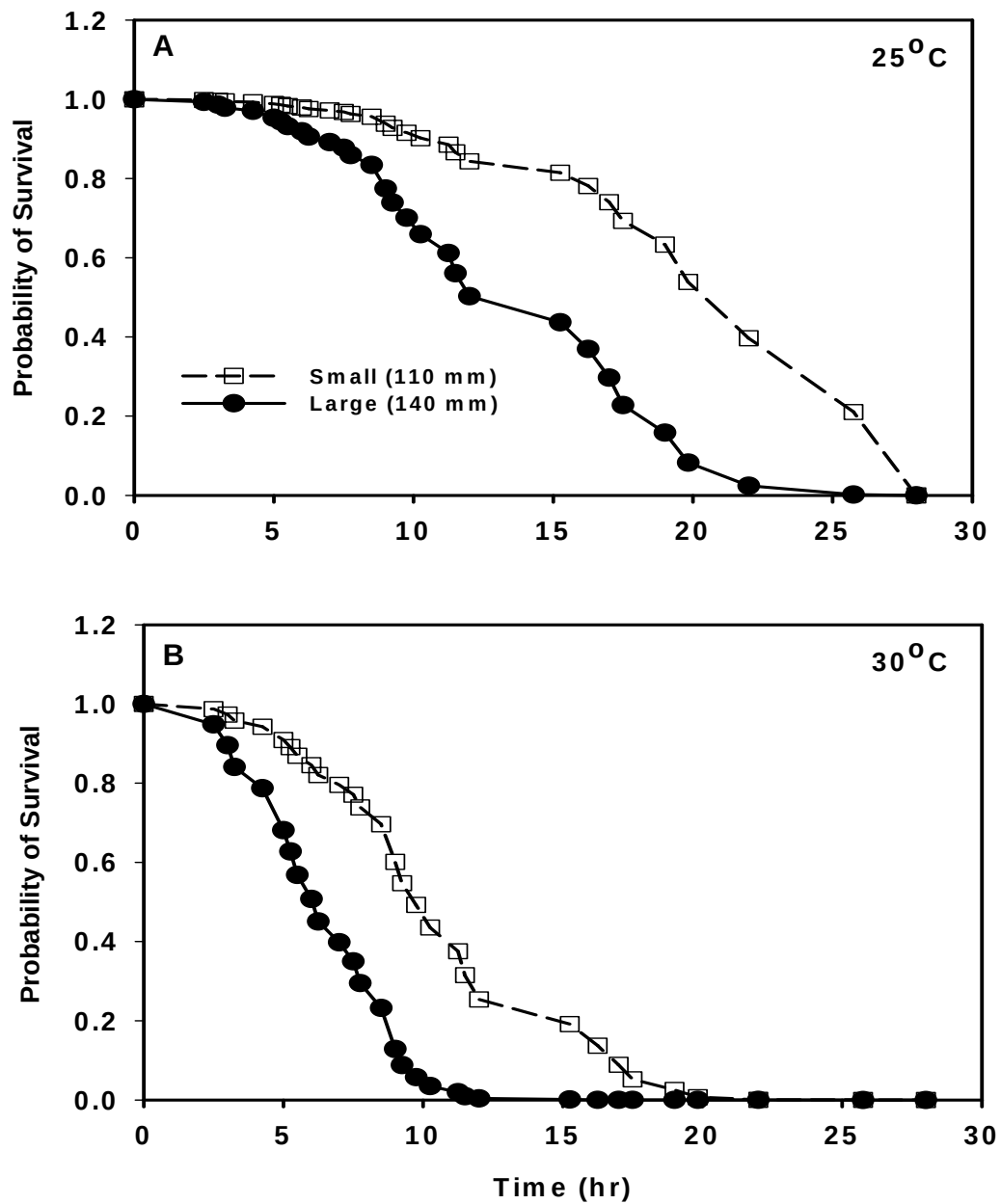
APPENDIX 1A

Blue crab survivorship functions from semi-parametric Cox proportional hazard models for 0.5 mg·l⁻¹ DO (A and C) and 1.0 mg·l⁻¹ DO (B and D) hypoxia treatments at 25°C (A and B) and 30°C (C and D). Probability of blue crab survival at each exposure time is shown for crabs collected from Bogue Sound (open squares) and the Neuse River (closed circles).



APPENDIX 1B

Blue crab survivorship functions from semi-parametric Cox proportional hazard models for crabs collected from the Neuse River and exposed to $0.5 \text{ mg}\cdot\text{l}^{-1}$ DO. Probability of crab survival at each exposure time is shown for an average sized small (CW = 110 mm; open squares) and large (CW = 140 mm; closed circles) crab at 25°C (A) and 30°C (B).



CHAPTER 2

ENVIRONMENTAL AND PHYSIOLOGICAL CONTROLS OF BLUE CRAB AVOIDANCE BEHAVIOR DURING EXPOSURE TO HYPOXIA

ABSTRACT

Generalizing the impacts of hypoxia on aquatic animal populations is difficult due to differences in behavioral and physiological responses among individuals, as well as the varying hydrodynamics of hypoxic events. Information on which environmental cues animals use to avoid hypoxia as well as how covarying variables and physiological acclimation influence avoidance behavior is lacking. Laboratory flume studies quantified the interacting effects of covarying hydrography (DO, temperature, and salinity), hydrodynamics (rate of DO change and current speed), and physiological state on avoidance behaviors of blue crabs (*Callinectes sapidus*) to hypoxia. Drops in DO stimulated increased movement rates, regardless of whether the change resulted in hypoxia. Faster rates of declining DO stimulated faster movement rates under hypoxic conditions because crabs spent less time in hypoxia when DO declined rapidly compared to when DO declined more slowly. Crabs also exhibited an increasing preference for the down-stream end of the flume with declining DO, suggesting that blue crabs may use current direction to orient away from hypoxia. Lastly, blue crabs possessing Hcy phenotypes with high O₂ affinity (i.e. hypoxia-tolerant) were less active under initial (< 1 hr) hypoxic conditions than conspecifics with the low O₂ affinity Hcy structure. This is the first study documenting a physiological mechanism underlying

individual-based differences in the behavioral response of an invertebrate to hypoxia. These results provide a mechanistic understanding of how physiological acclimation and the hydrodynamics of hypoxic events influence behavior of a mobile aquatic animal.

INTRODUCTION

Low dissolved oxygen (DO) is one of the most important environmental threats to coastal ecosystems today. The frequency, duration, and spatial extent of hypoxia ($\text{DO} < 2.0 \text{ mg}\cdot\text{l}^{-1}$) and anoxia ($\text{DO} < 0.2 \text{ mg}\cdot\text{l}^{-1}$) have increased worldwide over the past several decades (Diaz and Rosenberg 1995; Gray et al. 2002; Wu 2002). The ecological impacts of hypoxia on mobile animal populations are difficult to predict because mobile animals exhibit an array of behavioral and physiological responses to hypoxia that vary among individuals. When chronic hypoxia develops in the deepest portions of estuaries, mobile animals avoid relatively deep water and move into nearshore normoxic areas (Pihl et al. 1991; Eby and Crowder 2002; Bell and Eggleston 2005). The resulting habitat compression can cause decreased growth rates as inter- and intra-specific competition for prey increase (Lenihan et al. 2001), and elevated incidences of cannibalism as juvenile and adult conspecifics, which are normally spatially segregated, now overlap (Eggleston et al. 2005).

Behavioral responses of animals are least understood during relatively rapid episodic hypoxic conditions, when hypoxic bottom water upwells into ordinarily normoxic nearshore areas. During some hypoxic events, thousands of mobile animals seek to avoid hypoxia and form dense aggregations near the land-water interface, termed “Jubilees” (Loesch 1960). At the other extreme, hundreds of thousands of fish can die in a matter of hours from hypoxic events when they are trapped in hypoxic water (Paerl et al. 1998). Even for moderate hypoxic events that occur almost daily in estuarine systems (Bell et al. 2003; Reynolds-Fleming and Luetlich 2004), many individuals from a population can successfully avoid hypoxia while others are trapped in low DO for several hours, thereby leading to substantial

mortality in some cases (Breitburg 1992; Bell et al. 2003; Bell and Eggleston 2005). It is unknown why mobile animals exhibit such large variation in their behavioral and physiological responses to dynamic hypoxia. Understanding why this occurs may provide insight for identifying the sources of behavioral variability and predicting how and when hypoxia will alter ecosystem processes.

How aquatic animals detect and avoid hypoxia is still unknown, however, the blue crab (*Callinectes sapidus*) is a useful model species to begin examining hypoxic avoidance behavior because its physiological and behavioral responses to hypoxia are well-studied compared to other estuarine animals, and because it commonly occurs in hypoxic estuaries (Pihl et al. 1991; Eby and Crowder 2002; Bell and Eggleston 2005). Successful avoidance of upwelled hypoxic water depends on the ability of crabs to orient away from low DO and move faster than the encroaching hypoxic water. Blue crabs increase their movement rates after initial exposure to hypoxia, become quiescent shortly thereafter, and may bury under chronic hypoxic conditions (Lowery and Tate 1986; DeFur et al. 1990; Das and Stickle 1993, 1994). The environmental cue(s) that stimulate these responses are unknown.

Blue crabs may exhibit a threshold response by increasing their activity and avoiding DO concentrations below $2 \text{ mg DO} \cdot \text{l}^{-1}$ (Pihl et al. 1991; Das and Stickle 1994; Eby and Crowder 2002; Bell and Eggleston 2005), but this response may not accurately characterize their avoidance behavior. Relative rates of change in hydrography are known to affect behavior of fish and other crustaceans (Gerritsen 1982; de Vries et al. 1995; Forward et al. 1995). Moreover, changes in other abiotic factors (e.g. declining temperature and increasing salinity) covary with DO during hypoxic upwelling events (Luettich et al. 2000). Changes in

both temperature and salinity influence large-scale movement patterns of estuarine animals (Parker 1970; Vance et al. 1998; Meager et al. 2003); however, it is unknown how these abiotic variables interact with declining DO to affect movement of blue crabs.

Orientation mechanisms are also critical for successful avoidance of hypoxia. Free-ranging blue crabs exhibit weak directional bias towards the shoreline during hypoxic upwelling events (Bell et al. 2003). The prevailing hypothesis to explain this pattern is that blue crabs may use current direction as a proxy cue to orient away (i.e., down-current) from up-welled hypoxic water (Bell et al. 2003). Current direction is a critical component for blue crab orientation behavior when tracking the source of prey odor plumes (Weissburg and Zimmerfaust 1993, 1994; Weissburg and Dusenbery 2002), but its usefulness for avoidance of hypoxia is unknown.

Lastly, the physiological state of aquatic animals can influence behavior under hypoxic conditions (van Raaij et al. 1996; Timmerman and Chapman 2004; Bell et al. in review). Studying oxygen-carrying molecules (e.g hemoglobins in fish and hemocyanins in invertebrates) can be especially useful for understanding physiological adaptations to environmental stressors such as hypoxia because changes to these molecules are controlled directly by DO concentrations in the surrounding water (Powers 1980). In blue crabs, hemocyanin (Hcy) is an oligomer composed of six different kinds of monomer subunits. Two adaptive changes occur in crustacean hemocyanins during exposure to hypoxia: increased concentration, which boosts the oxygen carrying capacity of the hemolymph (Mangum 1997; Pirow et al. 2001; Timmerman and Chapman 2004; Seidl et al. 2005), and altered structure, which increases Hcy oxygen affinity (DeFur et al. 1990; Mangum 1997).

The oxygen affinity of Hcy increases when a subset of the six monomer subunits (subunit types one, two, and four) is preferentially used to produce the Hcy oligomer (DeFur et al. 1990); thus the relative proportions of the six subunits in hemolymph differ for hypoxia-tolerant and hypoxia-sensitive crabs (Bell et al. in review). Blue crabs whose hemolymph shows low proportions of subunits three, five, and six (i.e, high O₂ affinity Hcy structure) are better acclimatized to hypoxia, survive longer and are more active under chronic hypoxic conditions (> 1 hr), and are more frequently caught in hypoxic water than crabs with lower O₂ affinity Hcy structures (Mangum 1994). Whether Hcy concentration and structure affect avoidance responses to hypoxia is unknown. Understanding how physiological state influences behavior is key to bridging the gap between physiology and ecology (Brouwer et al. 2007), as well as predicting distribution, abundance and behavior patterns of mobile species in dynamic and stressful estuarine environments.

We used laboratory flume experiments to examine the effects of physiology (Hcy structure and concentration), temporal changes in hydrography (DO, temperature, and salinity), and hydrodynamics (current speed and rate of change in DO) on blue crab movement and burial behavioral patterns. We expected blue crab movement rates to increase with declining DO before decreasing with prolonged exposure to hypoxia, while burial behavior would generally increase with declining DO. We also hypothesized that these functional relationships would differ with concomitant changes in temperature and salinity, as well as between fast vs. slow current speeds and rates of change in DO. Lastly, we expected Hcy concentration and structure to differ between crabs that responded to hypoxia with slow- vs. fast-movement rates.

MATERIALS AND METHODS

Capture & Maintenance

Adult blue crabs were collected from Bogue and Back Sounds, NC and shipped overnight in coolers to our NC State University laboratory facilities in Raleigh, North Carolina, USA. Crabs were placed in holding tanks with recirculating artificial seawater. Temperature (23 – 25°C), salinity (12 – 15 psu), and DO (5 – 7 mg·l⁻¹) conditions in all tanks were monitored daily and remained constant but different from the salinity at the collection site during the holding period. Crabs were fed *ad libitum* daily with squid or shrimp and held in these tanks for a minimum of 24 h prior to beginning experiments to verify good condition after transport.

Experimental Procedures

Two experiments were conducted in a 903-l impeller-driven recirculating flume (2.25 m x 0.76 m x 0.47 m; (Vogel and LaBarbera 1978), which included a 95 cm x 76 cm x 47cm working section, the bottom of which was covered to a depth of 2.5 cm with artificial substrate (60 µm – 120 µm diameter Ballotini® impact beads). The sidewall of the working section of the flume was composed of clear glass. Behavioral observations were made through an aperture in a blind of black plastic film to minimize disturbance to subjects. A variable speed motor, located at the downstream end of the tank, turned a propeller that produced desired current speeds in the working section. The flow was generally laminar within the working section of the flume and formed a boundary layer at current speeds between 2 cm·s⁻¹ and 6 cm·s⁻¹ (Bell & Eggleston unpublished data). Changes in DO, temperature, salinity, and current speed during experiments were initiated at the downstream

end of the flume. Changes in these hydrographic variables did not produce spatial gradients within the working section; thus, crab behavioral patterns were due entirely to temporal changes in hydrography.

Only intermolt male and female crabs, and terminal molt females without eggs, were used in experiments. Crabs were measured, sexed, and bled for hemolymph (only for the rate of DO change/current speed experiment) before introduction to aquaria. A single adult crab (100 mm – 160 mm carapace width) was placed in the flume's working section and allowed to acclimate to the flume for 1.5 h. A clear, rigid plastic sheet was floated on the water surface to prevent oxygen diffusion into the flume and altering DO levels. After the acclimation period, behavioral observations were recorded during a 20-min control period of constant normoxic water quality conditions typical of mesohaline nearshore habitats during summer months prior to hypoxic upwelling events (6 mg·DO l⁻¹, 27 °C, and 12 psu). For both experiments (see below), a treatment was applied at the conclusion of the control period. A 5 x 4 grid of 19-cm x 19-cm cells was created in the substratum using marbles to mark the corners of each cell. Crab movements between cells were continuously recorded to quantify movement rates and document the location of crabs along the flume axis (i.e. upstream, middle, or downstream). We also recorded any instances of burial and swimming behavior.

Experiment 1: crab movement responses to covarying DO, temperature and salinity - For this experiment, a constant flow velocity of 6 cm·s⁻¹ was maintained throughout the control and experimental periods. This experiment tested crab movement responses to drops in DO and temperature, as well as increases in salinity, all of which co-occur during episodic hypoxic upwelling conditions in the NRE and Chesapeake Bay (Breitburg 1992; Luettich et

al. 2000; Bell et al. 2003). Nitrogen gas was bubbled into the downstream end of the flume to decrease DO from $6 \text{ mg}\cdot\text{l}^{-1}$ to $0.5 \text{ mg}\cdot\text{l}^{-1}$. An immersion chiller decreased temperature from 27°C to 25°C , and a super-concentrated artificial seawater solution was continuously added to the flume to increase salinity from 12 psu to 16 psu. These changes represented typical hydrographic changes that estuarine animals encounter in normoxic nearshore habitats during episodic hypoxic upwelling events (Breitburg 1992; Bell et al. 2003). DO, temperature, and salinity were continuously monitored with a YSI Model-85 instrument. This was a full factorial design so each level of the hydrographic variables (present vs. absent) was crossed. In experiments with changes to only temperature and salinity, the final values were reached in about 80 min and experiments were continued for an additional 10 min. For experiments in which DO was changed, experiments continued at the final DO concentration until crabs became quiescent (i.e., no movement for 10 consecutive minutes).

Experiment 2: crab movement responses to current speeds and rate of DO change - For this experiment, the current speed during the control period was set to $\sim 1 \text{ cm}\cdot\text{s}^{-1}$ but was increased during the experimental period to test crab movement responses to different current velocities (slow = $2.5 \text{ cm}\cdot\text{s}^{-1}$ or fast = $6.0 \text{ cm}\cdot\text{s}^{-1}$) and rates of change in DO (slow = $0.05 \text{ mg}\cdot\text{l}^{-1}\cdot\text{min}^{-1}$ or fast = $0.16 \text{ mg}\cdot\text{l}^{-1}\cdot\text{min}^{-1}$), which covary in the field during hypoxic upwelling events. These rates represent minimum and maximum measurements recorded in the NRE during hypoxic upwelling events (Luettich et al. 2000; Bell et al. 2003). This experiment was also a full factorial design with both levels of DO rate crossed with both current velocity treatment levels. Oxygen reductions ceased when the DO concentration reached $0.5 \text{ mg}\cdot\text{DO l}^{-1}$; therefore, the terminal DO concentration occurred after 35-40 min and 90-100 min for the

fast and slow rate of change treatments, respectively. After the terminal DO concentration was reached, crabs were exposed for another 40-60 min to observe when they ceased movement.

There are two possible mechanisms by which rate of DO change could influence avoidance behavior. If crabs respond directly to rate of DO change because fast rates of DO change represent a greater impending risk of hypoxic exposure, then under rapidly declining DO conditions we would expect crabs to move faster, emerge from burial, and spend more time at the down-stream end of the flume at higher DO concentrations compared to slow rate of change conditions. Alternatively, if blue crabs respond to cumulative hypoxia stress, we would expect similar movement rates during declining DO between fast and slow rates of DO change until hypoxic DO concentrations. At this point, crabs under fast rate of DO change conditions would have spent half as much time in hypoxic water than crabs in the slow rate of DO change treatment (~60 min vs. 25 min at or below 2.0 mg DO·l⁻¹) and they may be able to sustain faster movement rates (i.e., crabs respond to rate of DO change, but only indirectly).

Hemocyanin Analyses

A 0.1-ml hemolymph sample was collected at the base of the fifth periopod with a 22 GA needle on 1 cc syringe before the start of each experiment. Samples were placed in 0.6 ml polypropylene tubes and allowed to clot before being homogenized with a Teflon-tipped microtube pestle for 5 min and centrifuged at 15,000 rpm and 4°C for 15 min. The resulting supernatant was removed and stored at -70°C for later analyses.

To quantify hemocyanin concentration, hemolymph was diluted 1:60 with a Tris-Ca buffer (50 mM Tris HCl + 10 mM CaCl₂, pH = 8.0). Total protein and hemocyanin were measured by absorbance of duplicate samples against a blank of pure Tris-Ca buffer at wavelengths of 280 nm and 334 nm, respectively with a spectrophotometer. Hemocyanin and total protein concentration (mg·ml⁻¹) were calculated using extinction coefficients of 13.5 and 2.3 for absorbance measurements at 280 and 334 nm, respectively (Johnson et al. 1984). If hemocyanin and total protein measurements were not close (i.e, total protein:hemocyanin ratio > 2.0), samples were diluted 1:120 to obtain an accurate reading.

We used native, discontinuous polyacrylamide gel electrophoresis (PAGE) to quantify hemocyanin subunit structure. We diluted 25 µg of hemocyanin 1:60 (the volume of hemolymph needed to obtain this amount of Hcy from each crab was calculated from its Hcy concentration) with sample buffer (0.05 M Tris-HCl + 0.01 M EDTA + 10% glycerol + 0.002% Bromophenol blue tracking dye). Samples were incubated overnight at 4 °C to dissociate hemocyanin oligomers into the six constituent monomers (subunits 1-6). The 0.75 mm thick polyacrylamide gels (8 x 7.4 cm) consisted of a 3% stacking gel atop a 12.5% resolving gel (Hames and Rickwood 1990). The discontinuous buffer system used a Tris-Glycine (0.025 M Tris, 0.20 M Glycine; pH 8.1) upper chamber buffer and 0.025 M Tris-HCl (pH 8.9) lower chamber buffer. The gels were run at 4 °C (to minimize heating) and constant current (15 mA for 2 gels) using a BioRad MiniProtean II system until voltage reached 180 V (~ 7 h for 2 gels). The resolving gel was stained with Coomassie Blue R-250 for 40 min and then de-stained overnight with a 40% methanol, 10% acetic acid solution. Each gel contained two lanes of purified hemocyanin (Johnson et al. 1984) as a control. Each gel was

scanned using Lab Works image analysis software which identified the subunit bands and measured the optical density of each band. Subunits were identified as 1-6 based on their relative electrophoretic mobility from previous studies (DeFur et al. 1990; Mangum 1994). We then calculated the percent composition of each subunit band relative to the total amount of all subunits to reduce error associated with variation in the total amount of Hcy added to each lane, which can be substantial when using relatively small volumes of hemolymph.

Statistical Analyses

For both experiments, we used profile analysis from repeated measures MANOVA models to examine the functional relationship between three crab behavioral responses (movement rate, percent of time spent buried, and percent of time spent in the downstream end of the flume) and declining DO concentration (i.e. the within-subject factor). Profile analysis tests the statistical significance of overall polynomial model trends (i.e. linear, quadratic, cubic, etc.) for the within-subject factors, as well as interaction trends between the within-subject factors and explanatory variables (e.g. Experiment 1: changes in temperature and salinity; Experiment 2: rate of DO change, current speed) (von Ende 2001). To determine the most appropriate polynomial model, we proceeded from the highest (cubic – the most appropriate higher order polynomial to begin with for ecological data) to lowest order polynomial and stopped at the order of polynomial at which significance was found (von Ende 2001). We used analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) to test whether hemocyanin concentration and hemocyanin structure, respectively, differed for fast- and slow-moving crabs at each of the eight DO concentration classes (e.g. control, $5 \text{ mg}\cdot\text{l}^{-1}$, $4 \text{ mg}\cdot\text{l}^{-1}$, $3 \text{ mg}\cdot\text{l}^{-1}$, $2 \text{ mg}\cdot\text{l}^{-1}$, $1 \text{ mg}\cdot\text{l}^{-1}$, $0.5 \text{ mg}\cdot\text{l}^{-1}$, and chronic

anoxia exposure). Median movement rates for each DO class were used to classify crabs as slow- or fast-moving. Separate linear regression models were developed to describe the functional relationship between individual subunit concentrations and crab movement rate for those DO classes and subunits that showed significant differences in the MANOVA models. Since the ANOVA, MANOVA and linear regression analyses used several separate tests the critical alpha level to determine significance was adjusted using a Bonferroni correction factor ($\alpha_{\text{crit}} = 0.05/\text{no. of comparisons}$). Our data met assumptions of homogeneity of variance and normality using Levene's and Kolmogorov-Smirnov tests, respectively.

RESULTS

Movement rates

In both laboratory experiments, blue crab movement rates increased slightly with initial declines in DO before declining sharply with prolonged exposure to hypoxia (Fig. 1). Only rate of DO change, however, influenced the functional relationship between declining DO and movement rate; other factors such as temperature, salinity, and current speed had no effect. In the first experiment, movement rate slightly increased as DO declined to 3 mg DO·l⁻¹ then decreased with further drops in DO (Fig. 1A). There was a significant three-way interaction effect between DO, temperature, and salinity on crab movement rates (repeated measures ANOVA; $F = 6.27$, $df = 1$, $p = 0.022$), suggesting that the effect of temperature and salinity on the functional relationship between declining DO and crab movement rate was different. When separate repeated measures models for DO X temperature treatments and DO X salinity treatments were used to examine these potential interactive effects, there were no significant interactions between DO and salinity or DO and temperature on crab

movement rates ($df = 1$, $p > 0.05$). Thus, temperature and salinity did not exert a significant influence on crab movement rate, whereas DO did. The DO vs movement rate relationship (data pooled across temperature and salinity treatments) was best fit by a quadratic model ($F = 26.82$, $df = 1$, $p < 0.001$), which supports the idea that crabs initially increase and then decrease their movement rates with declining DO (Fig. 1A). The initial increase in movement rate was minor, however, as movement rates at 4 and 3 $\text{mg DO}\cdot\text{l}^{-1}$ were not significantly greater than the control ($df = 1$, $p > 0.05$). The decline in movement rate below 3 $\text{mg DO}\cdot\text{l}^{-1}$ was substantial as movement rate was significantly less than the control for DO concentrations less than 2 $\text{mg}\cdot\text{l}^{-1}$ and the chronic anoxia exposure ($df = 1$, $p < 0.05$; Fig. 1A).

Although blue crab movement rates were not affected by current speed (repeated measures MANOVA; $df = 1$, $p > 0.05$) the rate of change in DO concentration had a significant effect on the movement rate of blue crabs, with movement rates generally higher between 1.5 and 0.5 $\text{mg DO}\cdot\text{l}^{-1}$ under a fast rate of DO change conditions compared to a slow rate of change (Fig. 1B). There was a significant interactive effect of DO and rate of DO change on crab movement rates (repeated measures MANOVA; $F = 4.69$, $df = 1$, $p = 0.047$); indicating that the functional relationship between declining DO and movement rate differed between fast and slow rates of DO change. Under slow rate of DO change conditions, blue crab movement rates increased down to 3 $\text{mg DO}\cdot\text{l}^{-1}$, and declined below 1.0 $\text{mg DO}\cdot\text{l}^{-1}$. This pattern was best described by a quadratic model ($F = 5.54$, $df = 1$, $p = 0.03$); however; movement rates were not significantly different than the control at any DO concentration ($p > 0.05$) (Fig. 1B) suggesting that blue crabs exhibit a weak increase in movement rate with slowly declining DO. Conversely, under fast rate of DO change conditions, blue crab

movement rates continually increased down to $0.5 \text{ mg DO} \cdot \text{l}^{-1}$ then declined rapidly. A cubic curve best fit the relationship between fast declines in DO and movement rate (Fig. 1B; $F = 16.28$, $df = 1$, $p = 0.0009$) with movement rates significantly less than the control at $5 \text{ mg} \cdot \text{l}^{-1}$ ($F = 8.06$, $df = 1$, $p = 0.03$) and chronic anoxia ($F = 6.18$, $df = 1$, $p = 0.048$), and significantly greater than the control at $1.0 \text{ mg} \cdot \text{l}^{-1}$ ($F = 11.88$, $df = 1$, $p = 0.01$) and $0.5 \text{ mg} \cdot \text{l}^{-1}$ ($F = 14.7$, $df = 1$, $p = 0.008$). Therefore, blue crabs exhibited depressed movement rates during the initial rapid decline in DO but maintained much higher movement rates under hypoxic conditions than crabs experiencing a slower rate of DO change. Blue crabs became quiescent under chronic anoxia regardless of rate of DO change (Fig. 1B). In brief, blue crabs show a relatively strong response in activity to rapid changes in DO, and relatively weak response to relatively slow changes in DO (Fig. 1B).

There were also important qualitative changes in blue crab movement behavior with declining DO. When crabs became more active during hypoxic exposure, they usually walked with their legs fully extended and the bottom of their carapace several centimeters above the sediment surface as if searching for higher DO levels off-bottom. Even during pauses in movement, crabs maintained this “erect” posture. Crabs commonly climbed the grated structures at the upstream and downstream ends of the flume’s working section in an apparent attempt to reach more oxygenated water. When DO concentrations were hypoxic and crabs were still active, crabs appeared to spend somewhat more time climbing and were observed pushing up on the plastic sheet used to reduce diffusion of oxygen into the flume. Swimming rarely occurred and only after a crab had climbed to the top of a grate. Once crabs became quiescent in severe hypoxia or anoxia, they rarely buried (see below), but

instead rested atop the sediment surface for long periods of time (10-20 min), after which they moved very slowly around the flume for 1-3 min before resting again. When we tried removing crabs with a dip net from the flume at the end of the experiment (1 – 2 h of anoxic exposure) they attacked the net, and moved rapidly around the flume (at much faster rates than were ever recorded during our observation periods) while frequently changing direction, even though they were still exposed to DO concentrations below $0.5 \text{ mg DO} \cdot \text{l}^{-1}$. Therefore, blue crabs are capable of rapid evasive movement even under extreme anoxic stress.

Directionality

Blue crabs exhibited a weak trend for spending more time at the downstream end of the flume with declining DO (Fig. 2). In the first experiment in which the effects of covarying DO, temperature, and salinity on crab movement rates were assessed, mean percent time spent at the downstream end of the flume increased from 58% in the control period to 75% as DO levels declined (Fig. 2A), however, the trend was not statistically significant despite crabs spending more than 75% of their time at the downstream end of the flume (comprised 40% of the total available flume area) by the time conditions became anoxic (Fig 2A; repeated measures MANOVA; $df = 1$, $p > 0.05$). In the second experiment in which the effects of rate of change in DO and current speed on crab movement rates with declining DO was examined, blue crabs significantly increased the percent of time they spent in the downstream end of the flume from 45% to 60% as DO dropped ($F = 4.71$, $df = 1$, $p = 0.04$; Fig. 2B; repeated measures MANOVA; $df = 1$, $p < 0.05$). Percent of time spent in the downstream end of the flume was significantly greater than the control at DO concentrations below $2.0 \text{ mg DO} \cdot \text{l}^{-1}$ ($df = 1$, $p < 0.05$).

Burial

Blue crabs spent roughly 10-20% of their time buried under normoxic control conditions, but they spent progressively less time buried as DO declined to hypoxic concentrations (Fig. 3). In the first experiment, percent of time spent buried declined immediately as DO began to drop, and crabs almost never buried below 2 mg DO·l⁻¹ (Fig. 3A). The percent of time crabs spent buried was significantly less than that of the control at all DO concentrations at or below 2 mg·l⁻¹ and during chronic anoxia conditions (df = 1, p < 0.05). In the second experiment, crabs increased the percent of time they spent buried from about 18% to 25% during the initial drop in DO, steadily decreased their percent time spent buried until 2 mg DO·l⁻¹, after which crabs sharply reduced their percent time spent buried (Fig. 3B). The initial increase in percent time spent buried at 5 mg DO·l⁻¹ was significantly greater than the control (F = 9.44, df = 1, p = 0.02) and the subsequent decline was only significantly less than the control at 0.5 mg·l⁻¹ (F = 16.86, df = 1, p = 0.003) and under chronic anoxia conditions (F = 13.02, df = 1, p = 0.008) (Fig. 3A). Therefore, although there is considerable variability in the timing of emergence from burial with declining DO, nearly all blue crabs emerged from burial once DO reached 1 – 2 mg·l⁻¹ and remained unburied as DO concentrations declined to chronic anoxia (Fig. 3B).

Physiology

Hemocyanin structure, but not concentration, was correlated with blue crab behavior under hypoxic conditions. Although hemocyanin concentration was variable among individual crabs (mean = 47.02 mg·ml⁻¹ ± 2.81 SE; range = 16.96 mg·ml⁻¹ – 82.57 mg·ml⁻¹) it did not differ for crabs that displayed relatively fast versus slow movement rates under

hypoxic conditions. For example, there was no statistically significant difference in mean Hcy concentration between fast- and slow moving crabs at all levels of DO (all p values > 0.15 , Bonferroni adjusted $\alpha = 0.0063$). Conversely, Hcy composition did differ between slow- and fast-moving crabs (Fig. 4) at $1.0 \text{ mg DO}\cdot\text{l}^{-1}$ (MANOVA Wilks' Lambda $p = 0.0059$, Bonferroni adjusted $\alpha = 0.0063$). Crabs that were slow-moving when exposed to DO concentrations between $0.5 \text{ mg}\cdot\text{l}^{-1}$ and $1.5 \text{ mg}\cdot\text{l}^{-1}$ had higher relative concentrations of subunit one (univariate comparisons within MANOVA: $F = 4.93$, $df = 1$, $p = 0.035$), as well as lower relative concentrations of subunits two ($F = 8.46$, $df = 1$, $p = 0.007$) and four ($F = 4.30$, $df = 1$, $p = 0.047$) compared to fast moving crabs (Fig. 4), indicating that Hcy structure may influence blue crab avoidance responses to hypoxia. There were significant relationships between movement rates at $1.0 \text{ mg DO}\cdot\text{l}^{-1}$ and the proportions of Hcy subunits 1, 2 and 4 (linear regression: $df = 1$, all p -values < 0.01), with a negative relation for subunit 1 and a positive relationship for subunits 2 and 4 (Fig. 5). There was an 81% decline in movement rate ($89.5 \text{ cm}\cdot\text{min}^{-1}$ to $16.7 \text{ cm}\cdot\text{min}^{-1}$) with a 50% increase in the proportion of subunit one at $1.0 \text{ mg}\cdot\text{l}^{-1}$. Conversely, movement rates declined by 95% ($140 \text{ cm}\cdot\text{min}^{-1}$ to $7 \text{ cm}\cdot\text{min}^{-1}$) and 75% ($97 \text{ cm}\cdot\text{min}^{-1}$ to $24 \text{ cm}\cdot\text{min}^{-1}$) with a ~25% reduction in the proportion of subunits two and four, respectively. Therefore, Hcy subunit structure may influence crab behavioral patterns at moderately severe hypoxia concentrations.

DISCUSSION

It is generally unknown why mobile estuarine animal populations exhibit large variation in their responses (e.g., distribution and abundance patterns as well as mortality) to dynamic hypoxia. The results of this study suggest that (i) physiological state of a blue crab

influences its behavior under hypoxic conditions, (ii) although there is a suite of co-varying abiotic variables during hypoxic upwelling (i.e., temperature, salinity and DO), crabs respond most strongly to changes in DO, and (iii) blue crabs may orient downstream and away from the direction of hypoxic upwelling. The degree to which population distribution and abundance patterns, and mortality rates are altered during episodic hypoxic events may be a function of the hydrodynamics of the event and the hypoxia-tolerance of individuals within the population.

The avoidance responses observed in this study (increased activity, decreased burial, and down-current movement) consistently started with the onset of declining DO rather than at the 2 mg DO·l⁻¹ threshold of hypoxia commonly reported for many fish and crustaceans (Pihl et al. 1991; Wannamaker and Rice 2000; Eby and Crowder 2002; Bell and Eggleston 2005). Other evidence of DO change eliciting avoidance responses comes from laboratory experiments where estuarine fishes such as spot (*Leiostomus xanthurus*), Atlantic croaker (*Micropogonias undulatus*), white mullet (*Mugil curema*), and pinfish (*Lagodon rhomboides*) were given a choice of two zones with different DO concentrations. These fishes chose zones with relatively high DO irrespective of whether the lower DO zone was hypoxic (Wannamaker and Rice 2000). Increased activity by mobile species as DO decreases may in effect be a means of anticipating the likelihood of hypoxia exposure and may promote the successful avoidance of hypoxia. Blue crabs also maintain elevated movement rates, antennule flicking rates, and ventilation rates in hypoxic water when DO does not change much (Batterton and Cameron 1978; Lowery and Tate 1986; DeFur et al. 1990; Mangum

1997), suggesting that blue crab avoidance responses to hypoxia are stimulated by a combination of hypoxic DO concentrations and relative change in DO.

The strong differences in crab movement rate between fast and slow rates of DO change in this study indicate that the relative rate of DO change influences blue crab avoidance behavior. Crabs, however, did not move faster, emerge from burial, and spend more time at the down-stream end of the flume at higher DO concentrations in the fast rate of change treatment compared to the slow rate of change treatment. Therefore, blue crabs do not respond directly to rate of DO change. Instead, the elevated movement rates of crabs under fast rate of declines in DO conditions only under hypoxic conditions supports our alternative hypothesis that blue crabs respond directly to cumulative hypoxia exposure. The extra time crabs spent in hypoxic water when DO decreased slowly (~ 60 min at or below $2.0 \text{ mg DO}\cdot\text{l}^{-1}$ vs. 25 min when DO decreased rapidly) would have depleted the oxygen stored in their venous return system and initiated anaerobic metabolism (Lowery and Tate 1986; Mangum 1997), causing physiological stress to arise at higher DO concentrations. The influence of rate of DO change on crab movement behavior highlights the importance of knowing the hydrodynamics associated with exposure to stressors when trying to understand avoidance responses.

Although our flume study could not systematically test orientation behavior by changing the orientation of the flume (i.e., rotating it), or reversing the current direction to control for unknown movement biases, the increasing preference for the downstream end of the flume with declining DO suggests that blue crabs may use current heading as a directional cue to avoid hypoxic upwelling events. Crustaceans, including blue crabs, orient up-current when

presented with positive chemical cues (e.g., prey or conspecific odor) (Herrnkind 1983; Weissburg and Zimmerfaust 1994; Nevitt et al. 1995). Conversely, during hypoxic upwelling events estuarine animals experience a negative chemical cue (i.e., DO drop) and should orient down-current (i.e. shoreward) to escape hypoxia. We observed weak shoreward migration by free-ranging blue crabs during hypoxic upwelling events in a previous study, suggesting that they may use current direction as an orientation mechanism (Bell et al. 2003). After the completion of this study, however, we reanalyzed our biotelemetry data to account for our latest findings that crabs also respond to changes in DO and discovered a much clearer pattern of shoreward migration during conditions indicative of hypoxic upwelling events (G.W. Bell and D.B. Eggleston, unpublished data). Although more controlled studies are needed to assess the degree to which mobile animals may use current direction to avoid hypoxia, our findings after reanalysis of an existing dataset illustrate how understanding the behavioral mechanisms of avoidance responses can make it easier to detect population-level responses to stressors.

Although rarely studied, an animal's physiological state can also influence its behavioral responses to hypoxia (van Raaij et al. 1996). In our study, blue crabs with Hcys containing greater relative amounts of subunit one and lesser relative amounts of all other subunits, particularly subunits two and four, moved relatively slowly under hypoxic conditions, suggesting they exhibit a weaker avoidance response to hypoxia. The hemolymph of blue crabs acclimated to hypoxia show reduced amounts of subunits three, five, and six, which raises Hcy oxygen affinity (DeFur et al. 1990). Our slow-moving crabs had lower proportions of these three subunits than did fast-moving crabs. Moreover, the differences in

subunit proportions between slow- and fast- moving crabs is the same as the differences between hypoxia-tolerant and hypoxia-sensitive blue crabs exposed to hypoxia(Bell et al. in review). Therefore our slow-moving crabs had Hcys with relatively high O₂ affinity and were likely more tolerant than their fast-moving conspecifics. High Hcy O₂ affinity enhances the uptake of O₂ at the gills it also makes it more difficult to offload O₂ at the tissues; however, the increased activity and ventilation rates that occur during hypoxia exposure reduce pH at the tissues (Mangum and Weiland 1975; Batterton and Cameron 1978) and induce a Bohr shift that lowers the O₂ affinity of Hcy. This affinity lowering mechanism may be sufficient to counteract the elevated Hcy O₂ affinity of hypoxia-tolerant blue crabs.

Contrary to the findings that we report here, blue crabs with high O₂ affinity Hcy structures move more rapidly than those with the low O₂ affinity phenotype when exposed to chronic (30 h) hypoxia (Bell et al. in review). Under chronic hypoxia, the Hcy of hypoxia-tolerant crabs should extract more oxygen from the surrounding water which, in turn, would support greater activity (Bell et al. in review). During initial declines in DO to hypoxia (i.e., our present study), however, hypoxia-tolerant animals may use a “wait and see” behavioral response (i.e., less activity) compared to an alarm response by their less tolerant counterparts (van Raaij et al. 1996). The extent to which physiologically-induced differences in behavior and survival affect population-level patterns (e.g. distribution and abundance patterns; mortality rates) is unknown, but anecdotal evidence showing that blue crabs collected from hypoxic water express Hcy phenotypes indicative of hypoxia acclimation (Mangum 1994) suggests that inter-individual variation in physiological state may influence population-level responses to hypoxia.

Although individual variation in physiological stress responses can result in behavioral differences among individuals in higher vertebrates (Bohus et al. 1987) and some teleost fishes (van Raaij et al. 1996), such a response has not been documented for invertebrates. Our collective findings from this and a related study (Bell et al. in review) identify how Hcy structure, not Hcy concentration, may be an important physiological mechanism explaining inter-individual variation in blue crab avoidance behavior and survival under hypoxic conditions. Although increased Hcy concentration is a common physiological response to hypoxia exposure in crustaceans (Hagerman 1986; Mangum 1997; Brouwer et al. 2004), it results from reduced protein degradation, not increased protein synthesis (Brown-Peterson et al. 2005). There are substantial physiological costs associated with increased concentration of respiratory proteins. Increasing the number of Hcy molecules raises the colloidal osmotic pressure of hemolymph which can disturb fluid balance and desiccate tissues (Mangum and Johansen 1975). Moreover, increasing Hcy concentration could raise the viscosity of hemolymph, thereby raising the energetic cost of circulating it. Therefore, increased Hcy concentration may not be an adaptive physiological response to hypoxia rather; it may be the byproduct of another adaptive response--energy conservation via reduced protein synthesis and degradation.

Our experiments with blue crabs show how integrating physiology and ecology can identify sources of individual-level variation in behavioral responses to environmental stressors. Moreover, identifying specific environmental cues that mobile animals use when responding to disturbance events not only helps ascertain the avoidance capabilities of mobile animals, but is also essential to parameterize movement rules in simulation models that might be used

to assess the consequences of increasing frequency and magnitude of hypoxia on population dynamics. In particular, individual-based models can determine how variation in physiological state impacts population dynamics (Huston et al. 1988; DeAngelis et al. 1994) and are powerful tools to bridge the gap between physiology and ecology and determine the impact of environmental stressors on aquatic animal populations.

ACKNOWLEDGEMENTS

We would like to thank the following people for their valuable help with lab experiments and input on molecular techniques: G. Plaia, C. Huggett, J. Stephenson, J. Corales, A. Ullal, J-K. Seo, G. Godette, and M. Brouwer. Funding for this research was provided by research grants from the National Science Foundation (OCE-0221099) and the NC Blue Crab Research Program (01-POP-08) to D. B. Eggleston; the NC Blue Crab Research Program (03-BIOL-01), the National Sea Grant College Program, National Oceanic and Atmospheric Administration (Marine Biotechnology Program (Grant BR-01, NA16-RG-2251), and the USDA - NRI Competitive Grant Program (Project #2006-35204-17508) to E.J. Noga, as well as EPA STAR and NC Beautiful fellowships to G. W. Bell.

LITERATURE CITED

- Batterton, C. V. and J. N. Cameron (1978). "Characteristics of resting ventilation and response to hypoxia, hypercapnia, and emersion in the blue crab *Callinectes sapidus* (Rathbun)." J Exp Zool **203**: 403-418.
- Bell, G. W. and D. B. Eggleston (2005). "Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia." Mar Biol **146**: 761-770.
- Bell, G. W., D. B. Eggleston and E. J. Noga (in review). "Molecular keys unlock variable survival responses of blue crabs to hypoxia." Oecologia.
- Bell, G. W., D. B. Eggleston and D. L. Wolcott (2003). "Behavioral responses of free-ranging blue crabs to episodic hypoxia. I. Movement." Mar Ecol Prog Ser **259**: 215-225.
- Bohus, B., R. F. Benus, D. S. Fokkema, J. M. Koolhaas, C. Nyakas, G. A. Van Oortmerssen, A. J. A. Prins, A. J. H. De Ruiter, A. J. W. Scheurink and A. B. Steffens (1987). "Neuroendocrine states and behavioral and physiological stress responses." Prog Brain Res **72**: 57-70.
- Breitburg, D. L. (1992). "Episodic hypoxia in Chesapeake Bay: interacting effects of recruitment, behavior, and physical disturbance." Ecol Monogr **62**(4): 525-546.
- Brouwer, M., N. J. Brown-Peterson, P. Larkin, P. Patel, N. Denslow, S. Manning and T. H. Brouwer (2007). "Molecular and whole animal responses of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia." J Exp Mar Biol Ecol **341**: 16-31.
- Brouwer, M., P. Larkin, N. J. Brown-Peterson, C. King, S. Manning and N. Denslow (2004). "Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes sapidus*." Mar Environ Res **58**: 787-792.

- Brown-Peterson, N. J., P. Larkin, N. Denslow, C. King, S. Manning and M. Brouwer (2005). "Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*." Mar Ecol Prog Ser **286**: 203-215.
- Das, T. and W. B. Stickle (1993). "Sensitivity of crabs *Callinectes sapidus* and *C. similis* and the gastropod *Stramonita haemastoma* to hypoxia and anoxia." Mar Ecol Prog Ser **98**: 263-274.
- Das, T. and W. B. Stickle (1994). "Detection and avoidance of hypoxic water by juvenile *Callinectes sapidus* and *C. similis*." Mar Biol **120**: 593-600.
- de Vries, M. C., R. B. Forward, Jr. and W. F. Hettler (1995). "Behavioral response of larval Atlantic menhaden *Brevoortia tyrannus* (Latrob) and spot *Leiostomus xanthurus* (Lacepede) to rates of salinity change." J Exp Mar Biol Ecol **185**: 93-108.
- DeAngelis, D. L., K. A. Rose and M. A. Huston (1994). Individual-oriented approaches to modeling ecological populations and communities. Frontiers in Mathematical Biology. S. A. Levin. Berlin, Springer: 390-410.
- DeFur, P. L., C. P. Mangum and J. E. Reese (1990). "Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia." Biol Bull **178**: 46-54.
- Diaz, R. J. and R. Rosenberg (1995). "Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna." Oceanogr Mar Biol Ann Rev **33**: 245-303.
- Eby, L. A. and L. B. Crowder (2002). "Hypoxia-based habitat compression in the Neuse River Estuary: context-dependent shifts in behavioral avoidance thresholds." Can J Fish Aquat Sci **59**(6): 952-965.

- Eggleston, D. B., G. W. Bell and A. D. Amavisca (2005). "Interactive effects of episodic hypoxia and cannibalism on juvenile blue crab mortality." J Exp Mar Biol Ecol **325**: 18-26.
- Forward, R. B., R. A. Tankersley, M. C. de Vries and D. Rittschof (1995). "Sensory physiology and behavior of blue crab (*Callinectes sapidus*) postlarvae during horizontal transport." Mar Freshw Behav Phy **26**: 233-248.
- Gerritsen, J. (1982). "Behavioral response of *Daphnia* to rate of temperature change: possible enhancement of vertical migration." Limnol Oceanogr **27**(2): 254-261.
- Gray, J. S., W. R.S. and Y. Y. Or (2002). "Effects of hypoxia and organic enrichment on the coastal marine environment." Mar Ecol Prog Ser **238**: 249-279.
- Hagerman, L. (1986). "Haemocyanin concentration in the shrimp Carangon crangon (L.) after exposure to moderate hypoxia." Comp Biochem Physiol A **85**(4): 721-724.
- Hames, B. D. and D. Rickwood (1990). Gel Electrophoresis of Proteins, a Practical Approach. New York, Oxford University Press.
- Herrnkind, W. F. (1983). Movement patterns and orientation. The Biology of Crustacea. F. J. Vernberg and W. B. Vernberg. New York, Academic Press Inc. **7**: 41-105.
- Huston, M., D. DeAngelis and W. Post (1988). "New computer models unify ecological theory." Bioscience **38**(10): 682-691.
- Johnson, B. A., C. Bonaventura and J. Bonaventura (1984). "Allosteric modulation of *Callinectes sapidus* hemocyanin by binding of L-Lactate." Biochemistry-US **23**: 872-878.

- Lenihan, H. S., C. H. Peterson, J. E. Byers, J. H. Grabowski, G. W. Thayer and D. R. Colby (2001). "Cascading of habitat degradation: oyster reefs invaded by refuge fishes escaping stress." Ecol Appl **11**(3): 764-782.
- Loesch, H. (1960). "Sporadic mass shoreward migrations of demersal fish and crustaceans in Mobile Bay, Alabama." Ecology **41**(2): 292-298.
- Lowery, T. A. and L. G. Tate (1986). "Effect of hypoxia on hemolymph lactate and behavior of the blue crab *Callinectes sapidus* Rathbun in the laboratory and field." Comp Biochem Physiol A **85**(4): 689-692.
- Luettich, R. A., Jr., J. E. McNinch, H. W. Paerl, C. H. Peterson, J. T. Wells, M. Alperin, C. S. Martens and J. L. Pinckney (2000). Neuse River Estuary modeling and monitoring project stage 1: hydrography and circulation, water column nutrients and productivity, sedimentary processes and benthic-pelagic coupling, and benthic ecology, Water Resources Research Institute of the University of North Carolina: 172.
- Mangum, C. P. (1994). "Subunit composition of hemocyanins of *Callinectes sapidus*: phenotypes from naturally hypoxic waters and isolated oligomers." Comp Biochem Physiol B **108**(4): 537-541.
- Mangum, C. P. (1997). "Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*." Am Zool **37**(6): 604-611.
- Mangum, C. P. and K. Johansen (1975). "The colloid osmotic pressures of invertebrate body fluids." J Exp Biol **63**: 661-671.
- Mangum, C. P. and A. L. Weiland (1975). "The function of hemocyanin in respiration of the blue crab *Callinectes sapidus*." J Exp Zool **193**: 257-264.

- Meager, J. J., D. J. Vance, N. R. Loneragan and I. Williamson (2003). "Seasonal variation and environmental influences on juvenile banana prawn (*Penaeus merguensis*) abundance in a subtropical estuary (Logan River) of eastern Australia." Est Coast Shelf Sci **57**: 569-576.
- Nevitt, G. A., N. D. Pentcheff, K. J. Lohmann and R. K. Zimmerfaust (1995). "Evidence for hydrodynamic orientation by spiny lobsters in a patch reef environment." J Exp Biol **198**(10): 2049-2054.
- Paerl, H. W., J. L. Pinckney, J. M. Fear and B. L. Peierls (1998). "Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA." Mar Ecol Prog Ser **166**: 17-25.
- Parker, J. C. (1970). "Distribution of juvenile brown shrimp (*Penaeus aztecus ives*) in Galveston Bay, Texas, as related to certain hydrographic features and salinity." Contrib Mar Sci **15**: 1-12.
- Pihl, L., S. P. Baden and R. J. Diaz (1991). "Effects of periodic hypoxia on distribution of demersal fish and crustaceans." Mar Biol **108**: 349-360.
- Pirow, R., C. Baumer and R. J. Paul (2001). "Benefits of haemoglobin in the cladoceran crustacean *Daphnia magna*." J Exp Biol **204**: 3425-3441.
- Powers, D. A. (1980). "Molecular ecology of teleost fish hemoglobins: strategies for adapting to changing environments." Am Zool **20**: 139-162.
- Reynolds-Fleming, J. V. and R. A. Luettich, Jr. (2004). "Wind-driven lateral variability in a partially mixed estuary." Est Coast Shelf Sci **60**: 395-407.

- Seidl, M. D., R. J. Paul and R. Pirow (2005). "Effects of hypoxia acclimation on morpho-physiological traits over three generations of *Daphnia magna*." J Exp Biol **208**: 2165-2175.
- Timmerman, C. M. and L. J. Chapman (2004). "Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*)." Physiol Biochem Zool **77**(4): 601-610.
- van Raaij, M. T. M., D. S. S. Pit, P. H. M. Balm, A. B. Steffens and G. E. E. J. M. van den Thillart (1996). "Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia." Horm Behav **30**: 85-92.
- Vance, D. J., M. D. E. Haywood, D. S. Heales, R. A. Kenyon and N. R. Loneragan (1998). "Seasonal and annual variation in abundance of postlarval and juvenile banana prawns *Penaeus merguensis* and environmental variation in two estuaries in tropical northeastern Australia: a six year study." Mar Ecol Prog Ser **163**: 21-36.
- Vogel, S. and M. LaBarbera (1978). "Simple flow tanks for research and teaching." Bioscience **28**(10): 638-643.
- von Ende, C. N. (2001). Repeated-measures analysis. Design and Analysis of Ecological Experiments. S. M. Scheiner and J. Gurevitch. New York, Oxford University Press: 134-157.
- Wannamaker, C. M. and J. A. Rice (2000). "Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States." J Exp Mar Biol Ecol **249**: 145-163.

- Weissburg, M. J. and D. B. Dusenbery (2002). "Behavioral observations and computer simulations of blue crab movement to a chemical source in a controlled turbulent flow." J Exp Biol **205**(21): 3387-3398.
- Weissburg, M. J. and R. K. Zimmerfaust (1993). "Life and death in moving fluids: hydrodynamic effects on chemosensory-mediated predation." Ecology **74**(5): 1428-1443.
- Weissburg, M. J. and R. K. Zimmerfaust (1994). "Odor plumes and how blue crabs use them in finding prey." J Exp Biol **197**: 349-375.
- Wu, R. S. S. (2002). "Hypoxia: from molecular responses to ecosystem responses." Mar Pollut Bull **45**: 35-45.

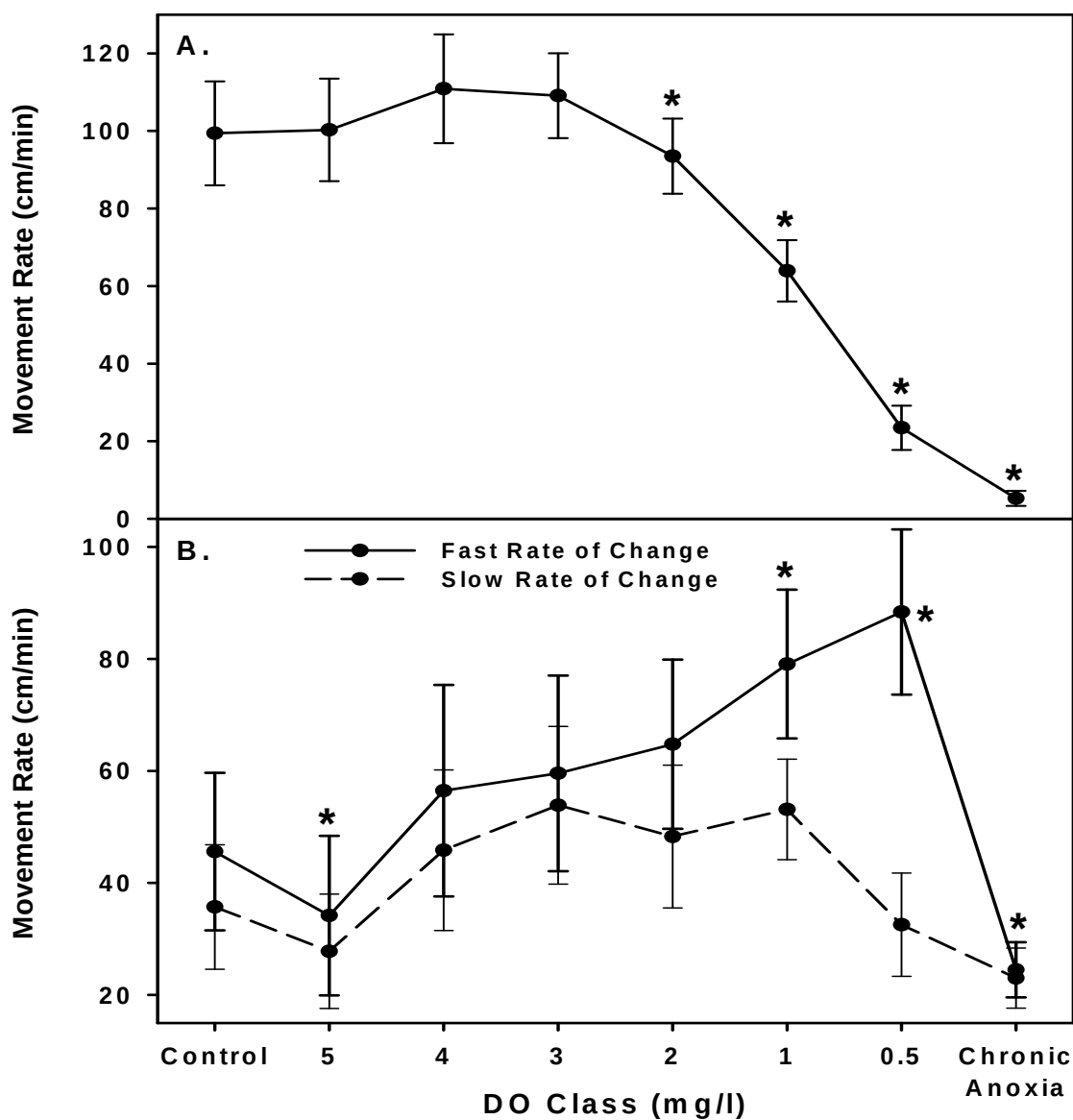


Figure 1 Change in blue crab movement rate with declining DO for experiments testing (A) interactive effects of DO, temperature, and salinity (data pooled across all treatments), and (B) effect of rate of DO change (data pooled across current speed treatments). Asterisks denote significant differences in movement rate at each DO concentration compared to the control.

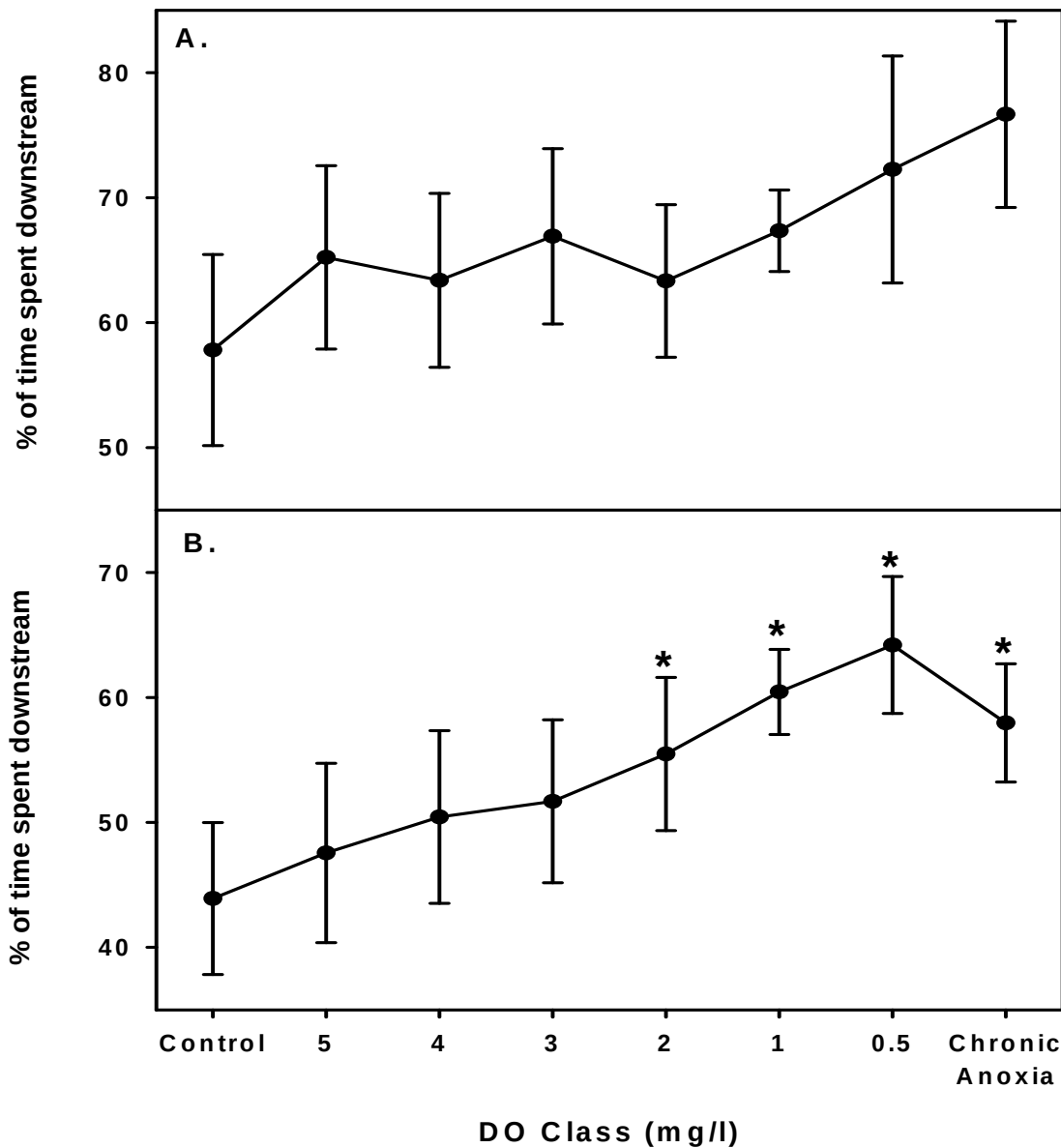


Figure 2 Change in the percent of time blue crabs spent in the downstream end of the flume as DO declined for experiments testing (A) interactive effects of DO, temperature, and salinity, and (B) effect of current speed vs. rate of DO change (data pooled across all treatments for both experiments). Asterisks denote significant differences in movement rate at each DO concentration compared to the control.

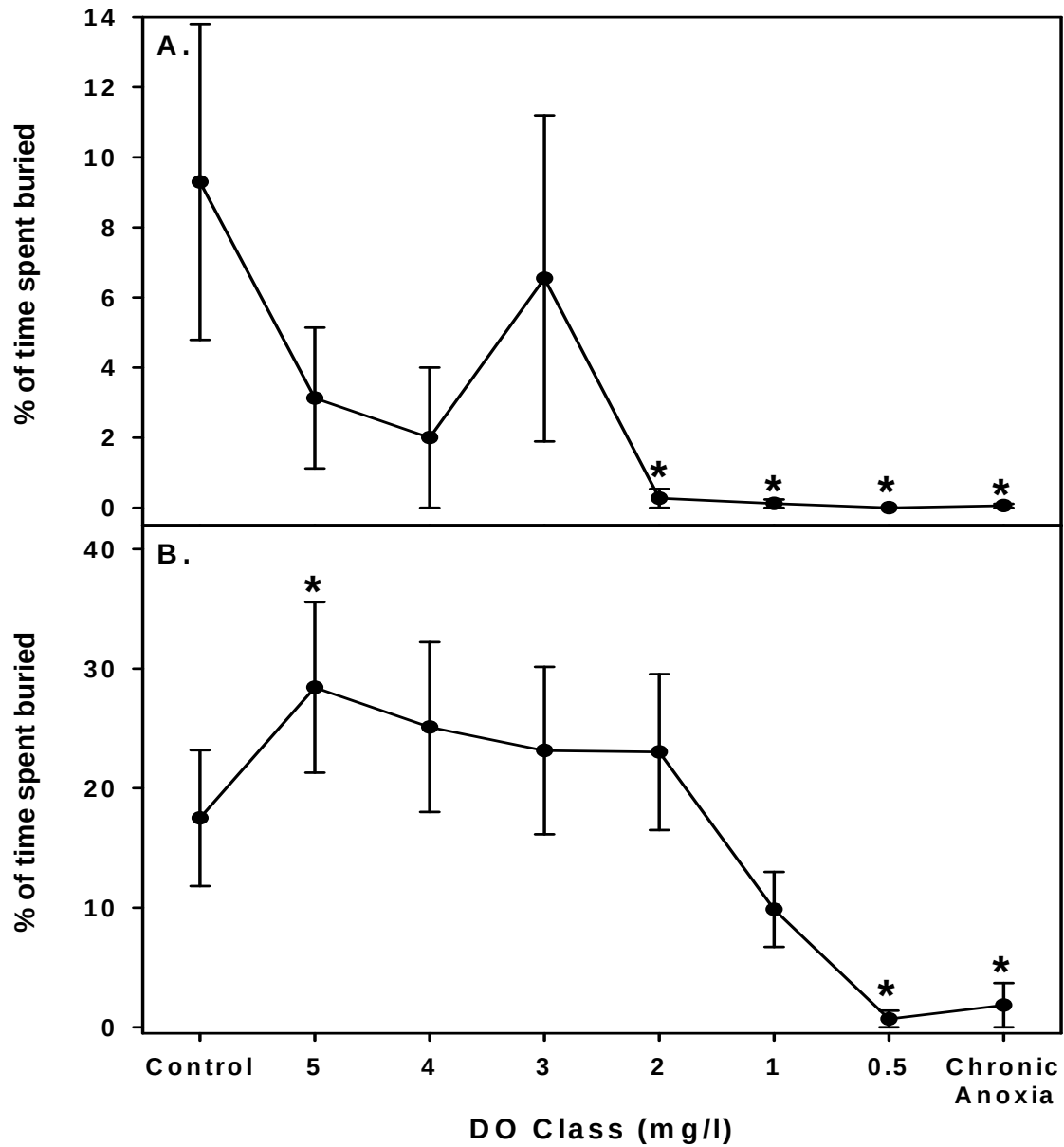


Figure 3 Change in the percent of time blue crabs spent buried as DO declined for experiments testing (A) interactive effects of DO, temperature, and salinity, and (B) effect of rate of DO change (data pooled across all treatments for both experiments). Asterisks denote significant differences in movement rate at each DO concentration compared to the control.

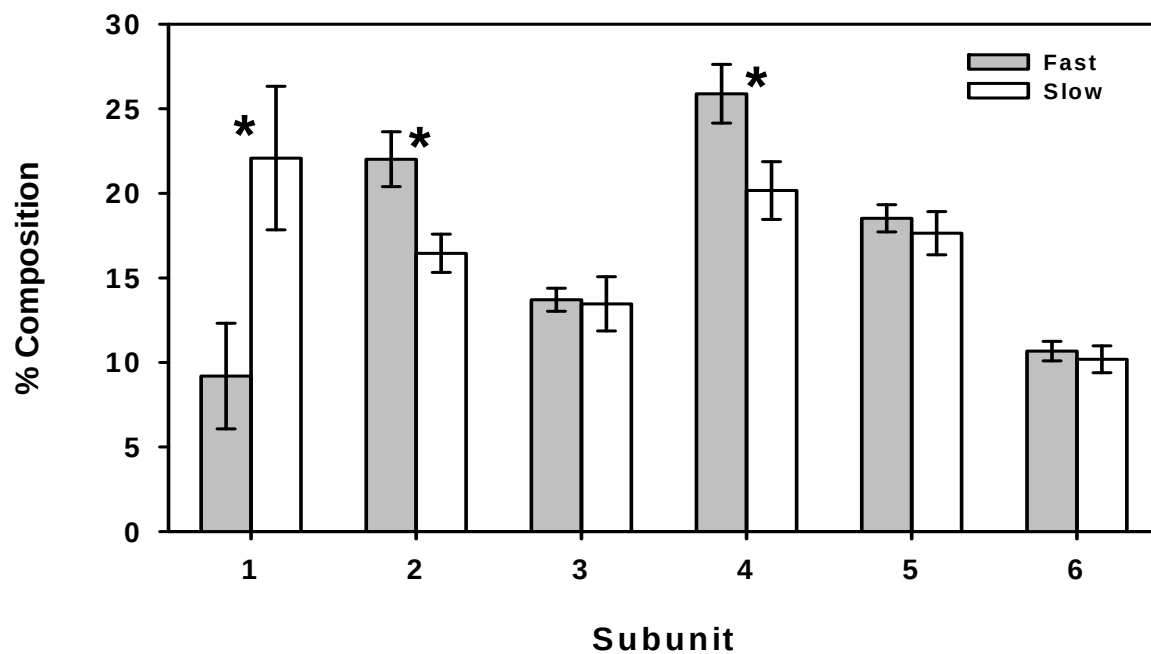


Figure 4 Comparison of Hcy subunit composition for fast- and slow-moving blue crabs when DO concentrations were $1.0 \text{ mg DO} \cdot \text{l}^{-1}$ (current speed vs. rate of DO change experiment). Asterisks denote subunits that were significantly different between fast- and slow-moving crabs.

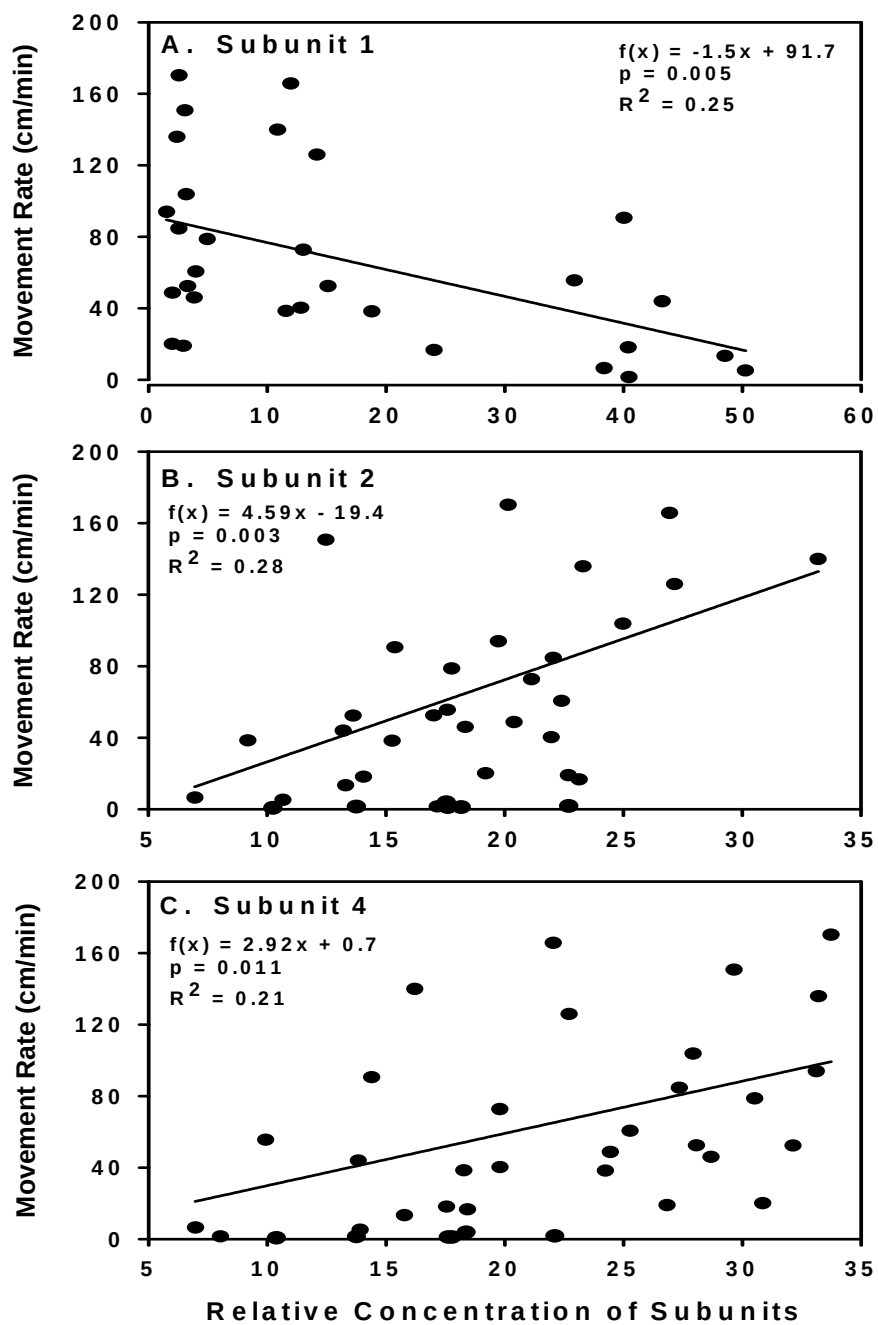


Figure 5 Functional relationship between relative proportions of each of the three significant Hcy subunits: (A) subunit 1, (B) subunit 2, and (C) subunit 4 and movement rate at 1.0 mg DO·l⁻¹.

CHAPTER 3

**EFFECTS OF PHYSIOLOGY AND HYPOXIA HYDRODYNAMICS ON BLUE
CRAB POPULATION DYNAMICS: INFERENCES FROM AN INDIVIDUAL-
BASED MODEL**

ABSTRACT

Episodic hypoxia impacts mobile aquatic animals directly when animals die from exposure to low dissolved oxygen (DO) and indirectly when they avoid intrusions of hypoxic water and aggregate in shallow nearshore habitats where increased competition and spatial overlap between predators and prey reduce growth and increase predation/cannibalism. It is difficult to assess the significance of these effects on population dynamics because episodic hypoxic events differ in their severity, duration, and hydrodynamics (i.e., current velocity and strength of the DO frontal boundary). Moreover, some individuals within a population can become acclimated to hypoxia, which affects their behavioral and survival responses to hypoxia. We developed a spatially-explicit, individual-based, population simulation model to determine the hydrodynamic conditions of episodic hypoxic events that cause the greatest impact on blue crab (*Callinectes sapidus*) population escape responses and mortality rates and determine how physiological acclimation (i.e., hypoxia-tolerant vs. -sensitive populations) may alter these population-level responses to hypoxia. We parameterized the model using empirical data from prior laboratory and field studies that quantified the functional relationships between DO hydrodynamics, physiology, behavior, and survival. Our model predicts that blue crab mortality is low during most episodic hypoxic events except for the most severe (i.e., minimum DO = 0.5 mg·L⁻¹) and longest duration (~ 30 hr)

events. Given the limited spatial extent of episodic events and how infrequently extreme events occur, the economic loss to the fishery and the impact to blue crab populations in coastal ecosystems is minimal. Escape responses of blue crabs and their resulting distribution and abundance patterns, were largely influenced by hypoxic upwelling hydrodynamics. In particular, indirect effects are likely to be greatest for long-duration events with a strong frontal DO boundary because these events cause the largest aggregations of crabs in shallow nearshore habitats. Since indirect effects are density-dependent and blue crab abundance has declined to historical lows in the past decade, indirect effects also do not pose a substantial threat to blue crab population dynamics. Mortality rates were 35% lower for hypoxia-tolerant than hypoxia-sensitive blue crab populations during hypoxic events suggesting that physiological acclimation influences population-level responses to stressors.

INTRODUCTION

Low dissolved oxygen (DO) continues to be a major global environmental problem as the number of hypoxic zones in coastal ecosystems has exponentially increased over the past several decades (Diaz and Rosenberg 1995, 2008). Coastal systems support many ecologically and economically important fishes and crustaceans that are impacted by hypoxia through two distinct pathways. Direct effects are initiated at the molecular and cellular levels when hypoxia alters the biochemical/physiological processes (e.g., neuroendocrine, gene expression, acid-base balance, etc.) of exposed individuals. The effects at these levels can lead to physiological acclimation, which may mitigate the impacts of hypoxia (DeFur et al. 1990; Mangum 1997; Bell et al. in review-b); metabolic impairment, which reduces performance (e.g. growth, reproduction, and immune function) (Holman et al. 2004; Brouwer et al. 2007); or mass mortality (Breitburg 1992; Paerl et al. 1998). Indirect effects are initiated when animals avoid hypoxia. For instance, animals lose access to resources in benthic habitats covered by hypoxia/anoxia (Buzzelli et al. 2002; Eby and Crowder 2002) and are restricted to marginal habitats where they can occur in high densities (Pihl et al. 1991; Craig and Crowder 2005). There, increased competition for resources leads to reduced growth (Eby et al. 2005) and spatial overlap between predators and their prey leads to increased cannibalism/predation (Lenihan et al. 2001; Eggleston et al. 2005). Evaluating the direct (i.e., mortality) and indirect (i.e., avoidance and subsequent predatory and competitive interactions) effects of hypoxia on population dynamics requires an integrative ecophysiology approach that accounts for responses at various levels of biological organization (Adams et al. 1989; Adams et al. 1998).

Linking the effects of hypoxia on lower levels of biological organization (e.g., molecular and cellular stress responses, physiological changes, and behavioral responses) with population dynamics is challenging because of the dynamic nature of hypoxia and individual variation in physiological acclimation. Chronic hypoxic and anoxic bottom water commonly forms during summer months in the deepest areas of many estuaries as water column stratification intensifies. The severity (i.e., minimum DO) and spatial extent of chronic hypoxic zones will vary depending on the degree of stratification (Buzzelli et al. 2002). Winds blowing across an estuary will cause hypoxic/anoxic bottom water to upwell into normoxic nearshore areas on leeward shorelines (Turner 1987; Breitburg 1990; Luettich et al. 2000). The speed with which hypoxic bottom water moves into nearshore areas is variable and depends on the strength of the winds (Luettich et al. 2000). These episodic events may also differ with respect to the structure of the frontal boundary between the hypoxic bottom water in the deep areas of estuaries and the normoxic nearshore waters. The frontal boundary can be sharp with DO going from normoxia to anoxia within a few tens of meters, or more diffuse (Eggleston et al. 2005). Upwelled hypoxic water may remain in nearshore habitats from hours to days before receding back to the deep basins (relaxation of hypoxia). Since these episodic hypoxic upwelling events are variable with respect to their severity, duration, speed, and strength of the frontal DO boundary, generalizing the ecological impacts across events is difficult without knowing the sensory capabilities that aquatic animals possess to detect low DO and the behavioral mechanisms they use to avoid it (Bell et al. in review-a).

The blue crab (*Callinectes sapidus*) is a useful model species to assess the relative importance of direct and indirect effects of hypoxia on population dynamics because it is

commercially and recreationally important, its physiological and behavioral responses to hypoxia are well-studied, and it is one of the most common species occurring in estuaries prone to hypoxia (Pihl et al. 1991; Eby and Crowder 2002; Bell and Eggleston 2005). Blue crabs respond to a suite of environmental cues associated with episodic hypoxic events. They become increasingly active in response to hypoxia and maintain their elevated movement rates under acute (< 1 hr) hypoxic exposures (Bell et al. in review-a), but reduce their activity during exposure to chronic and severe hypoxia. The magnitude of the increase in crab activity is controlled by the rate of DO change; crabs experiencing fast rates of DO decline move twice as fast in hypoxic water than crabs subjected to slow rates of DO change (Bell et al. in review-a). Blue crabs may also use water currents as a cue for the direction of encroaching hypoxic water and orient down-current (Bell et al. 2003; Bell et al. in review-a). The physiological state of aquatic animals, however, can modify their behavioral responses to hypoxia and influence survival (van Raaij et al. 1996; Timmerman and Chapman 2004; Bell et al. in review-b).

Although blue crabs use a suite of physiological responses to cope with hypoxia, changing the structure of their hemocyanin (Hcy) molecule, which binds oxygen at the gills and transports it to the tissues, is the only known mechanism that influences behavior and survival (DeFur et al. 1990; Mangum 1994; Bell et al. in review-b, in review-a). Blue crab Hcy is an oligomer protein composed of six different monomer protein subunits. Nonlethal exposures to hypoxia initiate the synthesis of new, high O₂ affinity Hcy molecules, with a subset of the six monomer subunits (DeFur et al. 1990). After approximately one week of exposure to low DO the relative proportions of the subunit types in the hemolymph change.

Blue crab populations in areas with severe and chronic hypoxia have a higher proportion of individuals with these high O₂ affinity Hcys than populations in areas less prone to hypoxia (Mangum 1994, 1997; Bell et al. in review-b), thus blue crabs can acclimate to hypoxia. Crabs with high O₂ affinity Hcy structures survive longer, are less active during initial acute exposures to hypoxia, but are more active under chronic hypoxic stress than conspecifics with low O₂ affinity Hcys (Bell et al. in review-b, in review-a). Hcy structure has characteristics that may make it a valuable bioindicator to assess the susceptibility of blue crab populations to the impacts of hypoxia including ease of collection and stability, spatial variation within and between local populations, and its influence on responses that may structure patterns at the population level such as survival and behavior (Noga et al. 2000; Adams et al. 2003; Brouwer et al. 2005). What is unknown, however, is whether blue crab Hcy structure influences population-level responses (e.g., mortality and distribution and abundance patterns) to episodic hypoxia.

Spatially-explicit, individual-based population simulation models provide a valuable framework to assess the consequences of direct and indirect effects of episodic hypoxia on population dynamics because they can integrate the hydrodynamic and physiological factors that influence the nature and magnitude of organismal responses to hypoxic disturbance. These mechanistic models are particularly useful for studying dynamic processes like hypoxic disturbance events, because they inherently account for variability in environmental conditions and individual-level differences in physiological state (Koehl 1989). Consequently, IBMs are a flexible tool that can be adapted to predict ecological impacts

across a variety of estuarine systems where the hydrodynamics of hypoxia and the susceptibility of populations to the impacts of hypoxia can vary.

We developed an individual-based, spatially-explicit, population model that simulated a variety of episodic hypoxic event scenarios spanning a wide range of conditions for several hydrodynamic factors, including severity, duration, upwelling speed, and frontal boundary DO gradient. This initial model simulated hypoxic events at small spatial (1 km²) and temporal scales (5 hr – 1 day) to: (i) identify which hydrodynamic factors exert the greatest effect on blue crab population escape responses and mortality rates during episodic hypoxic events, (ii) test whether physiological acclimation (i.e., hypoxia-tolerant vs. -sensitive populations) can alter these population responses, and (iii) provide a preliminary assessment of the consequences of hypoxia-induced mortality to blue crab population dynamics and the blue crab fishery. Connecting water quality to population dynamics of an ecologically and economically important estuarine organism is critical for directing water quality and fisheries management initiatives.

METHODS

Model description

The spatially-explicit individual-based model simulated episodic hypoxic events of varying severity (i.e., minimum DO), duration, speed, and frontal DO boundary structure as well as two blue crab populations with different degrees of physiological acclimation (i.e., percent of crabs with high O₂ affinity Hcy structure). These hydrodynamic and physiological factors influence the nature and magnitude of blue crab responses to hypoxic disturbance (Bell et al. in review-b, in review-a). The model was composed of a two-dimensional (2D)

spatial domain to simulate the bottom of an estuary, a crab state-matrix to record each crab's environmental conditions, and four main physical, demographic, and behavioral functions: (i) *Hydrodynamics*, (ii) *Survival*, (iii) *Movement rate*, and (iv) *Movement Distance and Direction* (Fig. 1). The crab state matrix stored attribute data for 500 individual blue crabs including: (1) each crab's Hcy phenotype (high and low O₂ affinity phenotypes), (2) its status (alive or dead), (3) its current DO conditions (e.g., [DO] and change in [DO]), (4) its location in the spatial domain, (5) its duration of hypoxic (DO < 1.5 mg·l⁻¹) exposure. The *Hydrodynamics* function was the principal model component that initialized DO conditions and blue crab locations in the spatial domain, as well as tracked the model time steps. During each 5-min time step, the *Hydrodynamics* function moved hypoxic water, updated each crab's environmental conditions, and subsequently passed the crab state matrix data to the demographic and behavioral functions. The *Survival* function evaluated each crab's hypoxia exposure history every 12th time step (i.e., 1 hr) and assigned a status to each crab based on survival probability functions (see below). The *Movement Rate* function assigned movement rates to crabs based on their hypoxia exposure history and current DO concentration. The *Movement Distance & Direction* function assigned each crab a movement direction based on user-defined directional biases (see below), used each crab's movement rate to calculate a movement distance, and assigned each crab to a new location in the spatial domain.

The 2D spatial landscape of the model resembled typical nearshore areas (1m – 4 m depth) of estuaries prone to episodic hypoxic events (e.g., Chesapeake Bay, Neuse River Estuary, and Mobile Bay, USA) (Breitburg 1990; Bell et al. 2003; Bell and Eggleston 2005). The

model was a cellular automata model and the landscape consisted of 0.5 m X 0.5 m grid cells that formed a 2 km section of shoreline (X-coordinate) that extended for 0.6 km away from shore (Y-coordinate). To simplify modeling of blue crab behavior and hypoxia hydrodynamics as a strict 2D process, we assumed a simple bathymetry with no complex benthic structures such as aquatic vegetation or oyster reefs that might alter the flow of hypoxic bottom water as it upwelled into nearshore areas. We assumed that crabs moved by walking on the bottom instead of swimming vertically (Bell et al. 2003). Hypoxia commonly intrudes into areas with simple bathymetry (Pihl et al. 1991; Bell and Eggleston 2005), therefore, our results are applicable to many parts of estuarine systems.

Model Parameterization

DO was spatially distributed as a gradient from normoxia ($6.0 \text{ mg}\cdot\text{L}^{-1}$) in shallow areas to hypoxia in deep areas. Hypoxic DO concentrations in nearshore areas differ among hypoxic events, therefore we ran model simulations with three different minimum DO concentrations (0.5 , 0.9 , and $1.2 \text{ mg}\cdot\text{L}^{-1}$) spanning the range of minimum DO concentrations observed in nearshore areas during hypoxic upwelling events (Breitburg 1990; Bell et al. 2003; Bell et al. in review-b). The structure of the frontal boundary between the relatively dense hypoxic bottom water in the deep areas of estuaries and the normoxic nearshore waters also differs among hypoxic events. In some cases, the frontal boundary can be sharp with DO going from normoxia to anoxia within a few tens of meters, in others it can be more diffuse (Eggleston et al. 2005). We ran model simulations with strong and weak frontal DO boundaries because the structure of the frontal boundary affects the rate of DO change that animals experience in nearshore areas during hypoxic upwelling events which, in turn,

influences blue crab avoidance behavior (Bell et al. in review-a). The DO depth gradients under strong and weak frontal boundary conditions were described by logistic equations since logistic curves were the best statistical models (see Appendix 1A & 2A) describing *in situ* spatial DO patterns along depth gradient transects from the NRE (Bell & Eggleston unpublished data). We assumed DO was homogeneously distributed in the alongshore direction (i.e., all X-coordinate cells with the same Y-coordinate were assigned the same DO concentration from the logistic equation). Although a homogeneously distributed frontal DO boundary is not truly indicative of field conditions, it is a reasonable simplification of how DO is spatially distributed at the scale of our model (Eggleston et al. 2005).

Hypoxia hydrodynamics

We were only interested in simulating the hydrodynamic processes that were important for controlling small scale ($\sim 1 \text{ km}^2$) spatiotemporal changes in DO concentrations during hypoxic events since changes in DO, as well as absolute DO concentrations, are the primary avoidance stimuli for blue crabs (Bell et al. in review-a). Therefore, it was not necessary to model any of the larger scale forcing mechanisms (e.g., Coriolis force, wind stress, pressure gradients, etc.) that influence the movement of hypoxic bottom water. Rather, we focused on simulating the advective and diffusive processes that alter nearshore spatial and temporal distribution of DO during hypoxic upwelling events. To determine if it was necessary to model both advection and diffusion, we first used the Reynolds equation to estimate the relative importance of inertial (i.e., advection) and viscous (i.e., diffusion) forces:

$$R_e = \frac{\nu_s \times D}{A_h}$$

Where v_s is the fluid velocity ($\text{cm}\cdot\text{s}^{-1}$), D is the horizontal distance, and A_h is the eddy diffusivity coefficient ($\text{cm}^2\cdot\text{s}^{-1}$). Large Reynolds numbers (i.e., $\gg 1$) indicate that advection is the major forcing mechanism, whereas small values suggest that diffusion is more important. Given that near-bottom upwelling velocities in the NRE range from two to four $\text{cm}\cdot\text{s}^{-1}$ (Luettich et al. 2000), that the distance over which hypoxia moves in our model is 60,000 cm, and that typical estimates of eddy diffusivity coefficients for wind-induced currents in stratified systems range from 125 to 2500 $\text{cm}^2\cdot\text{s}^{-1}$ (Wu and Tsanis 1995), we calculated Reynolds numbers ranging from 48 to 1920. These large values suggest that advection is the dominant force influencing the spatiotemporal distribution of DO in nearshore areas during hypoxic upwelling events (R. Luettich, UNC, Institute of Marine Science, pers. comm.); therefore, we ignored diffusion in our hydrodynamic model.

Since hypoxic upwelling events were modeled as a purely advective process, we simulated the movement of hypoxic bottom water by assigning each grid cell a new DO concentration at each 5-min time step that corresponded to the DO from a “seaward” cell in the Y-direction, and at a user-defined cell distance. Therefore, hypoxic water moved landward, perpendicular to the shoreline, at a constant velocity that was determined by the user-defined cell distance. Hypoxic water with DO concentrations less than $0.9 \text{ mg}\cdot\text{L}^{-1}$ was restricted to grid cells greater than 100 m from the shoreline so as to correspond with field observations (Breitburg 1990). Hypoxic water remained at the 100 m threshold for a specified period of time before the onset of relaxation, whereupon hypoxic water moved back towards the deep areas of the landscape. Prior to the onset of hypoxic relaxation events, the spatial distribution of crabs in the landscape was recorded to determine the escape response

of the crab population to hypoxic upwelling events. Model simulations concluded when the spatial distribution of DO was the same as the initial conditions, at which point the status (“alive” or “dead”) of individual crabs was recorded to determine the percent of the population that had died during the hypoxic upwelling and subsequent relaxation events. The duration of hypoxic events were controlled by manipulating the duration of time hypoxic water remained at the 100 m threshold and the velocity of hypoxic water during relaxation. We simulated hypoxic events with two different durations. Short duration hypoxic events were characterized by hypoxia lasting approximately 1 hr in the shallowest areas of the landscape (~ 100 m from shore) and 5 hr at mid-depths (~ 300 m from shore). This condition is the most common hypoxic upwelling scenario recorded in the NRE (Bell et al. 2003). We also simulated extreme duration hypoxic events when hypoxic water remained at the shallowest areas for approximately 5 hr and at the mid-depths for 30 hr (Bell et al. 2003). Model simulations of hypoxic upwelling events differed with respect to four factors: (i) upwelling velocity ($2 \text{ cm}\cdot\text{s}^{-1}$ vs. $4 \text{ cm}\cdot\text{s}^{-1}$), (ii) frontal DO boundary strength (strong vs. weak), (iii) duration (short vs. long), and (iv) minimum DO (0.5 , 0.9 , and $1.2 \text{ mg}\cdot\text{L}^{-1}$).

Movement rules (rate & direction)

Blue crab movement was updated during each 5-min time step by assigning each crab a movement rate and direction (Fig. 1). All crabs were assigned a baseline movement rate of $50 \text{ m}\cdot\text{hr}^{-1}$, which corresponded to the mean movement rate of free-ranging blue crabs in nearshore habitats of hypoxic estuaries (Bell et al. 2003). Crabs maintained their baseline movement rate until they experienced DO conditions that initiated avoidance behavior. Avoidance behavior was stimulated by exposure to $\text{DO} \leq 2 \text{ mg}\cdot\text{L}^{-1}$, or when crabs

experienced a drop in DO faster than $-0.05 \text{ mg}\cdot\text{L}^{-1}\cdot\text{min}^{-1}$, which corresponds to the minimum rate of DO change that elicited increased movement rates in a prior laboratory study (Bell et al. in review-a). There were two components to avoidance behavior, movement rate and direction. Since we were interested primarily in investigating crab avoidance behavior and understanding how hypoxia hydrodynamics and crab physiology influence population-level responses to hypoxia, we assumed that crabs did not interact with each other (e.g., cannibalism, agonism) in the model.

Blue crab movement rates during avoidance of hypoxic water are influenced by several factors including DO concentration, duration of hypoxic exposure, and Hcy phenotype (Bell et al. in review-a), therefore nonlinear statistical models were developed to describe the functional relationship between DO and movement rate for blue crabs with distinct Hcy phenotypes encountering different hypoxic exposure durations (see Appendix 2C). The forms of the curves were parameterized by fitting statistical models, chosen *a priori*, to laboratory data of blue crab movement rates under acute declining DO conditions (Bell et al. in review-a), as well as under chronic and constant hypoxic conditions (Bell et al. in review-b). The best statistical model to describe the functional relationship between DO concentration and crab movement rate for each Hcy phenotype and DO condition classification was chosen using AIC. Each statistical model that was selected had the lowest AIC value from among all models examined and a model probability greater than 0.60 (see Appendix 1C). Once the form of the relationship between DO and crab movement rate was determined using AIC and laboratory data, we altered the statistical model parameters so that maximum and minimum movement rates along the statistical curves were consistent with

movement rates observed for free-ranging blue crabs (see Appendix 2C). For example, blue crabs with low O₂ affinity Hcys exposed to a rapid drop in DO exhibited the largest increase in movement rate with declining DO; therefore we altered the logistic equation parameters such that the maximum movement rate corresponded to the upper quartile movement rate of free-ranging crabs during DO conditions indicative of hypoxic upwelling events (Bell et al. 2003).

During each 5-min time step, crabs either remained at their current position or moved in the direction of one of the eight neighboring cells. The direction that a particular crab moved was a stochastic process based on probabilities assigned to each of the possible movements in the X- and Y-directions (i.e., -1, 0, and 1). Since free-ranging blue crabs move randomly in nearshore areas under normoxic conditions (Wolcott and Hines 1996; Bell et al. 2003), crabs not exhibiting avoidance behavior were assigned an equal probability of moving in each of the three possible X- and Y-directions (i.e., random walk). Blue crabs, however, may avoid hypoxia by orienting down-current (i.e., inshore) during hypoxic upwelling events (Bell et al. 2003; Bell et al. in review-a); therefore, crabs avoiding hypoxia in the model were assigned directional probabilities that were biased towards the shoreline (i.e., biased random walk). We used blue crab orientation data from a field biotelemetry study (Bell et al. 2003) to parameterize these directional bias probabilities. All previous biotelemetry observations that were indicative of hypoxic upwelling events (e.g., crabs experiencing a rapid drop in DO, or crabs encountering hypoxia) (Bell et al. 2003) were extracted and the directional headings for each crab under these conditions were averaged to create a circular heading plot showing the frequency of headings under hypoxic upwelling events (see Appendix 3A). Based on this

frequency distribution, directional bias probabilities were assigned to each of the three X- and Y-directions (Appendix 3A). Each crab's movement rate ($\text{m}\cdot\text{hr}^{-1}$) was then converted to the corresponding distance (i.e., number of grid cells for a 5-min time step) such that a new X- and Y- coordinate could be assigned at each 5-min time step.

Survival

We parameterized survival in the IBM using data from a laboratory experiment where adult blue crabs were exposed to several hypoxic DO concentrations for 30 hr and the time of death recorded for each crab (Bell et al. in review-b). The data from this experiment were used in Cox proportional hazard models (Dixon and Newman 1991; Newman and Aplin 1992) to construct survival functions describing the probability of an individual crab surviving a hypoxic exposure time (hr) given its physiological state (Hcy O₂ affinity phenotype) and DO conditions. There were seven survival functions specified in the IBM (see Appendix 2B); three lethal DO concentrations (0.9, 0.6 and 0.3 mg DO·L⁻¹) for each of the two Hcy phenotypes (high and low Hcy O₂ affinity) and a 1.2 mg DO·L⁻¹ function for both Hcy phenotypes because Hcy O₂ affinity has no effect on survival above 0.9 mg DO·L⁻¹ (Bell et al. in review-b). We fitted *a priori* linear and nonlinear statistical models to the Cox proportional hazard model output to describe the relationship between hypoxic exposure time and probability of survival. Models with the lowest AIC value were selected and their probability of being the best model, from all tested statistical models, was always greater than 0.60 (see Appendix 2A).

Crab survival was assessed every hour (i.e., every 12th time step) to coincide with the time scale of observations made during a previous laboratory experiment (Bell et al. in review-b).

The *Survival* function stored a record of all prior hourly DO concentrations and calculated the mean DO concentration that each crab experienced during the preceding 12 time steps. The lethal threshold DO concentration for adult blue crabs is $1.2 \text{ mg}\cdot\text{L}^{-1}$ (Bell et al. in review-b); therefore, we assumed crabs that encountered a mean DO greater than $1.2 \text{ mg}\cdot\text{L}^{-1}$ survived the current time step. If crabs encountered DO concentrations less than $1.2 \text{ mg}\cdot\text{L}^{-1}$ during the preceding hour, the *Survival* function updated the total number of hours each crab spent in hypoxic water by one hour. The probability of each crab surviving its current hypoxic exposure was obtained by selecting the survival curve corresponding to each crab's Hcy phenotype and current DO and using its current hypoxic exposure time as the dependent variable in the survival curve equation. For crabs experiencing different hypoxic DO concentrations during their exposure history, the model computed a weighted average of the survival probabilities for each of the DO treatments that crabs encountered based on the percentage of time they spent at each lethal DO concentration.

It is also important to consider physiological recovery from environmental stressors when assessing the impacts of stressors on animal populations (Schreck 2000). We initially programmed a simple recovery function in the model to account for crabs that encountered lethal DO concentrations that were periodically interrupted by periods of normoxia. We assumed a constant rate of recovery, whereby each hourly time step of normoxic exposure raised the probability of survival by 0.07, because metabolite levels, altered by exposure to hypoxia (e.g., lactate, glycogen, glucose, etc.), return to normoxic levels at a relatively constant rate during post-hypoxic recovery (Lowery and Tate 1986; Hill et al. 1991). Intermittent exposures to normoxia were rare in the model as nearly all crabs encountering

hypoxia either remained in hypoxic water for the duration of the model simulation, or emerged from hypoxia and remained in normoxic water for the remainder of the model simulation. Moreover, a sensitivity analysis indicated that the percent of crabs dying from hypoxia exposure was unaffected by a $\pm 25\%$ change in the recovery rate parameter. Therefore, recovery from hypoxic stress was not an important process and the recovery function was irrelevant to our results.

Survival probability at the current time step is not independent of previous time steps. More specifically, the real probability of a crab surviving its current time step is the product of its survival probabilities from all prior time steps and its current time step (i.e., cumulative probabilities). Therefore, the probability of surviving the current time step must be adjusted upward to prevent underestimating survival and consequently inflating blue crab mortality rates. We applied the following conditional probability equation to calculate the probability of a crab surviving the current time step ($S_{(t)}$), given it survived its prior time step ($S_{(t-1)}$):

$$P(S_{(t)} | S_{(t-1)}) = \frac{P(S_{(hyp t)})}{P(S_{(hyp t-1)})}$$

where the numerator is the probability of surviving the current hypoxia exposure and the denominator is the probability of surviving the hypoxic exposure at the preceding time step. Thus, the probability of surviving each time step was larger than the probability of surviving the corresponding hypoxia exposure. Each crab's status ("alive" vs. "dead") was assigned for each hourly time step by choosing a random number (1 vs. 0) from a binomial distribution with probability of surviving equal to the conditional probability calculated from the above equation.

Model Simulations, Hypotheses, & Statistical analyses

Hydrodynamics

To determine which hydrodynamic characteristics are likely to have the greatest effect on blue crab population distribution patterns and mortality rates during hypoxic upwelling events, we conducted 24 model simulations, each with a specific combination of treatment levels for four features: (i) duration of a hypoxic event (short vs. long), (ii) minimum DO concentration (0.5, 0.9, vs. 1.2 mg·L⁻¹), (iii) hypoxic upwelling speed (slow vs. fast), and (iv) frontal boundary (strong vs. weak). We also conducted a “control” simulation of chronic hypoxic conditions in which a static, strong frontal boundary separated normoxic nearshore water from severely hypoxic (0.5 mg·L⁻¹) deep water for 30 hr. Each of these simulations was replicated 15 times because preliminary analyses suggested that this was the appropriate amount of replication to characterize within-treatment variation in both response variables. For these simulations, 70% of crabs had the high O₂ affinity Hcy phenotype to be consistent with wild populations of blue crabs in the NRE (Bell & Eggleston unpublished data). To assess the relative importance of the various hydrodynamic features on distribution patterns, we used the percent of the crab population that had successfully escaped hypoxic water at the peak of hypoxic upwelling events (i.e., immediately before hypoxic water began to recede) as a response variable. We tested eight alternative hypotheses (Table 1) that constituted various combinations of factors we expected to influence avoidance success. First, we hypothesized that crabs would be more successful at avoiding slower (i.e., speed factor) than longer hypoxic events (i.e., duration factor). We also expected that crabs would be better at avoiding hypoxia during events with fast rates of change in DO (i.e., interactive effect of

speed and frontal boundary) than slow rates of change because blue crabs can sustain faster movement rates when DO drops more rapidly (Bell et al. in review-b). Lastly, since blue crab movement rates increase with declining DO concentration during initial exposure to hypoxia, but decrease with declining DO under more chronic hypoxia conditions (Bell et al. in review-b, in review-a), we expected an interactive effect of minimum DO concentration and duration on crab avoidance success.

To assess the relative importance of different hydrodynamic characteristics on blue crab mortality, we tested six hypotheses (Table 2) that examined the effects of various combinations of three hydrodynamic factors (duration, minimum DO, and rate of change) on the percent of crabs that had died at the conclusion of each model simulation. We expected that percent mortality would be greatest for relatively long duration hypoxic events with low minimum DO concentrations. Since the probability of crab survival decays at different rates under different minimum DO concentrations (see Appendix 2B) we also hypothesized that there may be an interactive effect of minimum DO and hypoxic event duration on percent crab mortality. Lastly, since we expected hypoxic upwelling speed and frontal boundary to interactively affect blue crab escape responses, we hypothesized that percent mortality would show the opposite pattern to these factors because percent escape and percent mortality should be inversely related (i.e., lower escape success would correspond to greater mortality).

Physiology

To determine if blue crab populations with different hypoxia tolerances differ in their escape responses, which affects their distribution and abundance patterns as well as their mortality during episodic hypoxic events, we conducted 24 additional model simulations

(each simulation was again replicated 15 times) with the same hydrodynamic characteristics as the previous simulations, except that instead of 70% of the crabs in the population having the high O₂ affinity Hcy phenotype (i.e., hypoxia-tolerant population), no crabs had this phenotype (i.e., hypoxia-sensitive population). We calculated mean percent escape and mortality for each hydrodynamic treatment combination and tested whether percent successful escapes and mortality differed between hypoxia-sensitive and hypoxia-tolerant populations, as well as if differences in escape and mortality between the two populations varied across different levels of the hydrodynamic factors found to have the greatest effect on escape and mortality (Tables 3 and 4).

Sensitivity Analyses

We conducted model parameter sensitivity analyses to better understand which components of crab avoidance behavior may be most critical for successful avoidance and survival of crabs under dynamic hypoxic conditions. Sensitivity analyses entailed running a series of simulations in which we modified three model parameters (movement rate, Y-direction bias, and threshold DO concentration eliciting crab avoidance), one at a time, in increments of $\pm 5\%$ up to $\pm 25\%$ and then calculating a sensitivity index using the following formula (Crowder et al. 1994):

$$Sensitivity = \frac{\lambda_{(x+0.15x)} - \lambda_{(x-0.15x)}}{\lambda_{(x)} * (2 * 0.15)}$$

where λ is the model output in terms of either of the two response variables (e.g. % mortality and % escape), x is the model parameter being modified, and 0.15 is the proportion we used

to calculate the sensitivity index. Larger index values indicate that a parameter affects the model output more strongly than others.

Statistical Analyses

We used an information theoretic approach (Burnham and Anderson 2002) to identify which of the hydrodynamic factors were most important for altering crab population distribution and abundance patterns as well as mortality rates, and to determine if these patterns differed between crab populations with different tolerances to hypoxia. The information theoretic approach calculates statistics that estimate the relative likelihood of several competing hypotheses to explain patterns in response variables, rather than using traditional p-values that simply reject hypotheses. Although this approach uses residual sums of squares output (estimates the amount of variation not explained by a statistical model) from least squares ANOVA and regression models, it is inappropriate to quote least squares test statistics when using the information theoretic approach (Burnham and Anderson 2002). We first used least squares ANOVA to develop models that corresponded to the various alternative hypotheses we outlined earlier (Tables 1 - 4). Since the information theoretic approach uses ANOVA model output to compute statistical parameters (see below), the ANOVA assumptions must be met (Burnham and Anderson 2002). Frequency histograms and QQ-plots of the two response variables (% of crabs successfully escaping hypoxia and percent mortality) suggested the raw model output data were not normally distributed. For percent mortality, a log-transformation was sufficient to satisfy the normality assumption. There was a slight deviation from normality for the percent escape data since a moderate percentage of the data (18%) were zeros, thereby causing a small peak at the low end of the

frequency distribution. Since various transformations did not normalize the crab escape data, we used the raw data in our ANOVA models; however, ANOVA models are generally robust to such deviations from normality (Underwood 1981). Residual plots from each model indicated that variances were homogeneous; therefore, ANOVA assumptions were satisfied.

We used the residual sum of squares from each of the ANOVA model outputs to calculate Akaike's Information Criterion for small sample sizes (AIC_c) (Burnham and Anderson 2002). The AIC_c values for each set of ANOVA models were then used to compute several statistics that can be used to estimate the level of empirical support for each model. AIC differences (Δ_i) compare each model's AIC_c to the model with the lowest AIC_c to provide a relative ranking of the candidate models. The larger the AIC difference, the less likely a fitted model is the correct model given a set of plausible models. Although the model with $\Delta_i = 0$ is the most plausible model, some rules of thumb are available to judge the level of support for the alternative models ($\Delta_i = 0 - 2$: substantial support, $\Delta_i = 4 - 7$: considerably less support, $\Delta_i > 10$: nearly no support) (Burnham and Anderson 2002). Akaike weights (ω_i) estimate the probability of models being the best model from a set of models. Lastly, evidence ratios compare Akaike weights between pairs of models and these values estimate how much more likely one model is over another.

RESULTS

Hydrodynamics

Spatial Distribution & Escape

As expected, episodic hypoxic events shifted the distribution of blue crabs towards the shoreline, much more than chronic hypoxia conditions (Fig. 2). Under chronic hypoxic

conditions, crab encounters with hypoxic water were rare; crabs were distributed uniformly throughout the normoxic nearshore region and only 0.37% of crabs remained in hypoxic water at the end of simulations (Fig. 2A). During episodic hypoxic events, the percent of crabs in the population successfully escaping encounters with hypoxia was variable, ranging from 0% to 73%, depending on the hydrodynamics of the event. This range of values for crab avoidance of hypoxia is consistent with observations of escape success from free-ranging blue crabs (Bell et al. 2003). Thus our model reasonably portrays the escape behavior of blue crabs under varying hypoxia hydrodynamics, which suggests that our predictions of crab distribution and abundance patterns and mortality rates are realistic. The extent to which blue crab abundance increased in the shallowest areas of the landscape varied with respect to upwelling hydrodynamics. For example, crabs were most successful at avoiding the least severe ($1.2 \text{ mg DO} \cdot \text{L}^{-1}$), long duration, slow upwelling events that had a weak frontal boundary, with 68% of the crab population occurring in the shallowest, normoxic areas (Fig. 2B). Conversely, crabs were least successful at avoiding short duration, fast-moving hypoxic events that had a weak frontal boundary and $0.5 \text{ mg DO} \cdot \text{L}^{-1}$, with only 45% of the crab population occurring in normoxic water (Fig. 2C). Thus, stronger blue crab escape responses are indicative of greater shifts in crab distribution and abundance patterns.

All four of the hydrodynamic factors tested (minimum DO, hypoxic event duration, upwelling speed, and DO frontal boundary) affected blue crab population escape responses during episodic hypoxic events (see AIC results in Table 1 and Fig. 3). The effect sizes for these factors were different suggesting that some hydrodynamic factors had a greater affect than others on crab population escape responses, and consequently their distribution and

abundance patterns. The duration of hypoxic events had the strongest impact on blue crab escape responses but the magnitude of its effect depended on minimum DO concentration (i.e., severity) (Fig. 3A). For example, the percent of crabs successfully escaping hypoxia was always greater for long-duration hypoxic events compared to short-duration events with the difference in percent escape between long- and short-duration events increasing with increasing minimum DO (Fig. 3A). Minimum DO had a minor affect on escape compared to hypoxic event duration, regardless of hypoxic event duration (Fig. 3A). The frontal boundary strength had a moderate effect on blue crab distribution patterns compared to the duration of hypoxia; however, its effect depended on hypoxic upwelling speed. The percent of crabs escaping hypoxia was always greater when a strong frontal boundary was present compared to a weak frontal boundary; however, the difference in escape between weak and strong frontal boundary conditions was greatest when current speeds were slow (Fig. 3B). Current speed had almost no effect on the percent of crabs escaping hypoxia under weak frontal boundary conditions but crabs were more successful at escaping slow-moving hypoxic events compared to fast-moving events when a strong frontal boundary was present (Fig. 3B). Therefore, the duration of hypoxic upwelling events and, to a somewhat lesser extent, the strength of the frontal boundary appear to have the greatest impact on the ability of blue crabs to successfully escape hypoxia and consequently alter crab distribution and abundance patterns.

Mortality

In general, blue crab mortality was low during episodic hypoxic events, averaging $2.5\% \pm 0.5$ across the 24 hydrodynamic treatment combinations and ranging from 0.3% to 9.9%. All

four hydrodynamic factors had an affect on blue crab mortality during episodic hypoxic events (see AIC results in Table 2); however, some factors had a greater affect than others. The interactive effects of hypoxic event duration and minimum DO had the greatest impact on mortality. For example, percent mortality of crabs during short-duration hypoxic events increased four-fold from 0.4% to 1.7% as minimum DO decreased from 1.2 to 0.5 mg DO·L⁻¹; however, the percent difference in crab mortality across these treatments was low at 1.3% (Fig. 4A). Conversely, percent crab mortality increased by 6.1% with a decrease in minimum DO during long duration events (Fig. 4A), such that minimum DO (i.e., severity) had its strongest effect on mortality when the duration of hypoxic events was long (Fig. 4A).

The interactive effects of upwelling speed and frontal boundary had a relatively weak effect on blue crab mortality and the patterns we observed were inconsistent with our original hypotheses. We expected that percent crab mortality would be greater when a weak frontal boundary was present compared to a strong frontal boundary and when current speeds were fast compared to slow because crabs were less successful at escaping hypoxic events with a weak vs. strong frontal boundary and fast vs. slow current speeds (Fig. 3B). The results demonstrated that when a strong frontal boundary was present, percent crab mortality was higher under fast than slow current speeds, as expected. Under weak frontal boundary conditions, however, percent crab mortality was lower under fast current speeds compared to slow current speeds (Fig. 4B). The mechanisms underlying the interactive effects of frontal boundary strength and current speed on percent crab mortality are unclear, however, given that the differences in percent crab mortality among hypoxic event duration and minimum DO treatment levels were five times greater than for current speed and frontal boundary

treatment levels, it is unlikely that frontal boundary strength and current speed have much effect on blue crab mortality during episodic hypoxic events.

Physiology

Escape & Distribution

Blue crab populations that were hypoxia-sensitive (i.e., 0% of crabs had high O₂ affinity Hcys) and hypoxia-tolerant (i.e., 70% of crabs had high O₂ affinity Hcys) showed small differences in their escape response to episodic hypoxia. For example, only 2% more crabs in the hypoxia-sensitive population successfully avoided hypoxia compared to the hypoxia-tolerant population (Fig. 5A). Although the greatest difference in percent escape between these two populations occurred when hypoxic event duration was short (Fig. 5B) and when a strong frontal boundary was present (Fig. 5C), the differences in percent of crabs successfully escaping hypoxia were small. Moreover, a blue crab population's sensitivity to hypoxia had a minor influence on their escape response relative to hydrodynamics (see AIC results in Table 3). Therefore, the small difference in escape response between crab populations suggests that physiological acclimation has little effect on distribution and abundance patterns during episodic hypoxic events.

Mortality

In contrast to escape, mortality was greater for the hypoxia-sensitive blue crab population compared to the hypoxia-tolerant population. For example, mean percent mortality for the hypoxia-tolerant population was $2.5\% \pm 0.5$ (range = 0.3% to 9.9%), which was 35% less than percent mortality of the hypoxia-sensitive population (mean = $3.8\% \pm 0.9$; range = 0.3% to 16.6%) (Fig. 6A). Moreover, the hypoxia tolerance of blue crab populations explained

substantially more variation in mortality than the interactive effects of minimum DO and hypoxic event duration alone (see AIC results in Table 4). The difference in percent mortality between the hypoxia-sensitive and hypoxia-tolerant populations was most pronounced during long duration events. For example, percent crab mortality for the hypoxia-tolerant population was only 22% less than for the hypoxia-tolerant population during short hypoxic events (1.0% vs. 1.3%, respectively), but was 38% less during long duration events (3.9% vs. 6.2%, respectively) (Fig. 6B). Thus, physiological acclimation by individual blue crabs in a population can increase survival by ~ 35% during episodic hypoxic events.

Sensitivity

Blue crab escape and mortality responses were most sensitive to deviations in movement rate and the strength of orientation behavior, and were unaffected by the threshold drop in DO that elicits avoidance behavior (Fig. 7). For example, when the model parameter estimates for orientation strength and movement rate increased from -25% to +25% of the initial parameter values, average percent of crabs successfully escaping hypoxia doubled from ~19% to 39% (Fig. 7A). Conversely, the same change in the parameter value for the rate of DO drop required to elicit avoidance behavior caused no change in crab escape response (Fig. 7A). Moreover, the percent escape sensitivity index values for orientation strength (1.45) and movement rate (1.39) were two orders of magnitude greater than the threshold DO drop parameter (0.01). A similar pattern was evident for the mortality response. Changing the orientation strength and movement rate parameter estimates from -25% to +25% of their initial value decreased average percent mortality by 50% (Fig. 7B),

while modifying the parameter for rate of DO drop caused no change (Fig. 7B). The percent mortality sensitivity index values for orientation strength (1.98) and movement rate (1.78) were two orders of magnitude greater than the threshold DO drop parameter (0.07). Thus, animal movement rates and ability to orient away from low DO appear to be the most critical components of blue crab avoidance behavior that promote their survival during episodic hypoxic events.

DISCUSSION

Understanding and predicting the impact of hypoxia on population dynamics of estuarine organisms requires a mechanistic understanding of the behavioral and physiological mechanisms used by mobile animals to avoid and survive hypoxia. Using a spatially-explicit, individual-based population simulation model that was parameterized with laboratory and field data for our target species, we demonstrated that the mortality and escape responses of blue crab populations during episodic hypoxic events are influenced by a combination of hydrodynamics and physiology. For example, the duration of hypoxic upwelling events and the strength of the frontal boundary appear to have the greatest impact on the ability of blue crabs to successfully escape hypoxia and consequently alter crab population distribution and abundance patterns during episodic hypoxic events. Conversely, blue crab mortality was most affected by the duration and severity (i.e., minimum DO) of hypoxic events. Physiological acclimation to hypoxia had little effect on blue crab escape responses, however, mortality rates of hypoxia-tolerant crab populations were 35% lower than hypoxia-sensitive crab populations, indicating that physiological processes can influence population-level responses to hypoxia.

Hydrodynamic and physiological mechanisms

Blue crabs were most successful at avoiding hypoxia when episodic hypoxic event duration was long (~ 30 hr) vs. short (~ 5 hr) and when the frontal boundary was strong vs. weak, suggesting that blue crab populations will be more highly concentrated in the shallowest areas of nearshore habitats during long-duration events with strong frontal boundaries. Blue crabs maintain elevated movement rates for up to an hour after initial exposure to hypoxia and these movement rates nearly double when crabs are exposed to fast rates of DO change relative to slow rates of DO change (Bell et al. in review-a). Therefore greater avoidance success during long-duration events occurs because crabs have more time to escape hypoxia before it recedes to the deeper areas of estuaries. Furthermore, avoidance success will be relatively high when a strong frontal boundary is present because crabs experience a relatively rapid rate of DO change. In wind-driven systems, such as the NRE, a strong frontal boundary likely forms under calm wind conditions which slows water circulation, allows stratification to develop, and limits lateral mixing processes that break down the pycnocline. Therefore, “Jubilees” are more likely to form and indirect effects such as increased predation/cannibalism and competition are likely to be most severe when a period of a day or two of calm wind conditions is followed by strong and prolonged winds either from the southwest or northeast.

Physiological acclimation had little effect on the escape response of crab populations and consequently did not influence blue crab distribution and abundance patterns during episodic hypoxic events. Under certain hypoxic upwelling conditions, primarily during short-duration hypoxic events, the hypoxia-sensitive crab population was more successful at avoiding

hypoxia than the hypoxia-tolerant crab population. Hypoxia-sensitive crabs are nearly twice as active as hypoxia-tolerant crabs in hypoxic water during initial declines in DO (Bell et al. in review-a), therefore hypoxia-sensitive crab populations are more likely to successfully avoid short-duration hypoxic events than their hypoxia-tolerant counterparts. Conversely, during more prolonged exposures to hypoxia, hypoxia-tolerant blue crabs are more active than their less tolerant conspecifics (Bell et al. in review-b). Thus, during long-duration hypoxic events, hypoxia-tolerant crab populations compensate for their lack of an initial burst escape response by remaining relatively active in hypoxic water, which resulted in similar escape responses between the two crab populations.

Mortality of blue crab populations during episodic hypoxic events was affected by somewhat different hydrodynamic factors than their escape response. Blue crab mortality was generally low during chronic and most episodic hypoxic events; however, mortality increased with increasing duration of hypoxic events and decreasing DO concentration, with the primary potential threat to the population coming from the most severe ($0.5 \text{ mg DO}\cdot\text{L}^{-1}$) and longest duration ($\sim 30 \text{ hr}$) events that cause the highest mortality (5 – 10%). The magnitude of annual blue crab mortality that occurs in hypoxic estuaries may depend largely on the amount of rainfall, which in turn controls the degree of stratification and ultimately the spatial extent of hypoxic bottom water and severity of DO (Turner 1987; Stanley and Nixon 1992; Paerl et al. 1998; Buzzelli et al. 2002). During dry years, relatively low rainfall means that less freshwater enters estuaries to cover the more saline bottom water encroaching from coastal areas (Paerl et al. 1998). The relatively weak salinity stratification that results may cause patchy hypoxic bottom water to develop throughout the summer. During wet years,

increased rainfall causes strong stratification and extensive areas of severely hypoxic bottom water that can persist for months (Paerl et al. 1998). In US east coast estuaries, where blue crabs are most abundant, rainfall is relatively low during months of peak hypoxic bottom water formation (Paerl et al. 1998) such that dry conditions are probably the norm. This indicates that annual blue crab mortality in the two major ecosystems on the U.S. east coast, NC coastal waters and Chesapeake Bay, may be low with intermittent years of relatively high hypoxia-induced mortality when large rainfall events occur during summer months.

One of the potential reasons for low blue crab mortality during episodic hypoxia is the ability of blue crabs to acclimate to hypoxia. The sublethal exposures to hypoxia that free-ranging blue crabs commonly experience in estuaries (Bell et al. 2003) are likely due to short-duration events (< 5 hr), which in the NRE, can occur daily when winds relax in the evening causing a return seiche of hypoxic bottom water to the leeward side of the estuary (Reynolds-Fleming and Luettich 2004). This type of repeated daily exposure to hypoxia stimulates the production of high O_2 affinity Hcys (DeFur et al. 1990; Mangum 1997), which promotes blue crab survival during subsequent hypoxic conditions (Bell et al. in review-b). Hypoxia-sensitive crab populations (i.e., 0% of crabs had high O_2 affinity Hcys) suffered almost twice as much mortality during the severe events than the hypoxia-tolerant population (i.e., 70% of crabs had high O_2 affinity Hcys). Therefore, not accounting for physiological acclimation in this study would have systematically inflated our estimates of mortality. Although this bias may not be substantial during “average” years when we expect mortality from episodic hypoxia to be low ($\sim$ few thousand crabs, see below) it could be substantial during wet years when mortality may be two orders of magnitude greater ($\sim$ hundred

thousand crabs, see below). Although blue crab mortality of this magnitude is still minor compared to extraction due to fishing, our results demonstrate that physiological responses to hypoxia at the molecular level can affect subsequent mortality responses at the population level (Adams et al. 1989; Adams et al. 1998). Thus, molecular biomarkers, such as Hcy structure, can be useful tools for assessing the effects of stressors on aquatic animal populations.

Implications for other estuarine species

Mobile fishes including spot (*Leiostomus xanthurus*), Atlantic menhaden (*Brevoortia tyrannus*), bay anchovy (*Anchoa mitchilli*), and naked goby (*Gobiosoma bosc*) that also occur in hypoxia-prone systems are much less tolerant of hypoxia than blue crabs (Breitburg 1994; Shimps et al. 2005; Bell et al. in review-b). Consequently, episodic hypoxic events can cause greater mortality for these species compared to blue crabs. In the case of the naked goby, intrusions of hypoxic water can wipe out an entire cohort of young juveniles on oyster reefs in Chesapeake Bay (Breitburg 1992). Direct kills of spot and menhaden during episodic hypoxic events can be severe, visible, and dramatic when hundreds of thousands of dead fish wash up on the shorelines of the NRE during “fish kills” (Paerl et al. 1998). Although “fish kills” attract media attention and are of great public concern, the number of fish involved are only a small percentage of the total fish stocks in the estuary (Borsuk 2004). Therefore, hypoxia-induced mortality during episodic hypoxic events may not pose a substantial direct threat to the population dynamics of more sensitive species either.

Although episodic hypoxia may not have significant direct effects on mobile animal populations its indirect impacts on population dynamics via habitat compression (Pihl et al.

1991; Bell and Eggleston 2005), increased predator-prey overlap (Breitburg 1994; Lenihan et al. 2001), and altered diet composition (Pihl et al. 1992; Pihl 1994) may be more substantial. It is difficult to assess the significance of these effects on population dynamics of other species without knowledge of the underlying physiological and behavioral mechanisms that cause mobile animals to aggregate in nearshore habitats during episodic hypoxia events (Bell et al. in review-a). The environmental cues mobile animals use to avoid hypoxia are still poorly understood but our model's sensitivity analyses suggest that quantifying how changes in DO alter movement rates and identifying what directional cues animals use to orient away from low DO are the most critical behavioral components of avoidance responses that future work should address. Changes in these two parameters resulted in a 100% difference in blue crab population escape and mortality responses, therefore, animal behavior strongly influences population-level patterns (Shumway 1999). Moreover, quantifying these behavioral responses is critical for developing mechanistic models that can predict how changes in the severity, duration, and frequency of disturbances over time will impact ecological processes (Koehl 1989) which is particularly useful given the exponential rise in the number of global hypoxic zones (Diaz and Rosenberg 2008) and how global climate change may exacerbate hypoxic conditions.

Implications for the blue crab fishery and population dynamics

The potential ecological impact to blue crab populations and economic impact to the fishery will depend on the frequency and spatial extent of episodic hypoxic events as well as the density of crabs in the affected area. Atlantic and Gulf coast estuaries of the U.S. prone to hypoxic upwelling, could suffer six to ten of these severe events annually. Episodic

hypoxic events occur mainly from July through September and are triggered by wind reversals from weather fronts moving through these regions approximately every two weeks (Loesch 1960; Breitburg 1990; Bell et al. 2003; Reynolds-Fleming and Luetlich 2004). Based on estimates of the spatial distribution of bottom water hypoxia (Buzzelli et al. 2002), reports from local residents (Loesch 1960) and scientific surveys (G.W. Bell, pers. obs.) along the Atlantic and Gulf coasts of the U.S., we estimate that between 0.5 km and 8 km of shoreline can be affected by a single severe hypoxic upwelling event at the sites where they have been reported. Episodic hypoxic events are isolated to particular regions of estuaries including the upper NRE (Luetlich et al. 2000), Pamlico River (Stanley and Nixon 1992), the upper eastern shore of Mobile Bay (Loesch 1960), the Flag Pond study site in Chesapeake Bay (Breitburg 1990), as well as the York River (Pihl et al. 1991). Most recent published accounts of adult blue crab density for NC coastal waters and Chesapeake Bay are 0.01 crabs·m² (Sharov et al. 2003; Eggleston et al. 2004). Therefore, we estimate that direct mortality from episodic hypoxic events can kill between 500 and 24,000 blue crabs annually within each of the aforementioned regions.

Average annual blue crab mortality is likely near the low end of our estimates because “average” years are likely to have relatively less extensive areas of hypoxic bottom water (see above), which suggests that only a few thousand blue crabs die annually from hypoxia in the two most productive estuaries for blue crabs, Chesapeake Bay and NC coastal waters. During wet years, when hypoxic bottom water can be more extensive, annual blue crab mortality from episodic hypoxia might be as high as several tens to a hundred thousand crabs. Additional mortality from blue crabs trapped in crab pots during episodic hypoxic

events can occur; however, crabbers know the meteorological conditions indicative of hypoxic upwelling events and move their pots closer to shore prior to the onset of episodic events (Selberg et al. 2001). Thus, blue crab mortality inside crab pots during episodic hypoxic events is likely minor.

The economic and ecological impact of such mortality is probably small relative to the value of blue crab fisheries and the extraction of crabs by the fishery. For example, at the local level (i.e., NRE) where roughly one thousand crabs could die from hypoxia during an average year (i.e., lower quartile value from our range of estimates), the economic loss to each of the roughly 22 full-time watermen (Selberg et al. 2001) would be about \$150 given the current dockside value of three dollars per crab. At the ecosystem level (i.e., NC or Chesapeake Bay) the economic loss of a few thousand crabs would be on the order of magnitude of roughly ten thousand dollars of dockside value per year. The total dockside value of blue crabs in NC and Chesapeake Bay, however, is more than \$50 million each; therefore the economic loss from hypoxia to individual fishermen is small and is a minor percentage of the total revenue generated by the fishery. In terms of the relative ecological impact of hypoxia-induced mortality, the few thousands of blue crabs that annually die from hypoxia are a minor portion of the total number of blue crabs harvested by commercial crabbers. Based on the average landings data from Chesapeake Bay (Sharov et al. 2003) and NC (NC Division of Marine Fisheries trip ticket dataset) we estimate that annually there are 237 and 92 million blue crabs removed from these ecosystems, respectively. Therefore, mortality from hypoxia is likely a minor influence on blue crab population dynamics, relative to fishing disturbance.

Although it is impossible to fully quantify the impact of indirect effects on blue crab population dynamics without simulating the competitive and predatory interactions that cause decreased growth and increased mortality (Lenihan et al. 2001; Eby et al. 2005; Eggleston et al. 2005) our model suggests that these effects may not seriously affect blue crab populations. The model predicts that long-duration events (~ 30 hr) are required to form the aggregations in nearshore habitats necessary for indirect effects to occur. Since these events transpire only several times each year (Breitburg 1990; Bell et al. 2003) across narrow stretches of shoreline (see above) indirect effects may not be frequent enough and impact a large enough portion of the blue crab population to substantially alter population dynamics. Moreover, indirect effects are density dependent and blue crab abundance in Chesapeake Bay and NC has declined more than 80% over the past decade, therefore the low blue crab densities in these systems may limit competitive and cannibalistic interactions during episodic hypoxic events.

Despite our findings that episodic hypoxia does not substantially impact blue crab population dynamics; chronic hypoxia is a major threat to coastal ecosystems. Zones of chronic hypoxia have exponentially increased during the last half century and now affect a total area of more than 245,000 km² (Diaz and Rosenberg 2008). As the spatial extent, duration, and frequency of hypoxia continue to grow, the cascading effects from habitat compression and loss of benthic infauna likely have the greatest impact on higher level trophic animals. The near-complete loss of benthic infauna from “dead zones” that are important prey for economically important fish and crustaceans can reduce the amount of food energy for fisheries by as much as 106,000 metric tons of carbon (Diaz and Rosenberg

2008). Moreover, persistent hypoxia in coastal ecosystems diverts energy to microbial decomposition pathways, thereby depleting higher trophic level organisms, lowering the overall productivity of these systems, and making them less resilient to other stressors (Baird et al. 2004).

ACKNOWLEDGEMENTS

We would like to thank J.K. Craig and G. Hess for their valuable advice with modeling as well as C. Arellano and K. Gross for their statistical help. Funding for this research was provided by research grants from the National Science Foundation (OCE-0221099) and the NC Blue Crab Research Program (01-POP-08) to D. B. Eggleston, as well as EPA STAR and NC Beautiful fellowships to G. W. Bell.

LITERATURE CITED

- Adams, S. M., M. S. Greeley, J. M. Law, E. J. Noga and J. T. Zelikoff (2003). "Application of multiple sublethal stress indicators to assess the health of fish in Pamlico Sound following extensive flooding." Estuaries **26**(5): 1365-1382.
- Adams, S. M., K. D. Ham and R. F. LeHew (1998). A framework for evaluating organism responses to multiple stressors: mechanisms of effect and importance of modifying ecological factors. Multiple Stresses in Ecosystems. J. J. Cech, Jr., B. W. Wilson and D. G. Crosby. Boca Raton, CRC Press: 13-22.
- Adams, S. M., K. L. Shepard, M. S. Greeley, Jr., B. D. Jimenez, M. G. Ryon, L. R. Shugart and J. F. McCarthy (1989). "The use of bioindicators for assessing the effects of pollutant stress on fish." Mar Environ Res **28**: 459-464.
- Baird, D., R. R. Christian, C. H. Peterson and G. A. Johnson (2004). "Consequences of hypoxia on estuarine ecosystem function: energy diversion from consumers to microbes." Ecol Appl **14**(3): 805-822.
- Bell, G. W. and D. B. Eggleston (2005). "Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia." Mar Biol **146**: 761-770.
- Bell, G. W., D. B. Eggleston and E. J. Noga (in review-a). "Environmental and physiological controls of blue crab avoidance behavior during exposure to hypoxia." Biol Bull.

- Bell, G. W., D. B. Eggleston and E. J. Noga (in review-b). "Molecular keys unlock variable survival responses of blue crabs to hypoxia." Oecologia.
- Bell, G. W., D. B. Eggleston and D. L. Wolcott (2003). "Behavioral responses of free-ranging blue crabs to episodic hypoxia. I. Movement." Mar Ecol Prog Ser **259**: 215-225.
- Borsuk, M. E. (2004). "Predictive assessment of fish health and fish kills in the Neuse River Estuary using elicited expert judgement." Human and Ecological Risk Assessment **10**: 415-434.
- Breitburg, D. L. (1990). "Near-shore hypoxia in the Chesapeake Bay: patterns and relationships among physical factors." Est Coast Shelf Sci **30**: 593-609.
- Breitburg, D. L. (1992). "Episodic hypoxia in Chesapeake Bay: interacting effects of recruitment, behavior, and physical disturbance." Ecol Monogr **62**(4): 525-546.
- Breitburg, D. L. (1994). "Behavioral response of fish larvae to low dissolved oxygen concentrations in a stratified water column." Mar Biol **120**: 615-625.
- Brouwer, M., N. J. Brown-Peterson, P. Larkin, S. Manning, N. Denslow and K. Rose (2005). Molecular and organismal indicators of chronic and intermittent hypoxia in marine crustacea. Estuarine Indicators. S. A. Bortone. Boca Raton, CRC Press: 261-276.
- Brouwer, M., N. J. Brown-Peterson, P. Larkin, P. Patel, N. Denslow, S. Manning and T. H. Brouwer (2007). "Molecular and whole animal responses of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia." J Exp Mar Biol Ecol **341**: 16-31.

- Burnham, K. P. and D. R. Anderson (2002). Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. New York, Springer.
- Buzzelli, C. P., R. A. Luettich, Jr., S. P. Powers, C. H. Peterson, J. E. McNinch, J. L. Pinckney and H. W. Paerl (2002). "Estimating the spatial extent of bottom-water hypoxia and habitat degradation in a shallow estuary." Mar Ecol Prog Ser **230**: 103-112.
- Craig, J. K. and L. B. Crowder (2005). "Hypoxia-induced habitat shifts and energetic consequences in Atlantic croaker and brown shrimp on the Gulf of Mexico Shelf." Mar Ecol Prog Ser **294**: 79--4.
- Crowder, L. B., D. T. Crouse, S. S. Heppell and T. H. Martin (1994). "Predicting the impact of turtle excludor devices on loggerhead sea turtle populations." Ecol Appl **4**(3): 437-445.
- DeFur, P. L., C. P. Mangum and J. E. Reese (1990). "Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia." Biol Bull **178**: 46-54.
- Diaz, R. J. and R. Rosenberg (1995). "Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna." Oceanogr Mar Biol Ann Rev **33**: 245-303.
- Diaz, R. J. and R. Rosenberg (2008). "Spreading dead zones and consequences for marine ecosystems." Science **321**: 926-929.

- Dixon, P. M. and M. C. Newman (1991). Analyzing toxicity data using statistical models for time-to-death: an introduction. Metal Ecotoxicology: Concepts and Applications. M. C. Newman and A. W. McIntosh. Chelsea, MI, Lewis Publishers: 207-242.
- Eby, L. A. and L. B. Crowder (2002). "Hypoxia-based habitat compression in the Neuse River Estuary: context-dependent shifts in behavioral avoidance thresholds." Can J Fish Aquat Sci **59**(6): 952-965.
- Eby, L. A., L. B. Crowder, C. M. McClellan, C. H. Peterson and M. J. Powers (2005). "Habitat degradation from intermittent hypoxia: impacts on demersal fishes." Mar Ecol Prog Ser **291**: 249-261.
- Eggleston, D. B., G. W. Bell and A. D. Amavisca (2005). "Interactive effects of episodic hypoxia and cannibalism on juvenile blue crab mortality." J Exp Mar Biol Ecol **325**: 18-26.
- Eggleston, D. B., J. E. Hightower and E. G. Johnson (2004). Population dynamics and stock assessment of the blue crab in North Carolina. Final Report to the NC Sea Grant/Blue Crab Research Project for Projects 99-FEG-10 and 00-FEG-11: 230.
- Hill, A. D., A. C. Taylor and R. H. C. Strang (1991). "Physiological and metabolic responses of the shore crab *Carcinus maenas* (L.) during environmental anoxia and subsequent recovery." J Exp Mar Biol Ecol **150**: 31-50.

- Holman, J. D., K. G. Burnett and L. E. Burnett (2004). "Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Atlantic blue crab, *Callinectes sapidus* Rathbun." Biol Bull **206**: 188-196.
- Koehl, M. A. R. (1989). Discussion: from individuals to populations. Perspectives in Ecological Theory. J. Roughgarden, R. M. May and S. A. Levin. Princeton, Princeton University Press: 39-53.
- Lenihan, H. S., C. H. Peterson, J. E. Byers, J. H. Grabowski, G. W. Thayer and D. R. Colby (2001). "Cascading of habitat degradation: oyster reefs invaded by refuge fishes escaping stress." Ecol Appl **11**(3): 764-782.
- Loesch, H. (1960). "Sporadic mass shoreward migrations of demersal fish and crustaceans in Mobile Bay, Alabama." Ecology **41**(2): 292-298.
- Lowery, T. A. and L. G. Tate (1986). "Effect of hypoxia on hemolymph lactate and behavior of the blue crab *Callinectes sapidus* Rathbun in the laboratory and field." Comp Biochem Physiol A **85**(4): 689-692.
- Luetlich, R. A., Jr., J. E. McNinch, H. W. Paerl, C. H. Peterson, J. T. Wells, M. Alperin, C. S. Martens and J. L. Pinckney (2000). Neuse River Estuary modeling and monitoring project stage 1: hydrography and circulation, water column nutrients and productivity, sedimentary processes and benthic-pelagic coupling, and benthic ecology, Water Resources Research Institute of the University of North Carolina: 172.

- Mangum, C. P. (1994). "Subunit composition of hemocyanins of *Callinectes sapidus*: phenotypes from naturally hypoxic waters and isolated oligomers." Comp Biochem Physiol B **108**(4): 537-541.
- Mangum, C. P. (1997). "Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*." Am Zool **37**(6): 604-611.
- Newman, M. C. and M. S. Aplin (1992). "Enhancing toxicity data interpretation and prediction of ecological risk with survival time modeling: an illustration using sodium chloride toxicity to mosquitofish (*Gambusia holbrooki*). " Aquat Toxicol **23**: 85-96.
- Noga, E. J., R. Smolowitz and L. Khoo, H. (2000). "Pathology of shell disease in the blue crab, *Callinectes sapidus* Rathbun, (Decapoda: Portunidae)." Journal of Fish Diseases **23**: 389-399.
- Paerl, H. W., J. L. Pinckney, J. M. Fear and B. L. Peierls (1998). "Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA." Mar Ecol Prog Ser **166**: 17-25.
- Pihl, L. (1994). "Changes in the diet of demersal fish due to eutrophication-induced hypoxia in the Kattegat, Sweden." Can J Fish Aquat Sci **51**: 321-336.
- Pihl, L., S. P. Baden and R. J. Diaz (1991). "Effects of periodic hypoxia on distribution of demersal fish and crustaceans." Mar Biol **108**: 349-360.

- Pihl, L., S. P. Baden, R. J. Diaz and L. C. Schaffner (1992). "Hypoxia-induced structural changes in the diet of bottom-feeding fish and crustacea." Mar Biol **112**: 349-361.
- Reynolds-Fleming, J. V. and R. A. Luettich, Jr. (2004). "Wind-driven lateral variability in a partially mixed estuary." Est Coast Shelf Sci **60**: 395-407.
- Schreck, C. B. (2000). Accumulation and long-term effects of stress in fish. The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. G. P. Moberg and J. A. Mench. New York, CABI Publishing: 147-158.
- Selberg, C. D., L. A. Eby and L. B. Crowder (2001). "Hypoxia in the Neuse River estuary: responses of blue crabs and crabbers." North American Journal of Fisheries Management **21**(2): 358-366.
- Sharov, A. F., J. H. Volstad, G. R. Davis, B. K. Davis, R. N. Lipcius and M. M. Montane (2003). "Abundance and exploitation rate of the blue crab (*Callinectes sapidus*) in Chesapeake Bay." Bull Mar Sci **72**(2): 543-565.
- Shimps, E. L., J. A. Rice and J. A. Osborne (2005). "Hypoxia tolerance in two juvenile estuary-dependent fishes." J Exp Mar Biol Ecol **325**(2): 146-162.
- Shumway, C. A. (1999). "A neglected science: applying behavior to aquatic conservation." Environ Biol Fish **55**: 183-201.
- Stanley, D. W. and S. W. Nixon (1992). "Stratification and bottom-water hypoxia in the Pamlico River estuary." Estuaries **15**(3): 270-281.

Timmerman, C. M. and L. J. Chapman (2004). "Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*)."
Physiol Biochem Zool **77**(4): 601-610.

Turner, R. E. (1987). "The role of stratification in the deoxygenation of Mobile Bay and adjacent shelf bottom waters." Estuaries **10**(1): 13-19.

Underwood, A. J. (1981). "Techniques of analysis of variance in experimental marine biology and ecology." Oceanogr Mar Biol Ann Rev **19**: 513-605.

van Raaij, M. T. M., D. S. S. Pit, P. H. M. Balm, A. B. Steffens and G. E. E. J. M. van den Thillart (1996). "Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia." Horm Behav **30**: 85-92.

Wolcott, T. G. and A. H. Hines (1996). Advances in ultrasonic biotelemetry for animal movement and behavior: the blue crab case study. Methods and Techniques of Underwater Research, Proceedings of the American Academy of Underwater Sciences Scientific Diving Symposium, October 12-13, 1996. M. A. Lang and C. C. Baldwin. Washington, DC, Smithsonian Institution: 229-236.

Wu, J. and I. K. Tsanis (1995). "Numerical study of wind-induced water currents." Journal of Hydraulic Engineering **121**(5): 388-395.

Table 1 AIC parameters (K, RSS) and statistics (AIC_c , Δ_i , ω_i) used to assess the effects of hydrodynamic factors (*) on the percent of crabs successfully avoiding hypoxic upwelling events (n = 360). For each ANOVA model R^2 is the amount of variation explained, ‘RSS’ is the residual sums of squares, and ‘K’ is the number of parameters. The full ANOVA model, (in bold) was estimated to be the most likely model from the set of models. The probability of the full model being the correct model (ω_i) was 1.0, whereas all of the reduced ANOVA models had AIC differences (Δ_i) greater than 10 and AIC weights of zero indicating that there was little to no support for these alternative models.

Model	R^2	RSS	K	AIC_c	Δ_i	ω_i
DO min.*Duration interaction & Speed*Boundary interaction	0.94	9522.8	10	1199.8	0.00	1.00
DO min.*Duration interaction & Speed	0.66	49609.3	8	1789.7	590.0	0.00
DO min.*Duration interaction	0.62	56328.2	7	1833.3	633.6	0.00
Duration & Speed*Boundary interaction	0.89	16043.7	6	1379.2	179.4	0.00
Rate of change	0.32	100917.2	5	2039.1	839.4	0.00
Duration & Speed	0.62	56130.2	4	1825.9	626.1	0.00
Speed	0.05	141003.8	3	2155.4	955.7	0.00
Duration	0.58	62849.1	3	1864.5	664.8	0.00

*Hydrodynamic factors: DO min. (3 levels: 0.5, 0.9, 1.2 mg·L⁻¹), Duration (2 levels: short and long event duration), Rate of change = interactive effects of Boundary (2 levels: strong and weak frontal boundary) and Speed (2 levels: 2 and 4 cm·s⁻¹).

Table 2 AIC parameters (K, RSS) and statistics (AIC_c , Δ_i , ω_i) used to assess the effects of hydrodynamic factors (*) on the log-transformed percent of crabs dying during hypoxic upwelling and subsequent relaxation events (n = 360). For each ANOVA model R^2 is the amount of variation explained, 'RSS' is the residual sums of squares, and 'K' is the number of parameters. The full ANOVA model (in bold) was the best model (lowest AIC value and $\omega_i = 1$). All of the reduced ANOVA models had model probabilities (ω_i) equal to zero and AIC differences (Δ_i) greater than 10, indicating little to no support for these models.

Model	R^2	RSS	K	AIC_c	Δ_i	ω_i
DO min.*Duration interaction & Speed*Boundary interaction	0.88	3.13	10	-1687.6	0.0	1.00
Duration & Speed*Boundary interaction	0.43	14.98	6	-1132.3	555.3	0.00
DO min. & Speed*Boundary interaction	0.45	14.36	7	-1145.5	542.1	0.00
DO min.*Duration interaction	0.83	4.33	7	-1577.1	110.5	0.00
Duration	0.38	16.17	3	-1111.0	576.6	0.00
DO min.	0.40	15.55	4	-1123.0	564.6	0.00

*Hydrodynamic factors: DO min. (3 levels: 0.5, 0.9, 1.2 mg·L⁻¹), Duration (2 levels: short and long event duration), Rate of change = interactive effects of Boundary (2 levels: strong and weak frontal boundary) and Speed (2 levels: 2 and 4 cm·s⁻¹).

Table 3 AIC parameters (K, RSS) and statistics (AIC_c , Δ_i , ω_i) assessing the relative importance of hydrodynamic factors and hypoxia tolerance (*) on the percent of crabs successfully avoiding hypoxic events (n = 48). For each ANOVA model R^2 is the amount of variation explained, ‘RSS’ is the residual sums of squares, and ‘K’ is the number of parameters. The best model explaining variation in escape success was the simplest model (in bold) which only included hydrodynamic terms ($\omega_i = 0.53$). Although there was some support for alternative models containing the term for hypoxia tolerance ($\Delta_i \sim 2$), evidence ratios estimated that the simplest model was nearly three times more likely to be the best model compared to the next best model ($\omega_i = 0.19$) which included a main effect term for hypoxia tolerance. The simplest model was also four and six times more likely to be the best model compared to models with terms for interactive effects between hypoxia tolerance and boundary strength ($\omega_i = 0.13$) as well as hypoxia tolerance and event duration ($\omega_i = 0.08$).

Model	R^2	RSS	K	AIC_c	Δ_i	ω_i
Duration, Boundary, Population*Duration & Population*Boundary interactions	0.77	4874.4	7	238.6	4.37	0.059
Duration, Boundary, & Population*Duration interaction	0.76	5076.4	6	237.8	3.57	0.089
Duration, Boundary, & Population*Boundary interaction	0.77	4991.8	6	237.0	2.77	0.133
Duration, Boundary, & Population	0.76	5193.8	5	236.3	2.05	0.190
Duration & Boundary	0.76	5242.5	2	234.2	0.00	0.529

*Factors: Duration (2 levels: short and long event duration), Boundary (2 levels: strong and weak frontal boundary) and Population (2 levels: 0% tolerant individuals and 70% tolerant individuals).

Table 4 AIC parameters (K, RSS) and statistics (AIC_c , Δ_i , ω_i) used to assess the relative effects of hydrodynamics factors and hypoxia tolerance (*) on the percent of crabs dying during episodic hypoxic events (n = 48). For each ANOVA model R^2 is the amount of variation explained, ‘RSS’ is the residual sums of squares, and ‘K’ is the number of parameters. The reduced model that included interactive effects of minimum DO and duration as well as the main effect for hypoxia tolerance was the best model ($\omega_i = 0.51$). This model was 14.4 times more likely to be the best model compared to the simplest model, containing only hydrodynamic terms ($\omega_i = 0.035$). There was also substantial support ($\Delta_i < 2$) for the model that included the interaction between hypoxia tolerance and duration ($\omega_i = 0.324$, $\Delta_i = 0.89$) which was only 1.5 times less likely to be the best model compared to the model with the lowest AIC value.

Model	R^2	RSS	K	AIC_c	Δ_i	ω_i
DO min.*Duration, DO min.* Population, & Duration*Population interactions	0.91	0.82	11	-166.0	4.64	0.050
DO min.*Duration & DO min.* Population interactions	0.91	0.86	10	-167.1	3.54	0.086
DO min.*Duration & Duration* Population interactions	0.91	0.87	9	-169.8	0.89	0.324
DO min.*Duration interaction, & Population	0.91	0.91	8	-170.7	0.00	0.505
DO min.*Duration interaction	0.89	1.08	7	-165.3	5.33	0.035

*Factors: Duration (2 levels: short and long event duration), Boundary (2 levels: strong and weak frontal boundary) and Population (2 levels: 0% tolerant individuals and 70% tolerant individuals).

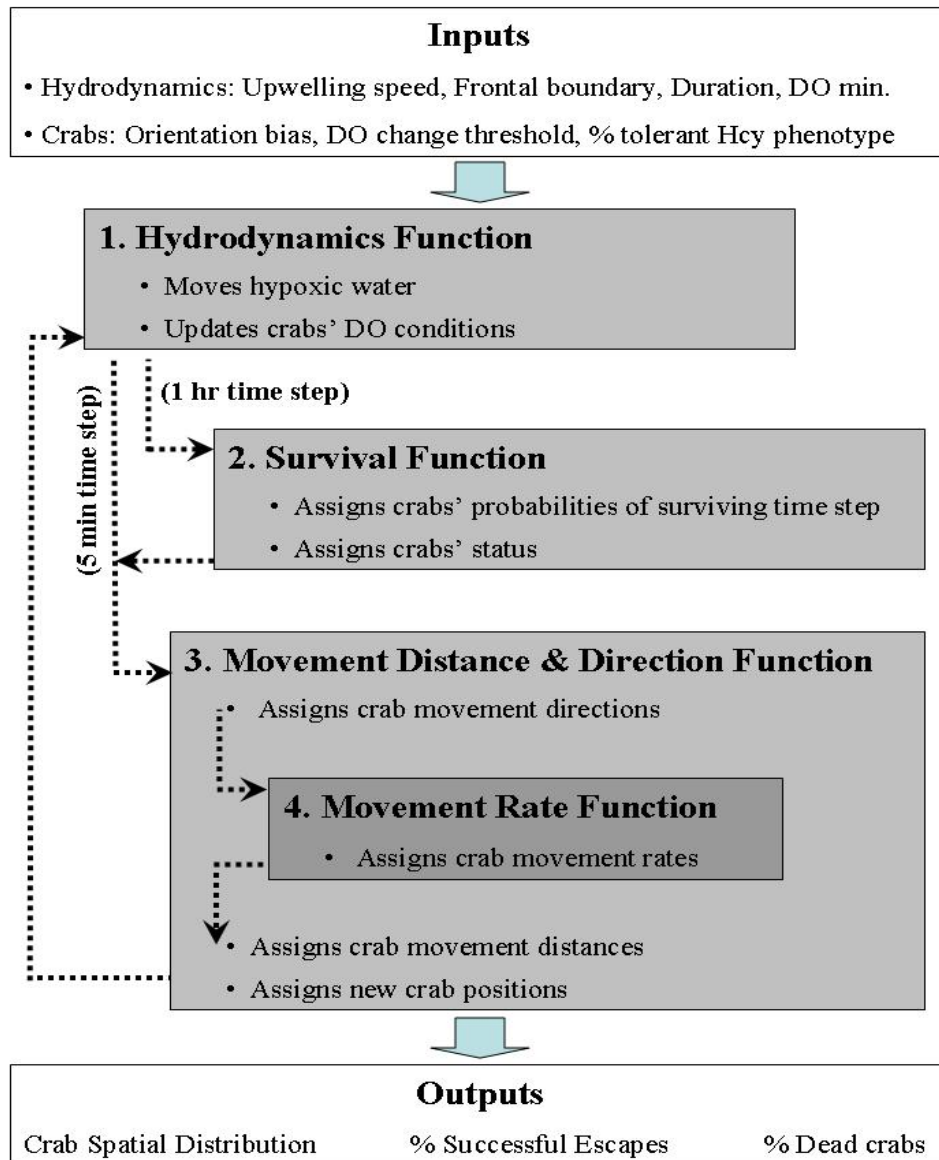


Figure 1 Conceptual diagram of model inputs, functions, and outputs. Arrows denote the path of each model step. Note that crab movement behavior is evaluated at each 5-min time step whereas crab survival is evaluated every 12th time step (i.e., every hour).

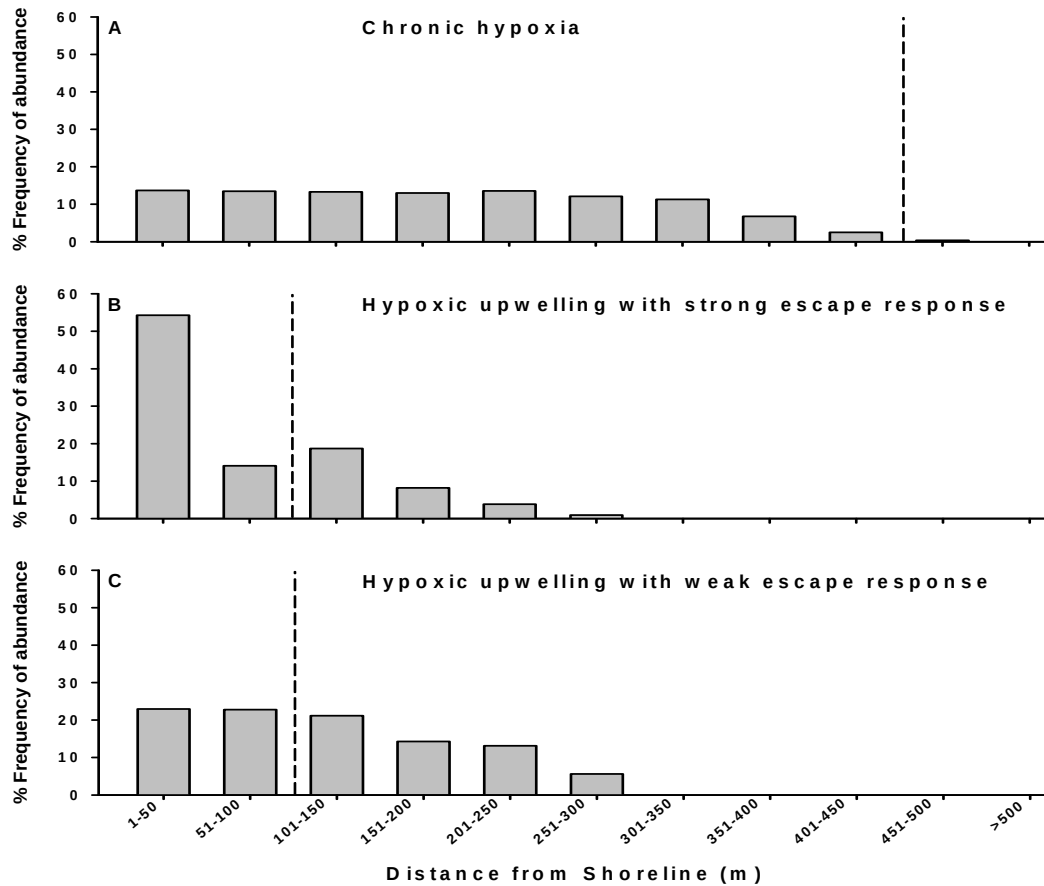


Figure 2 Percent frequency of blue crab abundance along a transect perpendicular to shoreline for (A) chronic hypoxic conditions, (B) hypoxic upwelling conditions in which crabs showed a strong escape response (long duration, slow upwelling speed, weak frontal boundary, and 1.2 mg/L), and (C) hypoxic upwelling conditions in which crabs showed a weak escape response (short duration, fast upwelling speed, weak frontal boundary, and 0.5 mg/L). Vertical dashed lines show the demarcation between hypoxic (≤ 2.0 mg DO·L⁻¹) and normoxic (> 2.0 mg DO·L⁻¹) in the spatial domain.

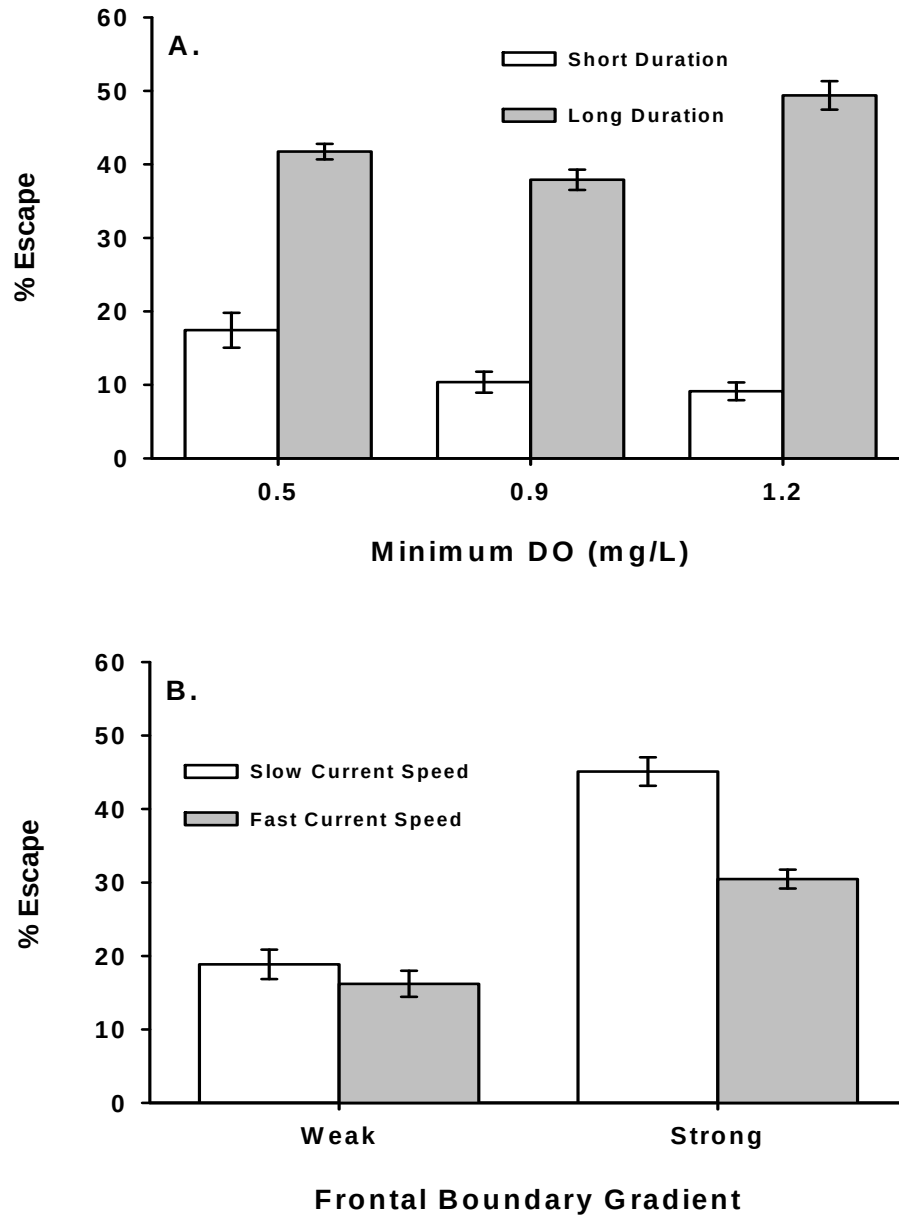


Figure 3 Interactive effects of: (A) hypoxic event duration and minimum DO, as well as (B) frontal boundary strength and upwelling velocity on the mean percent of crabs successfully avoiding hypoxic water ($\pm$ SE).

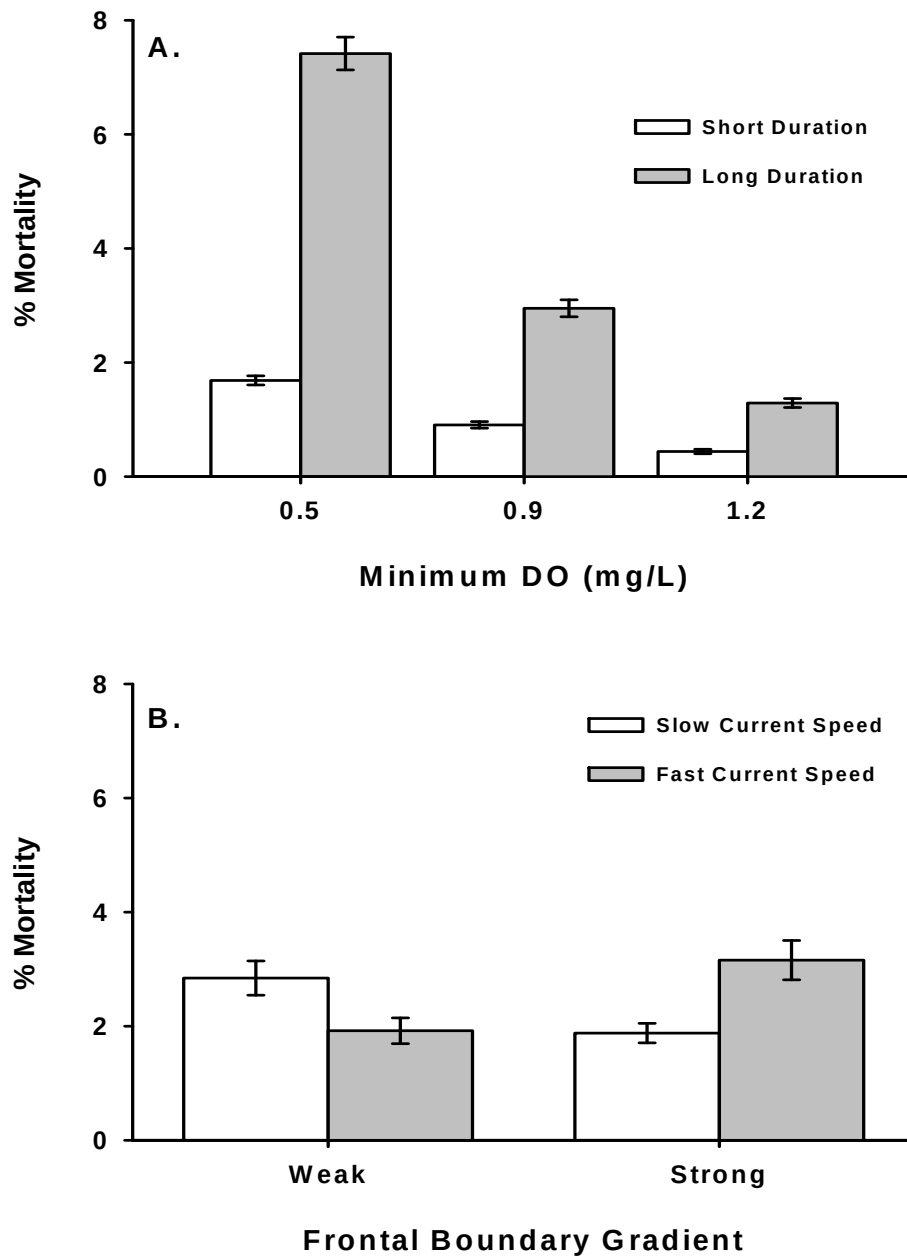


Figure 4 Interactive effects of (A) hypoxic event duration and minimum DO, as well as (B) frontal boundary strength and upwelling velocity on the mean percent of crabs dying ($\pm$ SE) during hypoxic upwelling events.

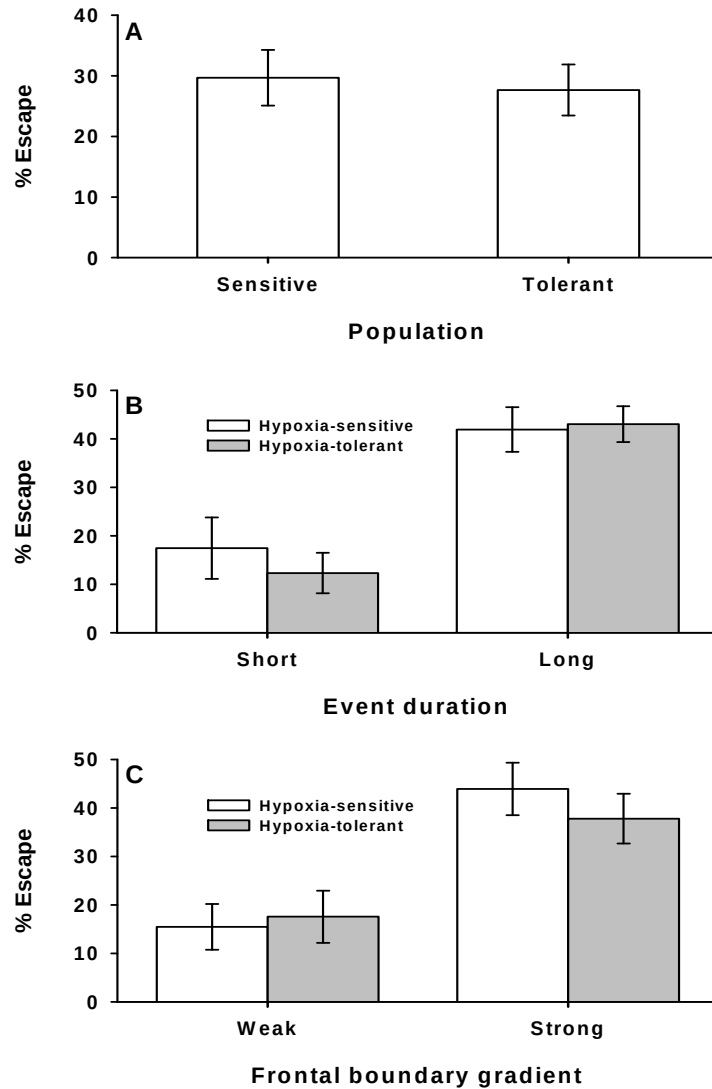


Figure 5 Effect of hypoxia tolerance on the escape response of blue crab populations during hypoxic events. (A) Mean percent of crabs successfully escaping hypoxia ($\pm$ SE) for hypoxia-sensitive (0% of crabs with the high O_2 affinity Hcy phenotype) and hypoxia-tolerant (70% of crabs with the high O_2 affinity Hcy phenotype) populations, irrespective of hydrodynamic factors. Interactive effects of hypoxia tolerance and (B) hypoxic event duration, and (C) frontal boundary on percent of crabs successfully avoiding hypoxia.

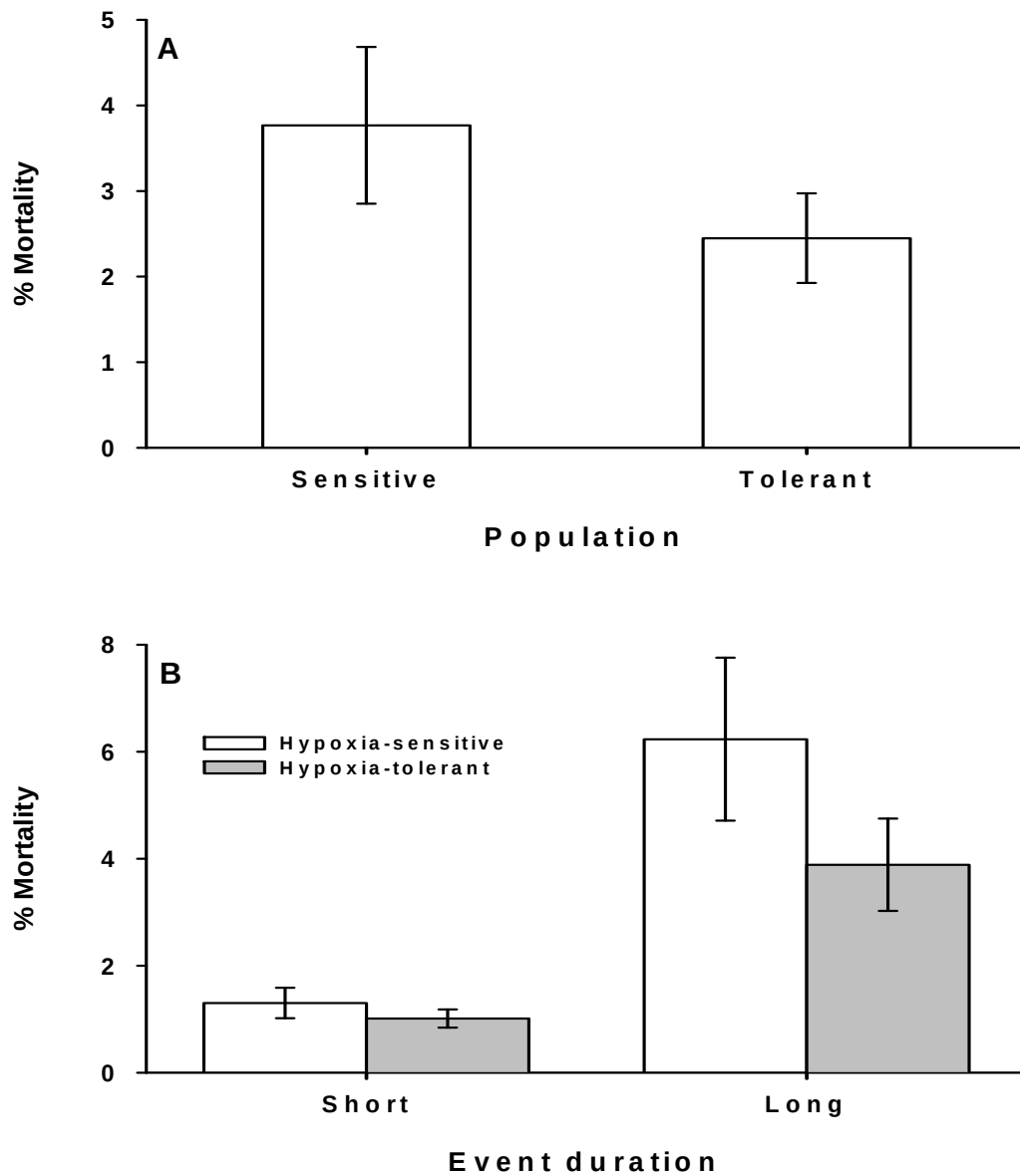


Figure 6 Effect of hypoxia tolerance on mortality of blue crab populations during hypoxic events. (A) Mean percent of crabs dying ($\pm$ SE) for hypoxia-sensitive and hypoxia-tolerant populations, irrespective of hydrodynamic factors. (B) Interactive effects of hypoxia tolerance and hypoxic event duration on percent of crabs dying during hypoxic events.

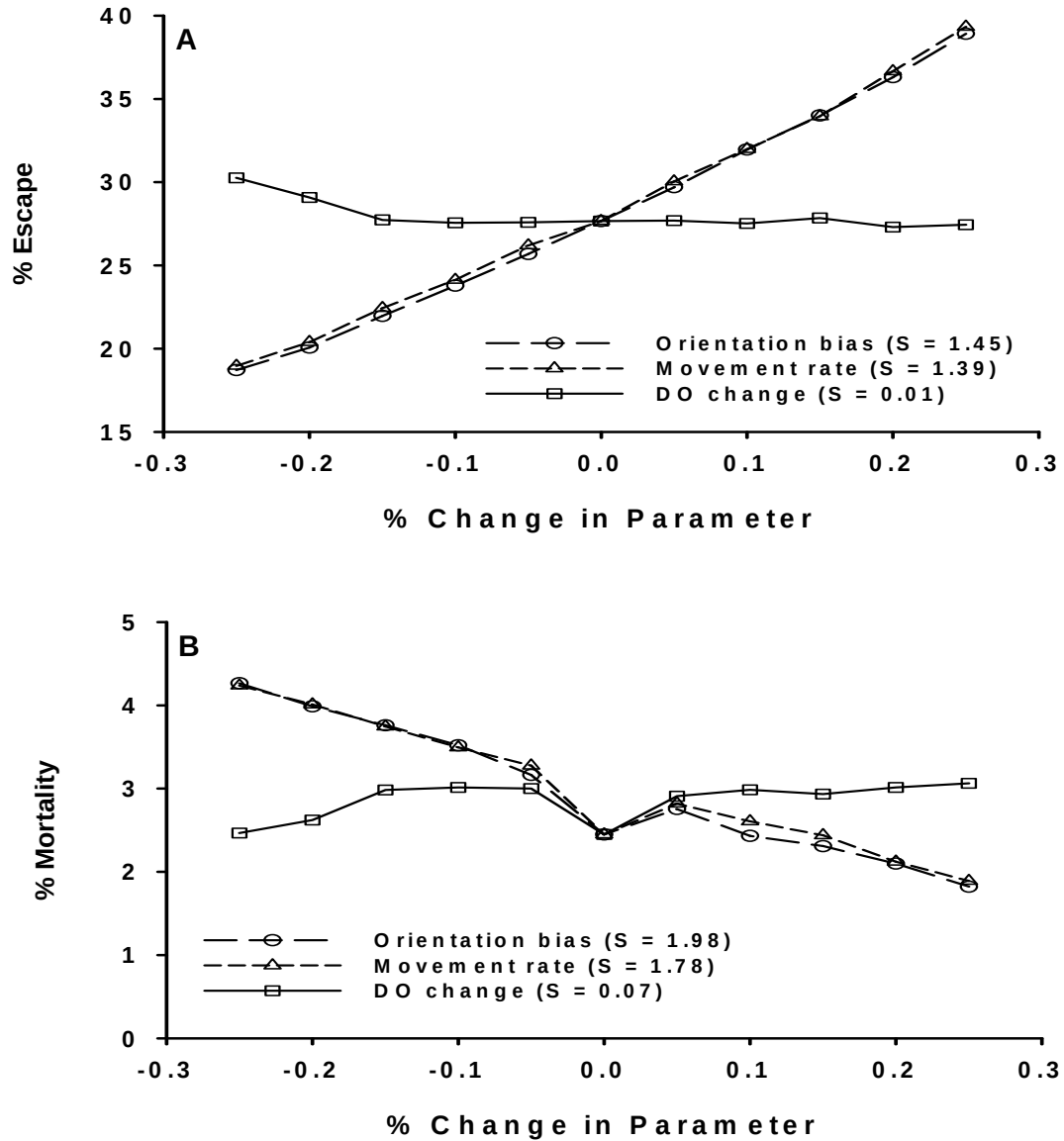


Figure 7 Sensitivity analyses showing how variation in three crab movement parameters: orientation bias (open circles & long dash line), movement rate (open triangles & short dash line), and threshold DO change that elicits avoidance responses (open squares & solid line) affect (A) percent of crabs successfully avoiding hypoxia and (B) percent of crabs dying during hypoxic events.

APPENDICES

APPENDIX 1A

Statistical model output and Akaike's Information Criterion (AIC) estimates for nonlinear models used to describe *in situ* spatial DO patterns along depth gradient transects. Each table shows the functional models tested for the two types of frontal boundaries (strong and weak). Shaded rows indicate the model selected to parameterize the spatial DO pattern in the IBM.

DO boundary = "weak"

n = 9

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Sigmoid	4	0.977	0.6825	-5.21	11.51	0.003
Sigmoid	5	0.994	0.1817	-5.12	11.60	0.003
Logistic	4	0.994	0.1899	-16.73	0.00	0.993
Power	3	0.933	1.9552	-2.94	13.79	0.001

DO boundary = "strong"

n = 9

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Sigmoid	4	0.978	1.0794	-1.09	5.03	0.069
Gompertz	4	0.979	1.0447	-1.38	4.74	0.080
Logistic	4	0.988	0.6171	-6.12	0.00	0.852

APPENDIX 1B

Statistical model output and Akaike's Information Criterion (AIC) estimates for linear and nonlinear models used to describe the functional relationship between hypoxia exposure time and survival probability from prior laboratory study. Each table shows the functional models tested for each DO and Hcy phenotype treatment combination. Hcy phenotype treatment levels were combined at 1.2 mg/L because Hcy phenotype does not affect survival above 1.0 mg DO·L⁻¹. Shaded rows indicate the model selected for each DO and Hcy phenotype treatment combination.

DO = 0.3 mg/L		Hcy Phenotype = Low O ₂ affinity				n = 35
Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Sigmoid	3	0.989	0.0377	-232.40	54.14	0.00
Sigmoid	4	0.994	0.1565	-180.02	106.52	0.00
Sigmoid	5	0.998	0.0069	-286.54	0.00	1.00
Gompertz	3	0.996	0.0163	-261.74	24.79	0.00
Gompertz	4	0.996	0.0140	-264.51	22.03	0.00
DO = 0.3 mg/L		Hcy Phenotype = High O ₂ affinity				n = 35
Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.994	0.0200	-256.98	26.17	0.00
Quadratic	3	0.995	0.0152	-264.19	18.97	0.00
Cubic	4	0.996	0.0142	-264.01	19.14	0.00
Sigmoid	4	0.986	0.0443	-224.19	58.96	0.00
Sigmoid	5	0.998	0.0076	-283.15	0.00	1.00
Gompertz	3	0.996	0.0132	-269.13	14.03	0.00
Gompertz	4	0.997	0.0114	-271.70	11.46	0.00
DO = 0.6 mg/L		Hcy Phenotype = Low O ₂ affinity				n = 36
Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.981	0.075	-217.89	73.39	0.00
Quadratic	3	0.993	0.0276	-251.49	39.78	0.00
Cubic	4	0.995	0.0196	-261.28	30.00	0.00

Sigmoid	3	0.981	0.0734	-216.28	75.00	0.00
Sigmoid	4	0.987	0.052	-226.15	65.13	0.00
Sigmoid	5	0.998	0.0079	-291.28	0.00	1.00
Gompertz	3	0.996	0.0159	-271.35	19.93	0.00
Gompertz	4	0.995	0.0182	-263.94	27.33	0.00

DO = 0.6 mg/L **Hcy Phenotype = High O₂ affinity** **n = 34**

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.916	0.1144	-189.22	86.76	0.00
Quadratic	3	0.994	0.0085	-275.20	0.78	0.40
Cubic	4	0.994	0.0077	-275.98	0.00	0.60

DO = 0.9 mg/L **Hcy Phenotype = Low O₂ affinity** **n = 35**

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.943	0.1075	-198.12	85.60	0.00
Quadratic	3	0.995	0.0087	-283.72	0.00	0.78
Cubic	4	0.995	0.0087	-281.16	2.56	0.22

DO = 0.9 mg/L **Hcy Phenotype = High O₂ affinity** **n = 35**

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.852	0.0508	-224.36	91.81	0.00
Quadratic	3	0.984	0.0057	-298.52	17.65	0.00
Cubic	4	0.991	0.0032	-316.17	0.00	1.00

DO = 1.2 mg/L **n = 35**

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.954	0.0105	-344.04	82.30	0.00
Quadratic	3	0.994	0.0014	-426.34	0.00	0.77
Cubic	4	0.994	0.0014	-423.89	2.45	0.23

APPENDIX 1C

Statistical model output and Akaike's Information Criterion (AIC) estimates for linear and nonlinear models used to describe the functional relationship between decreasing DO concentration and movement rate from prior laboratory experiments. Each table shows the functional models tested for different DO exposures (hypoxia and normoxia), exposure durations (0, <1, 1-2, and >2 hr), and Hcy phenotypes (high and low O₂ affinity).

DO exposure = normoxia Duration = 0 hr n = 23

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Quadratic	3	0.84	872.85	90.90	5.50	0.033
Cubic	4	0.88	656.55	87.31	1.91	0.200
Logarithmic	3	0.87	732.84	86.88	1.48	0.248
Logarithmic	4	0.89	604.27	85.40	0.00	0.519

DO exposure = hypoxia Duration < 1 hr Hcy Phenotype = Low O₂ affinity n = 8

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Quadratic	3	0.66	1744.39	55.08	4.29	0.089
Cubic	4	0.69	1576.74	63.60	12.81	0.001
Logarithmic	3	0.68	1620.42	54.49	3.70	0.119
Logarithmic	4	0.76	1203.97	61.44	10.65	0.004
Log-normal	3	0.72	1020.74	50.79	0.00	0.755
Log-normal	4	0.86	698.75	57.09	6.30	0.032

DO exposure = hypoxia Duration < 1 hr Hcy Phenotype = High O₂ affinity n = 8

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Quadratic	3	0.36	1296.92	52.71	5.77	0.034
Cubic	4	0.46	1112.78	60.81	13.87	0.001
Logarithmic	3	0.65	733.54	48.15	1.21	0.336
Logarithmic	4	0.75	505.01	54.49	7.55	0.014
Log-normal	3	0.69	630.83	46.94	0.00	0.615

Exposure = hypoxia Duration = 1-2 hr Hcy Phenotype = Low O₂ affinity n = 8

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Quadratic	3	0.99	18.51	18.71	0.00	0.846
Cubic	4	0.99	13.50	25.52	6.81	0.028
Logarithmic	3	0.94	110.38	33.00	14.29	0.001
Logarithmic	4	0.99	12.80	25.10	6.39	0.035
Log-normal	3	0.97	54.73	27.38	8.67	0.011
Log-normal	4	0.99	12.60	24.97	6.26	0.037
Single Exponential	2	0.95	93.39	26.06	7.35	0.021
Double Exponential	4	0.95	93.39	40.99	22.28	0.000
Simple Exponent	2	0.95	93.39	26.06	7.35	0.021

Exposure = hypoxia Duration = 1-2 hr Hcy Phenotype = High O₂ affinity n = 8

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Quadratic	3	0.51	569.72	46.13	4.32	0.060
Cubic	4	0.79	249.80	48.86	7.05	0.015
Logarithmic	3	0.71	332.12	41.81	0.00	0.519
Logarithmic	4	0.73	308.60	50.55	8.75	0.007
Log-normal	3	0.69	355.52	42.35	0.54	0.395
Log-normal	4	0.71	332.13	51.14	9.33	0.005

Exposure = hypoxia Duration > 2 hr Hcy Phenotype = Low O₂ affinity n = 8

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.55	276.75	34.75	1.08	0.354
Quadratic	3	0.61	240.50	39.23	5.56	0.038
Cubic	4	0.60	240.48	48.56	14.89	0.000
Single Exponential	2	0.60	241.66	33.66	0.00	0.608

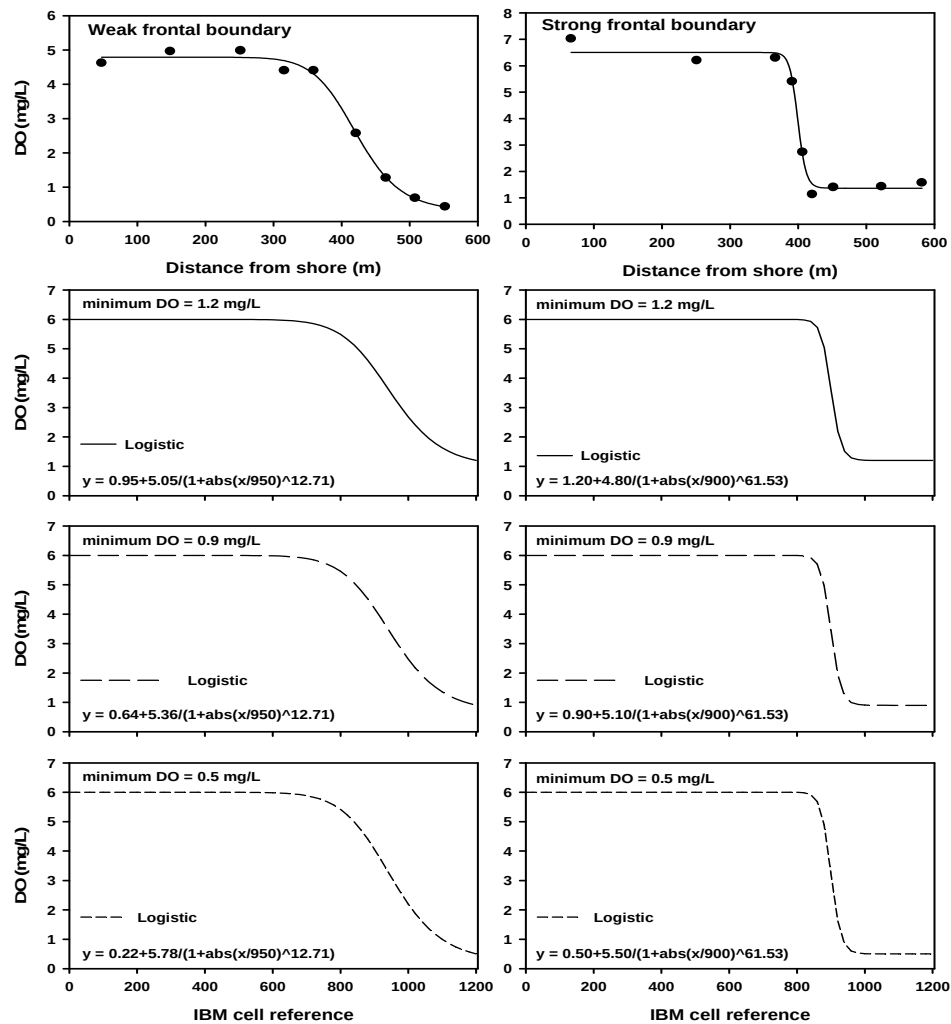
DO exposure = hypoxia Duration > 2 hr Hcy Phenotype = High O₂ affinity n = 8

Model	# of param.	R ²	Resid SS	AIC _c	ΔAIC	ω _i
Linear	2	0.67	540.21	40.10	2.78	0.106
Quadratic	3	0.67	536.36	45.64	8.32	0.007
Cubic	4	0.85	251.39	48.91	11.59	0.001
Log-normal	3	0.88	189.61	37.32	0.00	0.425
Log-normal	4	0.95	74.51	39.19	1.86	0.168

Sigmoid	3	0.85	249.03	39.51	2.18	0.143
Sigmoid	4	0.87	213.82	47.62	10.29	0.002
Logistic	3	0.82	293.99	40.83	3.51	0.074
Logistic	4	0.94	91.09	40.79	3.47	0.075

APPENDIX 2A

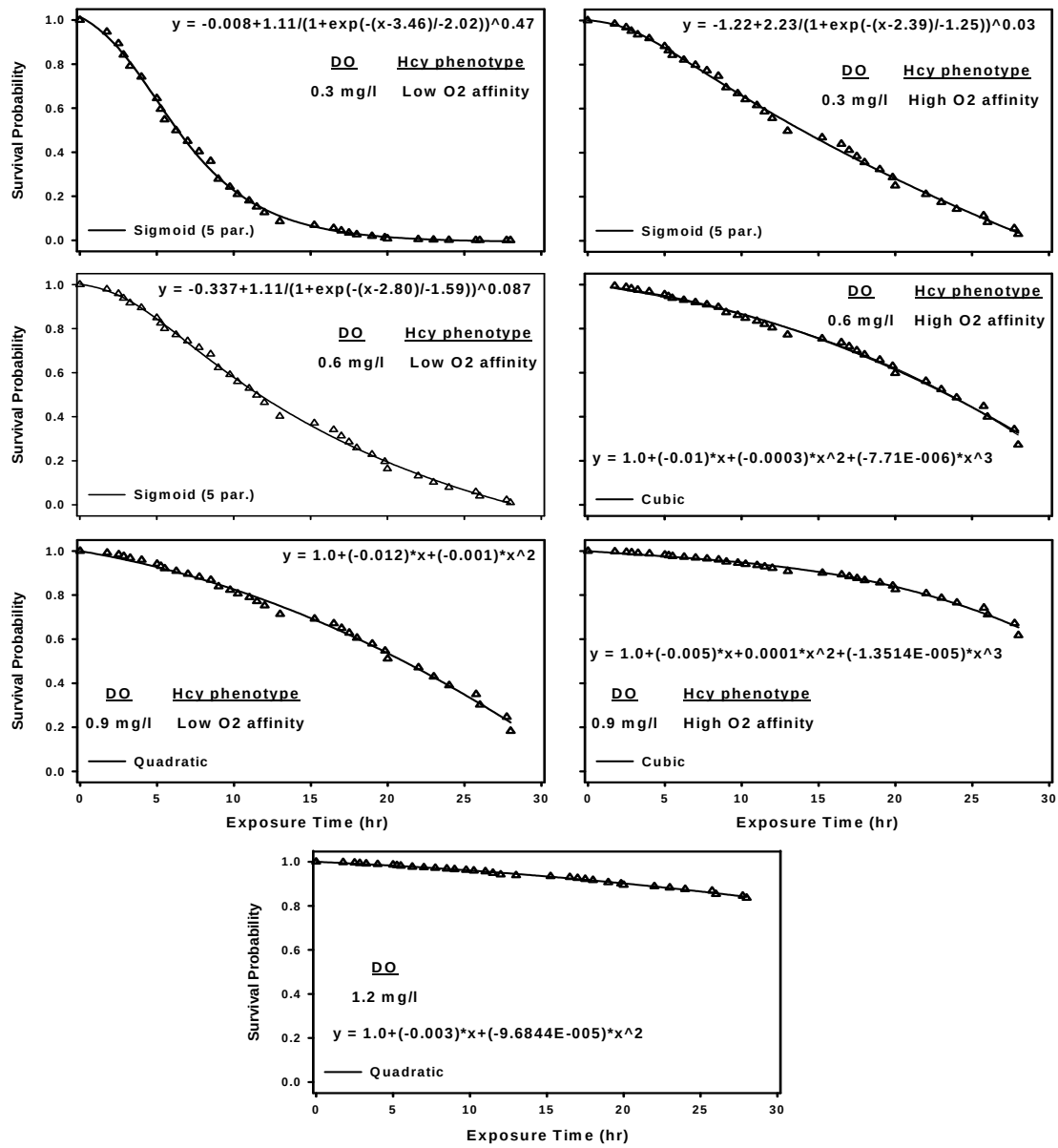
Logistic regression curves (best models from AIC – see Appendix 1A) describing the spatial distribution of DO in nearshore areas. Top two figures are empirical transect data from the Neuse River for two days when the frontal boundary between normoxic nearshore areas and deeper offshore water was classified as “weak” (left) and “strong” (right). Bottom three figures show logistic curves and equations used to parameterize the initial spatial DO concentration conditions in the IBM spatial landscape for the three minimum DO concentration treatment levels (1.2, 0.9, and 0.5 $\text{mg}\cdot\text{L}^{-1}$).



APPENDIX 2B

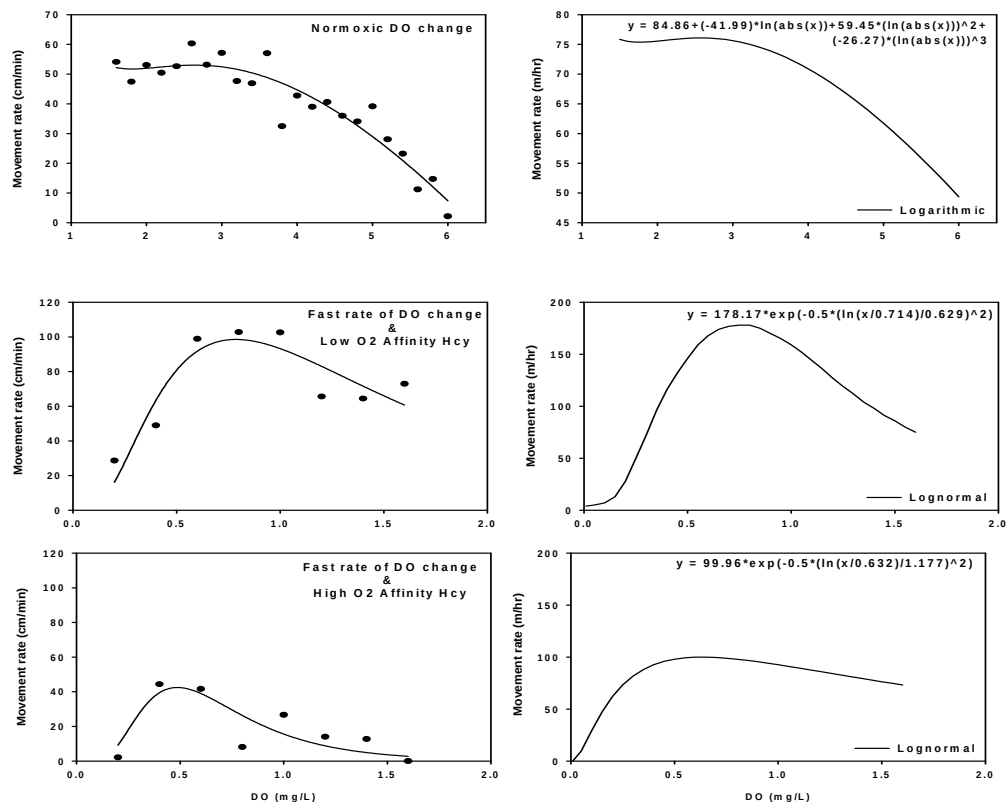
Cox proportional hazard model output from laboratory study used to parameterize survival probabilities in the IBM. Survival probabilities along a continuum of hypoxic exposure duration are shown for different DO concentration classifications and Hcy phenotypes.

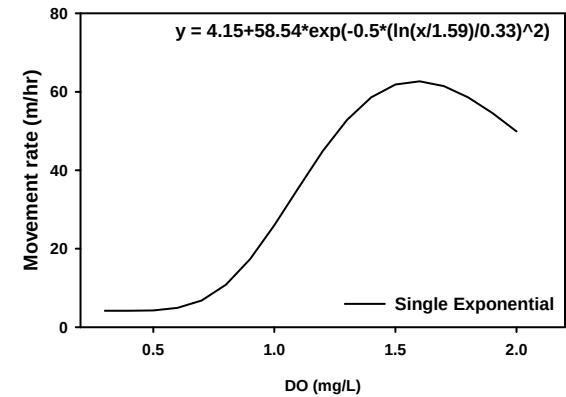
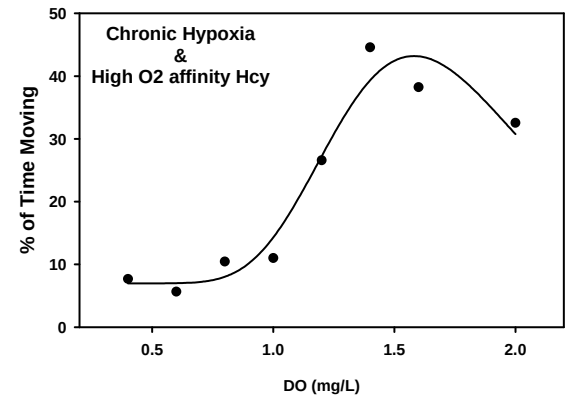
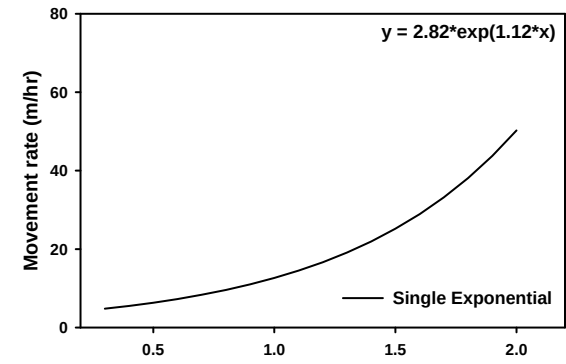
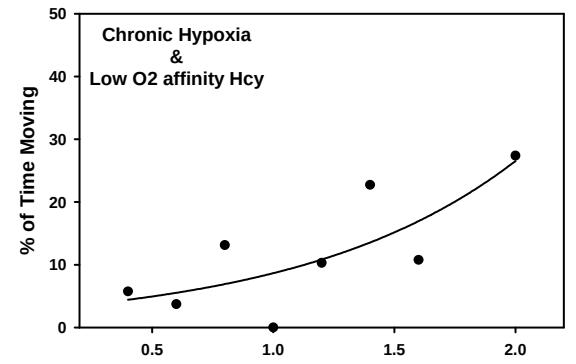
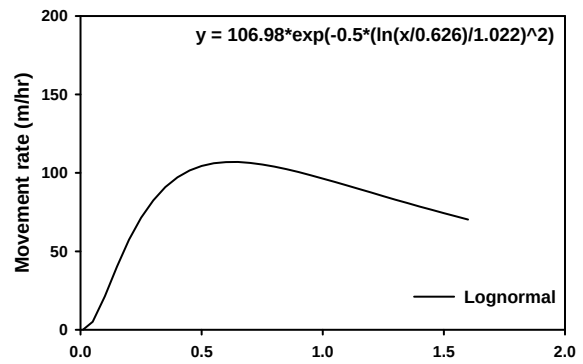
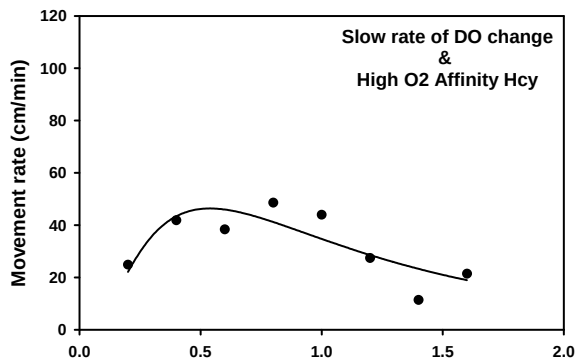
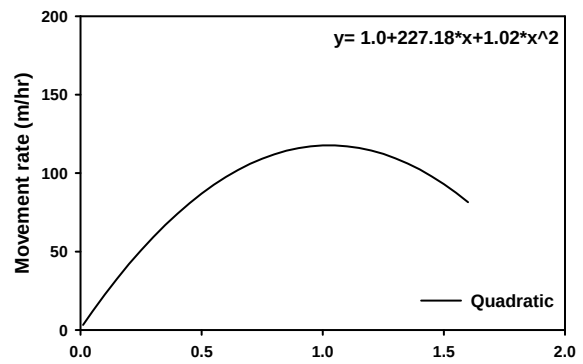
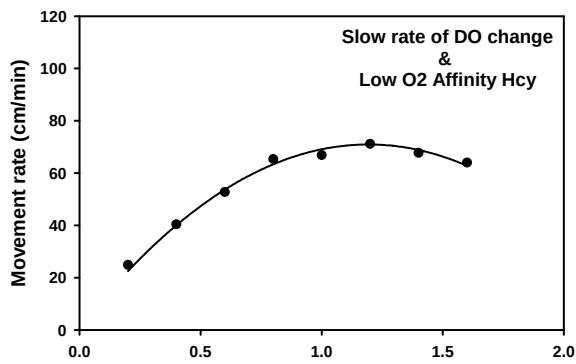
Curves are the best statistical models that fit the data based on AIC estimates (see Appendix 1A). The equation of each curve is also provided.



APPENDIX 2C

Functional relationships between DO concentration and movement used to parameterize blue crab movement rates in the IBM. First five figures on left are data from the flume laboratory study and describe changes in blue crab movement rates with declining DO under normoxic conditions and acute hypoxic exposure for crabs with high and low O₂ affinity Hcy phenotypes. Last two figures on the left are data from the survival laboratory study and show the percent of time crabs spent moving at various DO concentrations under chronically hypoxic conditions for crabs with both Hcy phenotypes. Curves in the left figures are statistical models with the lowest AIC values (see Appendix 1B). Curves in the right figures have the same form as the left figures but parameters have been scaled to describe blue crab movement rates in the IBM.





APPENDIX 3A

Heading frequency distribution of eight telemetered blue crabs during conditions indicative of hypoxic upwelling events in a section of the Neuse River Estuary. Directions of radiating bars shows crab headings and length of each bar is the number of crabs moving at that heading. Arrow indicates the mean heading of all crabs (21.0°) and outer arc shows 95% confidence interval of the mean heading ($349.3^\circ - 52.7^\circ$). To estimate the probability of crabs moving in each of the three possible X- and Y-directions (-1, 0, 1) in the model, a grid of nine cells (1 center cell and 8 surrounding cells) was superimposed over the heading frequency distribution and oriented to line up with the model landscape dimensions (bidirectional arrows). Heading observations were assigned to the eight surrounding cells and percent frequency (i.e., probability of moving in a specific direction) was calculated for each direction. The following probabilities were assigned to each possible direction: X(-1,0,1) = (0.272, 0.523, 0.205); Y(-1,0,1) = (0.682, 0.318, 0.00).

