TITLE: Acidification alters predator-prey interactions of blue crab *Callinectes sapidus* and soft-shell clam *Mya arenaria*

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ABSTRACT

Acidification due to anthropogenic CO_2 pollution, along with episodic or persistent acidification that occurs in coastal environments, will likely result in severe seasonal acidification in estuarine environments. Acidification decreases the fitness of individual species, but the degree to which predator-prey interactions will be impacted is largely unknown. This mesocosm study examined the effect of CO_2 acidification on crab-bivalve predator-prey interactions involving two commercially important Chesapeake Bay species, the blue crab *Callinectes sapidus* and the soft-shell clam *Mya arenaria*. In particular, the direct effects of CO_2 acidification on clam growth and behavior, and the indirect effects of CO_2 acidification on interactions between crabs and clams were examined. *Mya arenaria* were grown in CO_2-acidified water (pH 7.2) or ambient conditions (pH 7.8) for 30 d. To determine the effect of acidification on clam responsiveness to mechanical disturbance, a probe was slowly moved towards clams until they ceased pumping (a behavior to avoid detection by predators), and the distance between the probe and the clam's siphon was noted. Clams were exposed to predation by *C. sapidus*, which were held under acidified or ambient conditions for 48 h. *Callinectes sapidus* handling time, search time, and encounter rate were measured from video. Acidified clams had lighter shells than ambient clams, indicating that shell dissolution occurred. Acidification reduced the responsiveness of *M. arenaria* to a mechanical disturbance that simulated an approaching predator. As compared to ambient trials, crabs in acidified trials had higher encounter rates; however, this was offset by crabs taking longer to find the first clam in trials, and by increased occurrence of crabs eating only a portion of the prey available. As a result, there was no net change in predation-related clam mortality in acidified trials as compared

to ambient conditions. Understanding how acidification will impact food webs in productive estuarine environments requires an examination of the direct impacts of acidification on organism behavior and physiology, as well as indirect effects of acidification mediated through predator-prey interactions.

1. INTRODUCTION

In coastal ecosystems, many anthropogenic and natural processes combine to lower pH on various temporal and spatial scales. Runoff from land, especially acid sulfate soil runoff (Dove and Sammut, 2013), can reduce pH to < 5 for days (Macdonald et al., 2007) to months (Sammut et al., 1995). Seasonal acidification can result from upwelling (Feely et al., 2008), respiration (Feely et al., 2010), and eutrophication (Wallace et al., 2014). Estuaries have naturally low buffering capacity due to increased dissolved inorganic carbon (DIC) from detritus (Cai et al., 2011). As a result, coastal organisms encounter frequent and often extreme fluctuations in pH; for example, in Elkhorn Slough, California, the pH can vary from 8.1 to 7.4 over a tidal cycle (Hofmann et al., 2011). In addition, anthropogenic CO_2 emissions are expected to decrease open ocean pH by 0.3 to 0.4 units by the end of the century in a process known as ocean acidification (Orr et al., 2005; Solomon et al., 2007). All of these processes may be interacting to lower pH in some coastal systems (Feely et al., 2010).

Recent research suggests that CO_2 acidification may impact physiology, morphology, and behavior of coastal and estuarine species (Briffa et al., 2012; Dodd et al., 2015; Donohue et al., 2012). Major physiological changes in coastal species may include changes to internal pH, which has been observed in crustaceans (Donohue et al., 2012; Spicer et al., 2007) and fish (Esbaugh et al., 2012), and may negatively impact metabolic efficiency (Michaelidis et al., 2007; Pane and Barry, 2007). Acidification is expected to have negative effects on the morphology of many calcified organisms by inhibiting their ability to precipitate $CaCO_3$ to build their shells (Gazeau et al., 2007). For this reason, bivalve mollusks are expected to be some of the most sensitive organisms to changes in ocean pH, including common coastal bivalves such as oysters and

mussels (Amaral et al., 2012a; Gazeau et al., 2007; Hendriks et al., 2010). Acidification has been shown to alter the behavior of coastal organisms, influencing many processes including settlement behavior (Clements et al., 2016), shelter selection (de la Haye et al., 2011), homing (Devine et al., 2012), and predator-prey interactions (Bibby et al., 2007; Dodd et al., 2015).

The effect of acidification on predator-prey dynamics has been identified as an area of needed research (Parker et al., 2013). The majority of acidification studies on bivalves, and nearly all such studies related to predator-prey dynamics, focus on armored, reef-building species such as oysters or mussels (Glaspie and Seitz, in press; Kroeker et al., 2014; Parker et al., 2013). The parameters that have traditionally been used to study the effects of acidification on armored bivalves are calcification rate or shell strength, because a weakened shell means higher mortality rates for bivalves that cannot otherwise avoid predators, or increased investment in shell production that takes energy reserves from growth and reproduction (Amaral et al., 2012b; Sanford et al., 2014). However, thin-shelled or deep-burrowing bivalves that dominate in many estuaries (Beukema et al., 2010; Boesch, 1977; Hagy, 2002; Seitz et al., 2008) exhibit behavioral escape mechanisms that require a different set of traits than armored defense from predation, including traits that prevent detection by predators or features that promote burrowing or escape behavior (Vermeij, 1983). Few studies have examined the impact of acidification on bivalve burrowing or other avoidance behaviors (Clements et al., 2016; Schalkhausser et al., 2013), and even fewer have examined the impact of acidification on predator-prey interactions involving bivalves that exhibit behavioral escape mechanisms. For these reasons, the impact of acidification on predator-prey interactions involving thin-shelled bivalves is largely unknown.

To understand how acidification may impact the predator avoidance behavior of bivalves, research should examine acidification-related changes in the parameters of predator-prey

interactions. One such parameter is predator handling time (the time a predator spends manipulating and/or eating a prey item). Handling time is a major focus of studies involving armored bivalves because it reflects the consequences of shell thinning for predator-prey interactions (Amaral et al., 2012b; Dodd et al., 2015). However, handling time may not be as important for thin-shelled bivalves as other behaviors that reflect bivalve traits that prevent detection by predators, such as predator search time (the amount of time a predator spends foraging, or actively looking for prey) and encounter rate (the number of prey encounters over the search time). Search time may decline under acidification compared to ambient conditions (Dodd et al., 2015; Glaspie and Seitz, in press), possibly due to increased metabolic demand (Dissanayake and Ishimatsu, 2011) or altered cost-benefit analysis (de la Haye et al., 2011). Encounter rate is not calculated often in acidification studies (though see Appelhans et al., 2012; Dodd et al., 2015) and may change due to altered predator or prey behavior in acidified conditions (Cripps et al., 2011; de la Haye et al., 2012).

The goal of this study was to examine the effect of acidification on predator-prey interactions involving two commercially important species: a thin-shelled bivalve (the soft-shell clam *Mya arenaria*) and a crustacean predator (the blue crab *Callinectes sapidus*). This study was conducted in Chesapeake Bay, the largest estuary in the U.S. In the Bay, *C. sapidus* is a dominant species that can control prey resources (Eggleston et al., 1992). *Callinectes sapidus* is a generalist predator that grows up to 28 cm carapace width, is found along the Atlantic and Gulf coasts of North America (Williams, 1990), and is the main predator of bivalves in Chesapeake Bay (Hines et al., 1990). *Mya arenaria* is distributed on the east coast of North America from Virginia to Canada in estuarine waters (Baker and Mann, 1991), and has been introduced to the west coast of North America from California to Alaska (Strasser, 1999). *Mya arenaria* account

for about 12% of the value of U.S. commercial mollusk landings, which were worth more than $2.8 billion in 2014 (NMFS, 2015). They serve as biomass dominants in their native and introduced ranges (Strasser, 1999) and are a preferred prey item for many commercially important species such as *C. sapidus* (Eggleston et al., 1992; Hines et al., 1990). *Mya arenaria* is a deep-burrowing (greater than 30 cm), thin-shelled bivalve that avoids predators by achieving a spatial refuge (Abraham and Dillon, 1986; Hines and Comtois, 1985).

The objectives of the current study were to examine the direct effects of CO_2 acidification on *M. arenaria*, and the indirect effects of CO_2 acidification on predator-prey interactions between *M. arenaria* and *C. sapidus*. It was hypothesized that CO_2 acidification would have direct effects on clams through decreased growth (biomass and shell mass) due to rearing in acidified water, and decreased responsiveness to a simulated predator approach. It was hypothesized that indirect effects of CO_2 acidification would manifest through higher predation-related mortality of clams exposed to acidified crabs, and through lower handling time, lower search time, and higher encounter rates of *C. sapidus* feeding on acidified clams, as compared to those reared in ambient conditions.

2. MATERIALS AND METHODS

2.1. Growth conditions

This study was conducted in Gloucester Point, VA, from June through August 2015. Four 82-l rectangular tanks (76 x 33 x 33 cm) were filled with 8 cm sand (which is deep enough for clams < 30 mm to achieve maximum burial depth; Zwarts and Wanink, 1989) and 25 cm water

from the York River, VA, with ambient temperature and salinity (26.95 °C and 22.66, respectively). Juvenile *M. arenaria* clams, which were hand-collected in May 2015 from intertidal flats in the York River, were added to each tank, such that tanks contained 74-84 clams of average size 28.48 mm (SD 4.41 mm).

Two tanks were maintained at ambient pH with air bubbled through air stones. Ambient pH for the lower York River during the study period was 7.8, which is lower than controls used in other similar experiments (Dodd et al., 2015; Sanford et al., 2014). However, a pH of 7.8 is within the range of expected values for the York River, which is a respiration-dominated estuary (Raymond et al., 2000). The ambient pH used in this study was further validated using data from the CBVGIWQ monitoring station at the mouth of the York River, where pH was on average 7.94 (SD 0.15) during the study period (Fig. 1).

Two additional tanks were acidified with pure CO_2 mixed with air and maintained by an automated mini panel-mount pH controller (model PHCN-201 Omega Engineering Inc., Stamford, Connecticut), solenoid valve (model 4EKU5, Grainger, Chicago, IL), and pH electrode (model PHE-1411, Omega Engineering Inc., Stamford, Connecticut) calibrated using 4.00 and 7.00 buffers (PHA-4 and PHA-7, Omega Engineering Inc., Stamford, Connecticut). The pH was gradually lowered to 7.2 over six days and then maintained at 7.2 for three weeks for a total exposure period of four weeks. A pH of 7.2, or a total reduction of 0.6 pH units, is within the moderate range of pH reductions in similar experiments involving bivalves or crustaceans (Clements et al., 2016; Donohue et al., 2012; Fernández-Reiriz et al., 2012), but represents an extreme acidification scenario for the open ocean (IPCC, 2014).

Bivalves were fed 2.5 mL marine microalgae concentrate (Shellfish Diet 1800) per tank twice per day, and water was changed three times per week using filtered water from the York

River with ambient temperature and salinity. Temperature, salinity, dissolved oxygen, and pH were measured three times per week using a YSI (model 85, Yellow Springs Instruments, Yellow Springs, Ohio) and a pH probe (model PHE-1411, Omega Engineering Inc., Stamford, Connecticut). Total alkalinity (TA) was measured once per week using an Aquarium Pharmaceuticals carbonate hardness test kit (Ziegeweid et al., 2008). Seawater pCO_2 and calcite saturation state (Ω_C) were calculated from measured pH, TA, temperature and salinity with the program CO2SYS (Pierrot, 1998), using the Dickson (1990) values for the KSO_4 dissociation constant, the Uppstrom (1974) values for Total Boron, and an atmospheric pressure of 1.015 atm.

After a total of four weeks in acidified or ambient seawater, 10 clams were randomly chosen from both the acidified and ambient treatments. Clams were measured for shell length (mm), dried in a drying oven for 24 hours, and ashed in a muffle furnace at 550 °C for five hours. Ash-free dry weight (AFDW; dry weight minus ash weight, in g) was calculated as a measure of biomass, and ash weight (g) was calculated as a measure of shell mass. Both biomass and shell mass were standardized by dividing by shell length (g mm^{-1}). Due to logistical limitations and the low replication of rearing tanks (n = 2) these clams were pseudoreplicates. There was no reason to believe individual clam growth was impacted by other clams in the tank (i.e. there was no crowding and feeding rates were high); thus, clam biomass and shell mass measurements are treated as true replicates.

2.2. Clam behavior

After four weeks of growth in acidified or ambient conditions, 8 clams from each treatment were randomly selected and placed in a tank (76 x 33 x 33 cm) filled with 8 cm sand

and either filtered York River water (for ambient clams, pH 7.8) or filtered York River water that had been acidified with bubbled CO_2 (for acidified clams, pH 7.2). Clams were placed one per tank, 4 cm from the tank wall, siphon up, and pushed into the sand so they were completely covered. Clams were allowed time to resume pumping, usually about 15 minutes, before the start of the experiment.

At the start of the experiment a metal probe was inserted 2 cm into the sand at the opposite end of the tank from the clam. This probe simulated the approach of *C. sapidus*, which probes the sediment with the dactyls of its walking legs when foraging for infaunal prey (Blundon and Kennedy, 1982). The probe was slowly moved towards the clam at a rate of 1-2 cm s^{-1} until the clam ceased pumping (a behavior used to avoid predation; Smee and Weissburg, 2006), at which point the distance between the probe and the siphon (cm) was noted. This process was repeated three times for each clam, and the average distance of pumping cessation was calculated for each individual.

2.3. Predator-prey interactions

Callinectes sapidus (14 total) were collected from the York River in June 2015 via crab pots baited with frozen Atlantic menhaden (*Brevoortia tyrannus*) and left for 24-h soaks at ~4 m depth. All crabs were acclimated to the lab for one week or longer and fed fish or clam meat three times a week. Crabs were held individually or in pairs in 82-l tanks (n = 5, dimensions 76 x 33 x 33 cm) where they were exposed to either CO_2-acidified (pH 7.2) or ambient (pH 7.8) water and starved for 48 h prior to the start of the experiment. This time of exposure is long enough to produce a physiological response in other decapod crustaceans (Pane and Barry, 2007).

Clams were exposed to *C. sapidus* predation in 82-l tanks filled with 8 cm sand and 25 cm water from the York River at ambient temperature and salinity. Mesocosm chambers were set up in the same manner as growth tanks, so that tanks were either acidified with bubbled CO_2 or maintained at ambient pH conditions with bubbled air. Treatments and tank positions were randomized. No animals switched acidification treatments in the predator-prey experiment; all animals placed in acidified mesocosm tanks had been acclimated to acidified water, and all animals placed in ambient mesocosm tanks had been acclimated to ambient water. Cross-treatments (i.e. exposure of acidified clams to ambient crabs) depart from realistic "future" conditions. In addition, any observed response in such treatments may be due to the shock of improper acclimation (Widdicombe et al., 2010).

Four *M. arenaria* were placed in the sediment with their siphons up, away from the edge of the tank to avoid edge effects and were allowed 24 h to achieve a stable burial depth (Lipcius and Hines, 1986). Upon the start of the experiment, a crab was added to the mesocosm and allowed to feed for 48 h. After 48 h, predators were removed and surviving clams were counted. A different crab was used in each trial. There were seven replicates for each treatment (acidic and ambient) with crabs, and three replicate trials for each treatment without predators, which served as controls. No clams died in any predator-free controls, so clam mortality in treatment tanks was assumed to be from crab predation, and the predator-free controls will not be discussed further.

An IR-sensitive video with IR lights on 24 h d^{-1} was used to estimate search time, encounter rate, and handling time. Search time (h) was defined as the total cumulative time spent exhibiting foraging behavior, such as probing the sediment with legs or claws or lifting items to mouthparts. Encounter rate (hr^{-1}) was defined as the number of encounters (picking up and

consuming a bivalve) divided by the search time. All encounters led to a successful feeding throughout the course of the experiment. Handling time (h) was defined for the entire trial as the total cumulative time spent manipulating and/or eating a bivalve, divided by the number of encounters. The time it took for a crab to find its first clam (h) (i.e. time to first encounter), the amount of time crabs spent burrowed (h), and time spent exhibiting movements not related to foraging, such as agitated pacing behaviors or escape attempts (h), were also quantified.

2.4. Statistical design

Environmental variables (pH, alkalinity, pCO_2, Ω_C) were examined using linear mixed models, with treatment (acidified and ambient) as a fixed effect and tank number (2 levels, nested within treatment) as a random effects. pCO_2 was square-root transformed and Ω_C was quartic-root transformed for analysis. Parametric hypothesis testing (R package "pbkrtest") with 10,000 simulations was used to calculate p values for all linear mixed models. F-ratio tests were used to examine between-treatment differences in variability for environmental variables.

The following null hypotheses were tested: 1) clam biomass and shell mass in week four are no different in acidified than in ambient conditions; 2) clam behavior (distance from a disturbance upon cessation of pumping activity) is no different in acidified versus ambient conditions; 3) the proportion of clams eaten in acidified mesocosm trials is no different than in ambient trials; 4) the handling time, encounter rate, and search time of crabs are no different in acidified versus ambient mesocosm trials; 5) the time it took for a crab to find its first clam (time to first encounter) is no different in acidified versus ambient conditions; and 6) the proportion of time a crab spent exhibiting different activities (feeding, foraging, moving, and inactivity) in

acidified mesocosm trials is no different than in ambient trials. Two-sample comparisons for clam biomass, shell mass, and clam behavior were examined using non-parametric bootstrap hypothesis testing with 10,000 simulations and $\alpha = 0.05$ (Efron and Tibshirani, 1993), due to the inability to meet assumptions of normality and homogeneity of variance via transformations. For all two-group comparisons using non-parametric bootstrapping, Cohen's d is reported as a measure of effect size.

Number of clams eaten in acidified and ambient mesocosm trials was examined using linear mixed models, with treatment (acidified and ambient) as a fixed effect and tank number (2 levels, nested within treatment) and position (4 levels) as random effects. Handling time, encounter rate, and search time were square-root transformed to meet assumptions of normality and homogeneity of variance, and were examined using linear mixed models, with treatment (acidified and ambient) as a fixed effect and tank number (2 levels, nested within treatment) and position (2 levels) as random effects. Time to first clam encounter was also examined using linear mixed models with the same structure. Due to the low sample size ($n = 4$) and the variable nature of the handling time, encounter rate, and search time data, $\alpha = 0.10$ was used to interpret statistical significance for handling time, encounter rate, search time, and time to first encounter.

Analysis of the number of mesocosm trials with all of the clams eaten, a portion of the clams eaten, and none of the clams eaten was completed using a chi-square test with Monte Carlo simulation of p values due to the presence of zeros in the contingency table. Analysis of proportional crab activity data (e.g. percent of time spent inactive) was completed using a chi-square test. All analyses were completed using R statistical software (R Core Team, 2015).

3. RESULTS

3.1. Growth conditions

Temperature, salinity, and dissolved oxygen were fairly consistent among treatments, but were variable throughout the course of the study due to natural fluctuations in source water conditions (Table 1). Average pH during the study was lower ($t = 13.51$, $p < 0.001$) and alkalinity was higher ($t = -4.34$, $p = 0.05$) in acidified tanks than in ambient tanks (Table 1). The maximum pH observed throughout the experiment was 8.1 in the ambient treatment, and the minimum was 6.9 in the acidified treatment. pCO_2 (as calculated from CO2SYS) was higher ($t = -14.37$, $p = 0.001$) and more variable ($F = 4.93$, $p < 0.001$) in acidified tanks than in ambient tanks. Calcite saturation state (Ω_C) was lower ($t = 10.74$, $p = 0.001$) and less variable ($F = 12.57$, $p < 0.001$) in acidified tanks than in ambient tanks.

Sources of variability in pH throughout the experiment are likely related to variability in source water pH and buffering capacity (from tidal mixing and respiration), because incoming water was not scrubbed using a buffer (Fig. 1). The CO_2 delivery method produced minor fluctuations once the target pH was reached, and both ambient and acidified tanks had similar variability in pH ($F = 2.06$, $p = 0.08$) and alkalinity ($F = 2.07$, $p = 0.08$). Measured and calculated values of carbonate variables were similar to those reported in other mesocosm studies (Dodd et al., 2015).

At the end of the experiment, there was no difference in biomass of clams grown in acidified (0.003 g mm^{-1} ± 0.001 SD) or ambient (0.004 g mm^{-1} ± 0.001 SD) conditions ($p = 0.86$, $d = 0.14$). However, there was a difference in shell mass between clams grown in acidified and

ambient treatments ($p = 0.03$, $d = 0.24$). Clams grown in ambient conditions had a shell mass of 0.027 g mm^{-1} on average (95% CI [0.020, 0.034]), whereas acidified clams had a shell mass of 0.020 g mm^{-1} on average (95% CI [0.016, 0.024]).

3.2. Clam behavior

Upon exposure to a mechanical disturbance used to simulate a predator (a probe moving through the sand at a steady rate towards a buried clam), clams that had spent four weeks in CO_2-acidified water allowed the probe to get closer before reacting (by ceasing pumping behavior) than clams that were grown in ambient conditions ($p = 0.01$, $d = 0.41$). Clams grown in ambient conditions reacted when the predator-simulating probe was 29.6 cm away on average (95% CI [17.9, 41.4]), whereas acidified clams did not react until the probe was 11.1 cm away on average (95% CI [6.3, 15.9]).

3.3. Predator-prey interactions

There was no difference in average number of clams eaten per tank between acidified and ambient treatments ($p = 1.0$), averaging 2.9 (SE = 0.7) in the acidified treatment and 2.9 (SE = 0.5) in the ambient treatment. In the ambient treatment, crabs either ate all of the available clams (occurred 5 times), or none of them (occurred 2 times; Fig. 2). In the acidified treatment, crabs either ate all of the acidified clams (occurred 3 times), or a portion of the clams available (occurred 4 times); however, there was never a trial where an acidified crab failed to find and consume at least one acidified clam (Fig. 2). There was a significant difference in the frequency

of occurrence of these events (all clams eaten, a portion of the clams eaten, and no clams eaten) between the two treatments ($\chi^2 = 6.5$, $p = 0.04$).

Handling time for crabs preying on clams grown in the ambient treatment was not different from handling time for crabs and clams in the acidified treatment ($t = 0.34$, $p = 0.97$; Fig. 3a). The encounter rate for trials with acidified clams was greater than the encounter rate for trials with ambient clams ($t = -1.93$, $p = 0.09$; Fig. 3b). The search time for crabs in trials with acidified clams was not significantly different than the search time for crabs in trials with ambient clams ($t = 1.70$, $p = 0.13$; Fig. 3c). Time to first encounter (prey capture) was significantly greater for crabs in acidified trials than in ambient trials ($t = -2.24$, $p = 0.09$). Crabs spent 0.474 h on average searching for the first clam in ambient trials (95% CI [0.050, 0.898]), and 4.513 h on average searching for the first clam in acidified trials (95% CI [0.976, 8.049]).

There was no significant difference between acidified and ambient crabs in the proportion of time crabs spent engaging in various activities such as feeding, foraging, other movement (non-foraging related), and resting ($\chi^2 = 0.09$, $p = 0.99$). Acidified crabs spent an average 55% of the time burrowed or resting still (95% CI [25%, 84%]), and 43% of the time exhibiting agitated, non-foraging related movement patterns or escape behavior (95% CI [13%, 73%]; Fig. 4). Ambient crabs spent an average 71% of the time burrowed or resting still (95% CI [50%, 91%]), and 23% of the time exhibiting non-foraging related movement patterns (95% CI [2%, 44%]; Fig. 4).

4. DISCUSSION

Acidification affected some aspects of clam growth but not others. After the acclimation period, there were no differences in *M. arenaria* biomass between acidified clams and clams grown under ambient conditions, which was contrary to our hypothesis. It is possible that four weeks was not enough time to see a meaningful difference in clam biomass. However, other similar studies have observed changes in growth due to acidification in less than a month (Sanford et al., 2014). Four weeks in acidified water was sufficient to produce significant declines in shell mass as compared to ambient conditions, supporting our hypothesis. Shell thinning or weakening has been observed for other armored mollusks, including bivalves (Amaral et al., 2012b) and gastropods (Bibby et al., 2007), but had never been determined for this commercially and ecologically important species. Even though thin-shelled species like *M. arenaria* do not rely on their shell to defend from predators, shell growth and integrity are still important. *M. arenaria* must grow quickly to achieve a burial depth refuge from predation (Zaklan and Ydenberg, 1997), and must be strong enough to withstand pressure from sediments (Dorgan, 2015; Savazzi and Sälgeback, 2004). There are likely energetic costs associated with maintaining growth and shell integrity that could not be sustained in acidified conditions.

This decrease in shell mass of acidified clams in relation to ambient conditions did not affect the predator's handling time, which was similar for both acidified and ambient clams. Handling time may not be a sensitive indicator of acidification for thin-shelled bivalves, because it tends to respond to anti-predator traits, such as thick shells, found in armored bivalves. Alternatively, since handling time is an indirect function of acidification and involves direct effects of acidification on both crabs (motor ability, stress) and clams (shell thinning), these effects could be in opposition, therefore canceling out any noticeable impact of acidification on handling time. For example, even though the periwinkle *Littorina littorea* experienced shell

weakening and green crabs *Carcinus maenas* experienced claw muscle weakening under acidification, encounters between the two species led to no net change in handling time (Landes and Zimmer, 2012). Physiological studies on the impact of acidification on motor control and stress response of crustaceans are necessary to fully understand this relationship for crabs preying on thin-shelled prey.

Although shell thinning may not increase risk of predation for *M. arenaria*, acidification may negate some traits that protect clams from predation by altering predator avoidance behavior. Cessation of pumping is a behavior that aids the clam in avoiding detection by predators (Hay, 2009; Nakaoka, 2000; Smee and Weissburg, 2006; Weissburg and Zimmer-Faust, 1993). Bivalves that are unable to respond to mechanical disturbance (such as *C. sapidus* foraging behavior) may experience increased encounters with predators, and thus increased mortality. Decreased clam predator avoidance behavior was a likely mechanism behind *C. sapidus* encounter rates that were nearly three times higher in acidified trials as compared to ambient trials.

Despite higher encounter rates in acidified trials as compared to ambient trials, there was no net increase in predator-related mortality for acidified clams as compared to ambient clams. Acidification-related changes in predator behavior may have compensated for increased prey encounter rates. Acidified crabs took 9.5 times longer to find the first clam than ambient crabs. While acidified crabs were always able to find at least one clam during the trial, they also exhibited increased incidence of failing to find all of the available clams as compared to ambient trials. This evidence indicates that an overall lack of interest in foraging or an inability to forage effectively may be a consequence of acidification for *C. sapidus*.

Briffa et al. (2012) suggest acidification by CO_2 can directly influence the behavior of predators in three ways. The first is by making predatory behaviors such as foraging more costly by altering metabolic processes in the predator. Marine crustaceans experience direct physiological consequences of acidification, including decreased extracellular pH (Donohue et al., 2012), which may influence metabolism and energy budget. While acidified crabs did tend to spend less time foraging than ambient crabs (not statistically significant), any decrease in foraging behavior did not coincide with a decrease in non-foraging related movements such as cleaning, aggressive behaviors, walking, or swimming, indicating these behaviors were unlikely to be especially costly under acidification.

A second way acidification influences predator behavior is through predator avoidance of acidified areas (Briffa et al., 2012). Little is known regarding the avoidance behavior of marine crustaceans exposed to acidification. However, crabs are commonly found in acidified portions of estuaries experiencing acid-sulfate soil acidification (Amaral et al., 2011; Russell and Helmke, 2002). In the current study, this mechanism is an unlikely cause of the observed alterations in *C. sapidus* behavior because acidified *C. sapidus* did not spend a significantly greater amount of time pacing or attempting to escape than ambient crabs.

A third way acidification might directly influence behavior of predators is through the disruption of information-gathering and decision-making processes (Briffa et al., 2012). Low pH reduces the ability of some organisms, such as reef fish, to sense their environment and make decisions that maximize their fitness (Cripps et al., 2011; Devine et al., 2012). *Callinectes sapidus* relies heavily on olfaction to forage, and when these senses are removed crabs are either unable to detect the presence of prey or unable to orient themselves towards the source of the chemical signal (Keller et al., 2003; Weissburg and Zimmer-Faust, 1994). Inability to detect or

process chemosensory information is a possible mechanism behind observed shifts in crab behavior, such as longer time to first encounter and increased incidence of consuming only a portion of the prey in acidified versus ambient trials. Altered decision-making under acidification has been invoked as a mechanism explaining observed changes in foraging behavior of other decapods. Mud crabs *Panopeus herbstii* spent less time before giving up an unsuccessful predation attempt when they were acidified, as compared to controls, despite no changes in relative activity level of the crabs (Dodd et al., 2015). Hermit crabs *Pagurus bernhardus* were less capable of tracking odor sources under acidification, as compared to controls (de la Haye et al., 2012).

One indirect mechanism by which predator behavior may be influenced by CO_2 acidification involves predators avoiding prey that is lower quality due to acidification. Extreme stress, such as changes in temperature, salinity, or acidification, may lead to changes in prey tissue condition (Mitra and Flynn, 2005). In particular, acidification that leads to bivalve shell dissolution, as observed in the current study, may necessitate allocation of more resources to shell growth and less to tissue maintenance (Hiebenthal et al., 2013, 2012; Lannig et al., 2010). There is some support for this mechanism, since changes in crab foraging behavior has been observed for both acidified (current study, de la Haye et al., 2012; Dodd et al., 2015) and non-acidified crabs (Glaspie and Seitz, in press); however, this mechanism requires further research on the impact of acidification on prey quality and the implications for predator foraging behavior.

There are several limitations of the current study that may impact interpretation of results. Due to time and space requirements, the current mesocosm experiment included 82 l tanks and n = 7 replicates for each treatment. The tank size was as large as or larger than experimental arenas

in similar studies, and the number of replicates used was in the range of replication used in similar studies (Amaral et al., 2012b; Dodd et al., 2015). However, animal behavior in tanks may not be entirely representative of that in natural systems, due to the constraints imposed on the enclosed organisms (Brockmann, 1990) and the inability to replicate natural phenomena such as water column structure/currents (Carpenter, 1996) or protection from predation supplied by complex habitats such as seagrass (Orth et al., 1984).

This experiment used water directly from the York River that was not manipulated using pH scrubbers or chillers; thus, the clams in both treatments (acidified and ambient) experienced natural fluctuation in temperature, salinity, dissolved oxygen, pH, and alkalinity throughout the experiment. While this variability may be viewed as a limitation for acidification studies in open-ocean environments, which are relatively constant, variability is a meaningful component of estuarine acidification studies, which must address the effect of multiple stressors on an organism's survivability (Fabry et al., 2008). The relatively extreme pH used in this study (as compared to open-ocean acidification predictions) is also a function of the estuarine environment, where high dissolved inorganic carbon (DIC) from detritus, increased respiration in highly productive coastal regions, pollution, and stratification, low salinity, and low buffering capacity all result in much lower and more variable pH and total alkalinity than in the open-ocean environment (Cai and Wang, 1998; Feely et al., 2010; Ringwood and Keppler, 2002).

The acclimation period in this study (4 weeks for *M. arenaria* and 48 hours for *C. sapidus*) was relatively short considering the time scale of ocean acidification. However, these time scales have biological relevance for near-shore or estuarine systems. In the Chesapeake Bay, extremely low pH (lowest 1% of measurements) occurred during a 30-40 day period in the summer (Figure 1), suggesting the 30 day acidification of clams in the current study was

sufficient to examine combined impacts of anthropogenic CO_2 acidification and seasonal acidification due to respiration and stratification. In addition, previous studies suggest the shallow-water Dungeness crab *Cancer magister* have the capacity to regulate hemolymph pH and recover metabolic efficiency over a 24 h acclimation period, even with a pH reduction of 0.32 units (Pane and Barry, 2007), indicating that 48 h was a sufficient acclimation period for *C. sapidus*. Lastly, short-term laboratory mesocosm studies may provide direction for long-term acidification studies in more natural experiments such as in-situ observational studies in CO_2 vent systems (e.g. Hall-Spencer et al., 2008) and open-ocean CO_2 enrichment experiments (e.g. Gattuso et al., 2014).

5. CONCLUSIONS

A mesocosm experiment was conducted to determine the effects of estuarine acidification on predator-prey interactions involving the thin-shelled bivalve *M. arenaria* and the crab *C. sapidus*. The direct effects of acidification on thin-shelled clams may be offset by indirect effects of acidification on predator-prey interactions with their crustacean predators. Under a scenario of acidification, *C. sapidus* encountered *M. arenaria* prey at higher rates, due at least in part to reduced predator avoidance behavior by clams. However, acidified crabs took more time to find the first clam and had increased incidence of consuming only a portion of the prey available in experimental mesocosms, as compared to ambient conditions, resulting in no net increase in predation-related mortality for clams. In estuaries, which are some of the most productive areas in the world, anthropogenic CO_2 acidification combined with natural processes such as respiration may produce extreme acidification events that have direct impacts on the physiology

and behavior of a variety of organisms. However, an understanding of the indirect impacts of acidification mediated by predator-prey interactions is necessary to make viable predictions and take conservation actions that may preserve these resources for future generations.

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TABLES

Table 1. Environmental variables expressed as means (and standard deviation) over the course of the 4-week grow-out period for *Mya arenaria* in ambient (n = 2) and acidified (n = 2) tanks. Measurements were taken 3 times per week per tank for pH, temperature, salinity, and dissolved oxygen (DO); measurements were taken twice per week per tank for total alkalinity (TA). pCO_2 and calcite saturation state (Ωc) were calculated from pH and TA using CO2SYS.

	Ambient	**Acidified**
pCO_2 (µatm)	1284.36 (744.79)	6463.53 (1652.85)
Ωc	3.88 (1.47)	1.18 (0.41)
pH	7.79 (0.19)	7.17 (0.13)
TA (µmol/kgSW)	2928.03 (239.10)	3500.07 (344.11)
Temperature (° C)	26.95 (1.48)	26.69 (1.27)
Salinity	22.66 (0.48)	22.67 (0.37)
DO (mg/L)	5.42 (0.59)	5.03 (0.48)

FIGURES

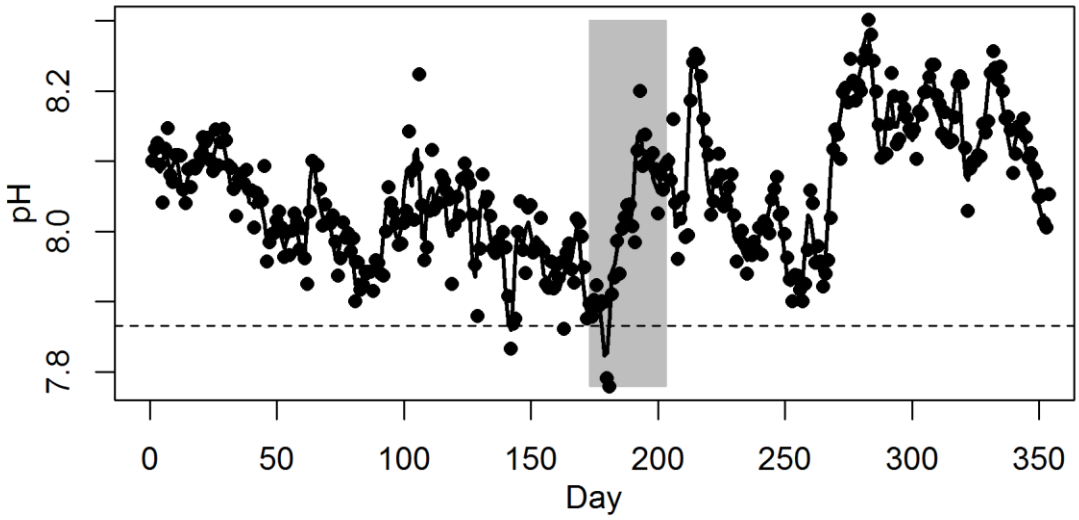

Figure 1. Average daily pH by Julian day for 2015 at the York River Goodwin Islands station CBVGIWQ (NERRS). Shaded region represents the study period, and dashed line represents the lowest 1% of pH measurements for the year. Trend line is the 3-day moving average for the time series.

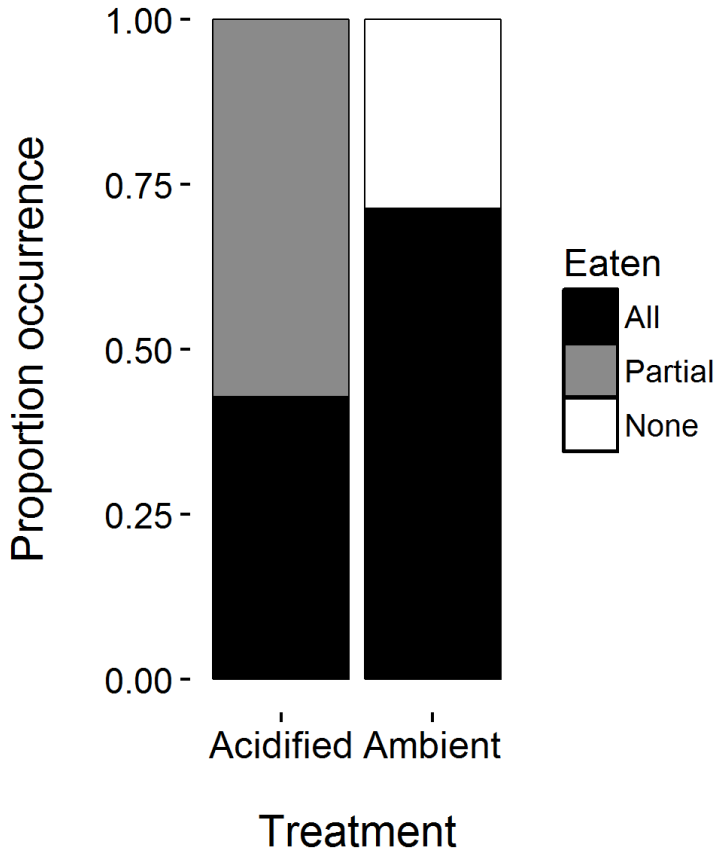

Figure 2. Foraging success for acidified and ambient crabs feeding on clams. Proportion of the trials in which all (black), some (partial, gray), or none (white) of the clams (*Mya arenaria*, 4 total in each trial) were eaten for crabs (*Callinectes sapidus*) and clams in acidified or ambient water, n = 7.

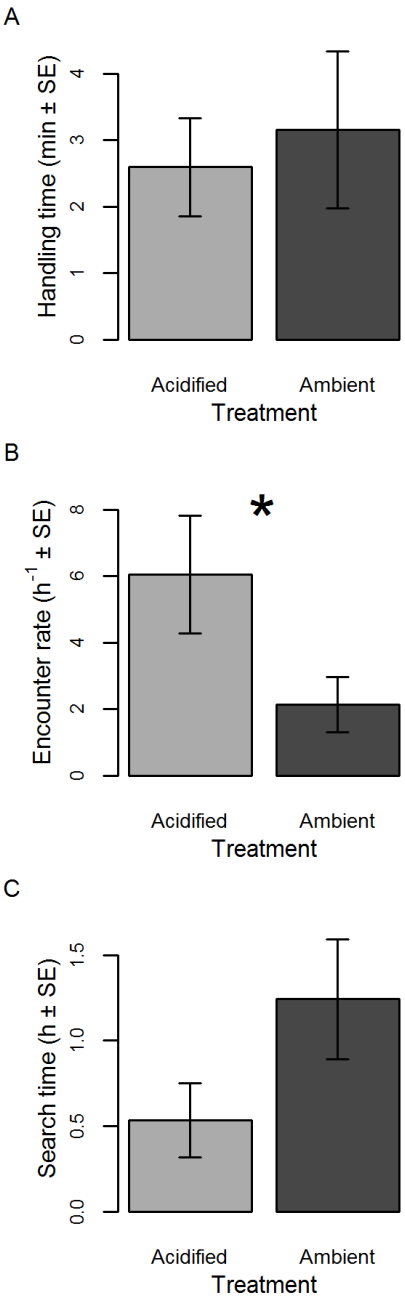

Figure 3. Crab behavior for acidified and ambient crabs feeding on clams. Means (± 1 SE) for blue crab *Callinectes sapidus* a) handling time, b) encounter rate, and c) search time when exposed to acidified or ambient water and prey *Mya arenaria*. Asterisk denotes significant difference at $\alpha = 0.10$; n = 4.

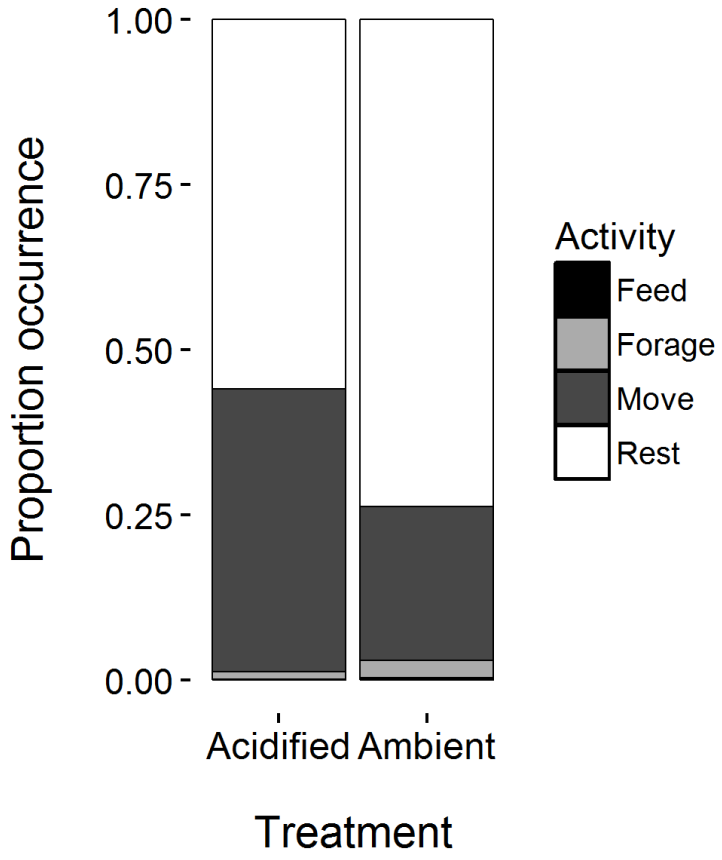

Figure 4. Relative amount of time crabs spent exhibiting various behaviors. Proportion of the time crabs spent feeding (black), foraging (light gray), exhibiting non-foraging movement (dark gray), and inactive (white) in acidified or ambient water, n = 4.