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NOTE

Survival and osmoregulation of the purple marsh crab (*Sesarma reticulatum*) at varying salinity and pH

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Abstract: Overfishing of top predators along the western Atlantic coastline has led to a trophic cascade in salt marshes, with increases in herbivorous purple marsh crab (*Sesarma reticulatum* (Say, 1817)) abundances in North American estuaries leading to overgrazing of cordgrass (*Spartina alterniflora* Loisel.) and shoreline erosion. To evaluate potential physiological limits on the range of *S. reticulatum* within an estuary, we evaluated survival and physiological tolerance of *S. reticulatum* from the Ashepoo–Combhee–Edisto (ACE) River Basin in South Carolina, USA, to combinations of salinity (5‰ and 30‰) and pH (pH 6.6, 7.6, and 8.6) challenges, representative of estuarine extremes. Survival, haemolymph ion concentrations, and gill Na^+,K^+-ATPase (NKA) and vacuolar-type H^+-ATPase (VHA) activity were measured after a 48 h exposure to each experimental condition. Survival was nearly 100% and osmoregulatory control was maintained across estuarine salinity and pH ranges. *Sesarma reticulatum* appeared to be robust to all potential combinations of salinity and pH stressors examined in this study, and therefore are likely unrestricted in their fundamental niche based on these stressors throughout an estuary.

Key words: purple marsh crab, *Sesarma reticulatum*, osmoregulation, salinity, pH, estuary.

Résumé : La surpêche de superprédateurs le long de la côte ouest de l'Atlantique a mené à une cascade trophique dans les marais salés dans laquelle des augmentations de l'abondance du crabe violet des marécages (*Sesarma reticulatum* (Say, 1817)) herbivore dans les estuaires nord-américains ont entraîné une surconsommation de spartine (*Spartina alterniflora* Loisel.) et l'érosion des rives. Afin d'évaluer les limites physiologiques potentielles de l'aire de répartition de *S. reticulatum* dans un estuaire, nous avons évalué la survie et la tolérance physiologique de *S. reticulatum* du bassin versant des rivières Ashepoo–Combhee–Edisto (ACE) en Caroline du Sud (États-Unis) en réponse à différentes combinaisons de salinité (5 ‰ et 30 ‰) et de pH (6,6, 7,6 et 8,6) représentatives d'extrêmes estuariens. Le taux de survie, les concentrations hémolymphatiques d'ions et l'activité de la Na^+,K^+-ATPase (NKA) et de la H^+-ATPase de type vacuolaire (VHA) dans les branchies ont été mesurés après une exposition de 48 h à chacune des conditions expérimentales. La survie était de près de 100 % et l'osmorégulation était maintenue dans toute la fourchette de salinités et de pH estuariens. Les *S. reticulatum* ont semblé résister à toutes les combinaisons possibles de facteurs de stress associés à la salinité et au pH examinés dans l'étude; par conséquent, ces facteurs de stress ne limiteraient vraisemblablement pas la niche fondamentale de l'espèce dans un estuaire. [Traduit par la Rédaction]

Mots-clés : crabe violet des marécages, *Sesarma reticulatum*, osmorégulation, salinité, pH, estuaire.

Introduction

Salt marsh habitat loss along the western Atlantic coast has become an increasing problem in the last 40 years (Bertness et al. 2014). The pronounced vegetation die-off is believed to be due to changes in trophic structure (Silliman and Bertness 2002; Bertness and Silliman 2008). Shifts in the abundance of one or several key species within a community are known to have cascading effects on populations of other species and broader impacts on the ecosystem. Top-down effects result from predator removal, which increases herbivore populations and consequently decreases vegetation cover (Hughes et al. 2013). In salt marshes especially, this decrease in vegetation can lead to erosion and habitat loss (Nyman et al. 1994). We can therefore reasonably expect the spatial scale of estuarine habitat loss to be based on both (*i*) the spatial scale of predator removals and (*ii*) key environmental variables that influence the physiological limits of the herbivore.

The purple marsh crab (*Sesarma reticulatum* (Say, 1817)) is an herbivorous crab native to salt marshes along the east coast of North America, ranging from Massachusetts to southern Florida (Abele 1973). In the past 20 years, commercial and recreational fishing of natural predators to *S. reticulatum* (e.g., blue crab (*Callinectes sapidus* M.J. Rathbun, 1896), striped bass (*Morone saxatilis* (Walbaum, 1792)), and Atlantic cod (*Gadus morhua* L., 1758)) has resulted in the rapid overpopulation of *S. reticulatum* in many salt marsh ecosystems along the eastern coast of the United States (Silliman and Bertness 2002; Bertness and Silliman 2008). With fewer predators, root and aboveground biomass consumption of smooth cordgrass (*Spartina alterniflora* Loisel.) by *S. reticulatum* has risen dramatically, resulting in severe salt marsh erosion (Holdredge et al. 2009). This loss of salt marsh habitat has severe consequences not only for the health of the ecosystem, but also for the economic and recreational use of the shoreline and coastal waterways. If such primary consumption remains uncontrolled by natural predators, then the extent of herbivory by *S. reticulatum* and subsequent potential salt marsh erosion could extend throughout the coastal environment where *S. reticulatum* is found. *Sesarma reticulatum* is highly mobile and displays low habitat fidel-

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ity (Seiple and Salmon 1982), suggesting that expansion of these populations will depend largely on the physiological capacity of *S. reticulatum* to survive in the dynamic salt marsh environment.

Salt marshes are coastal habitats often adjacent to estuaries which undergo daily fluctuations in environmental factors due to river outflow and tidal influence. To survive in an estuarine habitat, many organisms, including crabs, have a suite of physiological adaptations that allow them to tolerate fluctuations in their abiotic environment. These adaptations set the physiological niche for these organisms (Basset et al. 2013) and define the habitat range over which they may be found. In an estuary, the daily rising and retreating tide produces large fluctuations in salinity (between 2‰ and 32‰; Bulger et al. 1993). The lower salinity tolerance limit of most estuarine invertebrates overlaps the upper salinity tolerance limit of most freshwater invertebrates, between 2‰ and 4‰ (~50–100 mosmol·kg^{-1}; Bulger et al. 1993). Conversely, the upper salinity tolerance for most estuarine invertebrates overlaps the lower salinity tolerance limits for many stenohaline (i.e., obligate) marine species, between 24‰ and 27‰ (~700–800 mosmol·kg^{-1}). For an osmoregulating estuarine invertebrate (such as *S. reticulatum*), these lower and upper salinity boundaries describe the theoretical lower and upper limits of osmoregulatory control, within which *S. reticulatum* is able to maintain internal osmotic pressure by actively regulating ion and water balance. Within this range, *S. reticulatum* hyper-osmoregulates to maintain an internal osmolality around 700 mosmol·kg^{-1} (at or above that of the environment) (Foskett 1977; Staton and Felder 1992). Estuarine organisms unable to meet this hyper-osmoregulatory demand might experience excessive ion loss to and water gain from the more dilute environment. There is evidence that the degree of osmoregulatory ability in *S. reticulatum* is habitat- and population-specific (Staton and Felder 1992), and therefore could reasonably be affected by other fluctuating environmental factors.

The mechanism of hyper-osmoregulation in estuarine crabs is accomplished by a suite of primary and secondary ion transport proteins on the gill epithelium. The activity of the Na^+,K^+-ATPase (NKA) in mitochondria-rich cells produces a local electrogenic gradient favoring ion (primarily Na^+ and Cl^-) uptake across the gill epithelium (Lucu et al. 2000). In many euryhaline crabs, the apically located vacuolar-type H^+-ATPase (VHA) too is utilized in ion (Na^+) uptake from dilute environments (Tsai and Lin 2007). Alongside changes in salinity, shifts in local environmental factors such as the input from agricultural runoff, products of organic matter decomposition, and poor water mixing can cause fluctuations in local ambient pH between pH 6.0 and pH 8.5 (Berounsky and Nixon 1993; Feely et al. 2010). Physiological processes of acid–base balance and nitrogen excretion may also be under frequent acute stress (Tsai and Lin 2007). Compensation for these pH challenges by estuarine invertebrates, although much less studied than osmoregulation, is also reported to be fulfilled in part by the acid (H^+) excretion path associated with VHA activity (Weihrauch et al. 2001).

Research on salinity acclimation and acid–base regulation in crabs typically looks at the effects of these stressors in isolation. However, the multitude of simultaneous changes in the abiotic estuarine environment is an example of the need for more multiple stress analyses in ecophysiology research. Estuarine and migrating organisms continuously experience an array of new environmental challenges, and multiple stressor analyses offer a larger window through which we can study the physiology associated with these environmental challenges. When the physiological analysis is more comprehensive of the natural environmental stressors, we can draw more powerful ecological inferences from these physiological data (Todgham and Stillman 2013).

This study investigated the effects of varying both ambient salinity and pH levels, representative of hypothetical limits experienced along an estuarine gradient, on the osmoregulatory capacity of *S. reticulatum* collected from the field. Although physiological responses to changes in salinity in *S. reticulatum* have been documented (Foskett 1977; Staton and Felder 1992), to our knowledge no other studies have evaluated responses to simultaneous salinity and pH changes, representative of what these animals may encounter in an estuarine environment. Ecological inferences made from these multiple stressor data could be useful for predicting the potential range of *S. reticulatum* populations within estuaries. Presuming that *S. reticulatum* is well adapted to the environment it inhabits, we predicted that survival and osmoregulatory capacity of *S. reticulatum* would be robust to changes in ambient pH and salinity between observed estuarine limits. This would be evidenced by the maintenance of osmoregulatory control at dilute salinities, regardless of ambient pH, and would indicate the potential for successful habitation by *S. reticulatum* (and thus, cordgrass consumption and shoreline erosion) throughout estuaries if the current decline in its natural predators persists.

Materials and methods

Field location and animal collection site

We collected adult *S. reticulatum* (48 male, 48 female; carapace width = 18.7 ± 0.2 mm; mass = 3.7 ± 0.1 g) from the top 0.25 m of sediment along a 125 m stretch of muddy berm of the intertidal region found near the junction of Mosquito Creek and the Ashepoo River (UTM zone 17: 361792, 3602357). These estuaries are in the Ashepoo–Combhee–Edisto (ACE) River Basin in South Carolina, the second largest watershed in the continental United States that includes 1400 km^2 of riparian and estuarine habitats and 540 km^2 of protected estuarine habitat (SC DNR and NOAA, Coastal Services Center 2000). These undeveloped stretches of salt marshes are characterized by the presence of *S. alterniflora*, a prominent food source for *S. reticulatum* across its range (Haines and Montague 1979).

Handling and sampling protocol

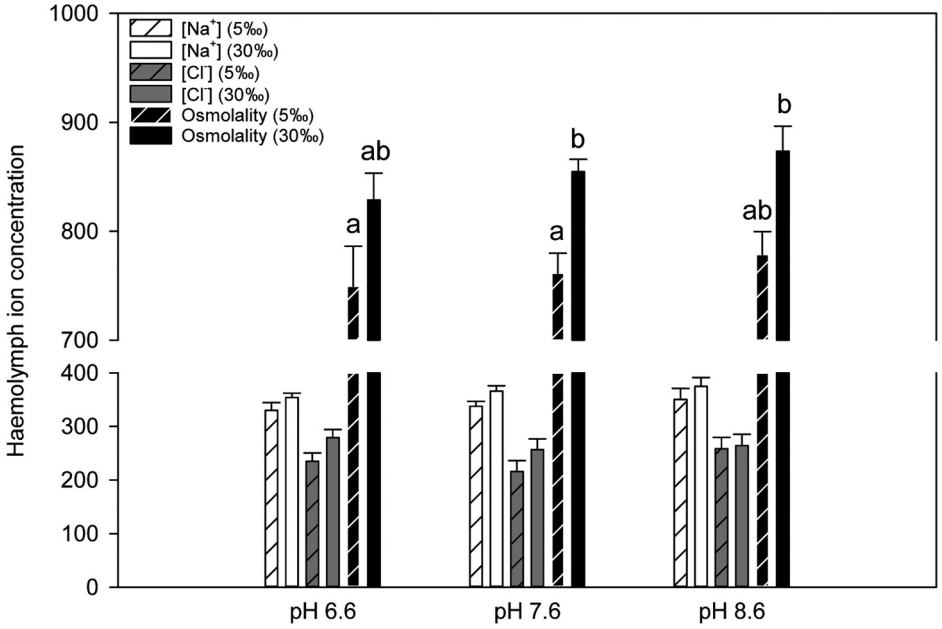
Immediately after collection, we placed *S. reticulatum* in individual porous holding vessels (0.35 L) to reduce direct handling, eliminate unwanted interactions between individuals, and allow for proper water mixing and exposure. We acclimatized *S. reticulatum* over 36 h to water conditions that were representative of the water at the site of collection (18 °C, pH 7.6, 30‰). Following acclimatization, we randomly assigned individuals to treatments (n = 18) where they were acutely exposed to one of six combinations of two salinity (5‰ and 30‰) and three pH (pH 6.6, 7.6, and 8.6) experimental water conditions. This range of salinities was chosen to represent salinities near the limits of the ACE River Basin according to water-quality data accessed through the National Estuarine Research Reserve System (NOAA NERRS 2017). Individuals were held under these conditions at 18 °C for 48 h. We created diluted salinity treatments from natural seawater using dechlorinated fresh water and manipulated pH to acidotic or basic conditions by the addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH), respectively. We checked pH hourly and maintained levels within 0.1 units of the target pH.

At the conclusion of the acute exposure, we euthanized crabs by destroying the dorsal ganglion and immediately sampled for haemolymph and gill. We drew hemolymph from the venus sinus by puncturing the arthrodial membrane between fourth and fifth pereiopods using a 22-gauge needle coated with a 10% sodium citrate anticoagulant. We removed all gill arches from the left side, flash-frozen haemolymph and gill samples in liquid nitrogen, and stored samples at –80 °C until analysis.

Haemolymph ion analysis

We measured all haemolymph ion concentrations in duplicate following manufacturer protocols: haemolymph [Cl^-] using a digital chloridometer (ChloroChek; Wescor, Inc., Logan, Utah, USA), haemolymph [Na^+] by emission flame photometry (Jenway PFP7; Bibby Scientific Ltd., Staffordshire, UK), and total haemolymph

Fig. 1. Haemolymph Na^+ (white bars; n = 8–12) and Cl^- (gray bars; n = 18–22) concentrations and total osmolality (black bars; n = 8–12) in the purple marsh crab (*Sesarma reticulatum*) after a 48 h exposure to varying combinations of pH and salinity conditions. Salinity is indicated by hatched (5‰) or solid (30‰) patterns. Data are presented as mean ± SE. Letters indicate differences in total osmolality across varying ambient conditions (two-way ANOVA and Tukey's post hoc analysis). There were no significant differences in [Na^+] or [Cl^-] between experimental conditions (Tukey's post hoc analysis).



osmolality using a vapor pressure osmometer (VAPRO 5600; Wescor, Inc., Logan, Utah, USA).

Tissue analysis

We prepared excised gill samples on ice in a ground glass manual homogenizer (#410; Will Scientific, Inc., Rochester, New York, USA) in 500 μL of a homogenizing medium (250 mmol·L^{-1} sucrose, 10 mmol·L^{-1} EDTA, 50 mmol·L^{-1} imidazol, 0.1% sodium deoxycholate; pH 7.5), then centrifuged at 4000*g* for 2 min. We spectrophotometrically (595 nm) measured protein content of the crude homogenates using the Bradford protein assay (BioRad Laboratories, Richmond, California, USA) with a bovine serum albumin standard.

We assayed NKA and VHA activity in duplicate by incubating 10 μL of homogenized gill in 300 μL of solution designed to either activate (assay solution A) or inhibit (assay solution B) the enzyme. Assay solution A contained 100 μmol·L^{-1} NaCl, 12.5 μmol·L^{-1} KCl, 5 μmol·L^{-1} $MgCl_2$, 0.1 μmol·L^{-1} EDTA, 15 μmol·L^{-1} imidazole, and 0.04 μmol·L^{-1} Na_2-ATP. Assay solution B was identical to solution A, except KCl was omitted and 1 mmol·L^{-1} of an enzyme-specific inhibitor (ouabain inhibits NKA, bafilomycin inhibits VHA) was added. After incubation at 18 °C for 30 min, we stopped the reaction by adding 500 μL of an ice-cold stopping solution (39.6 mL of 0.538 mol·L^{-1} HCl, 1.29 g of ascorbic acid, 2.13 mL of 10% ammonium molybdate, and 3.3 mL of 20% SDS). We spectrophotometrically (850 nm) measured inorganic phosphate (P_i) liberated via hydrolysis of ATP during incubation as a phosphomolybdate complex. We determined NKA- and VHA-specific ATPase activity by calculating the difference in P_i liberation between uninhibited and respective enzyme-inhibited solutions.

Statistical analysis

To test whether the survival of individuals was associated with the treatment exposure, we used a χ^2 analysis. To assess the main effects of salinity and pH and any potential interactions between the two, on [Na^+], [Cl^-], osmolality, and NKA and VHA activity as univariate response variables, we used two-way analysis of variance (ANOVA) testing. The data conformed to the equal variance assumption of ANOVA based on F_{max} tests. We ran Tukey's post hoc analyses to determine significant differences between treatments following significant ANOVA results. We conducted all statistical analyses using R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria; available from https://www.r-project.org/).

Results

Survival

We observed high survival rates (>99%) in all experimental conditions. Survival was not significantly different between treatments ($\chi^2_{[5]} = 5.035$, $P = 0.4116$). The single mortality occurred in the 30‰ salinity and pH 6.6 experimental treatment. Irrespective of the combination of pH and salinity stressors, crabs appeared to be fully responsive after the 48 h exposure with no visible or physiological signs of poor health.

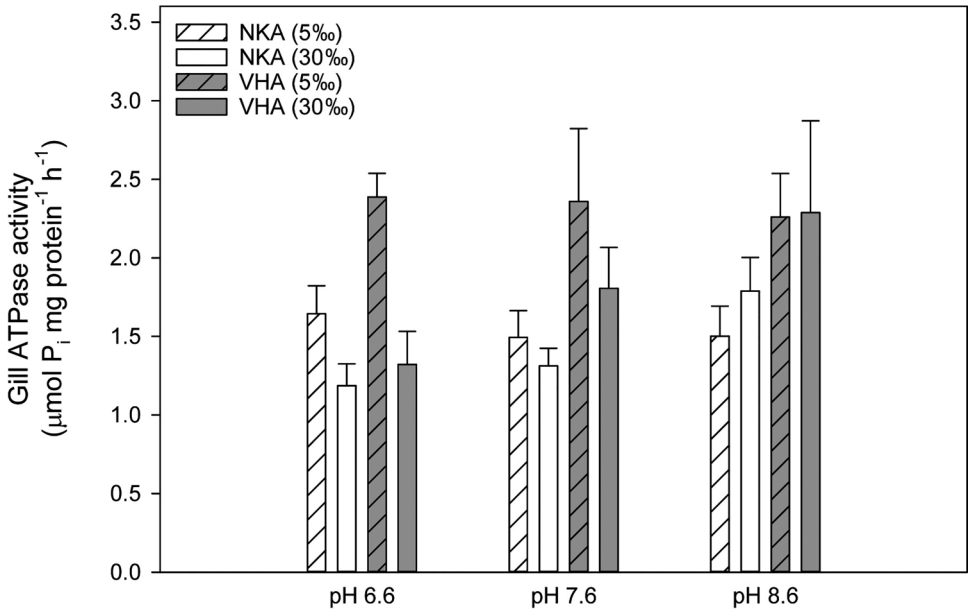
Haemolymph ions

Haemolymph [Na^+] was slightly (although not significantly) higher in all 30‰ salinity treatments compared with the 5‰ salinity treatments at the equivalent pH (Fig. 1). There was no significant effect on haemolymph [Na^+] by pH ($F_{[2,35]} = 1.02$, $P = 0.370$), salinity ($F_{[1,35]} = 3.99$, $P = 0.054$), or the interaction of pH and salinity ($F_{[2,35]} = 0.01$, $P = 0.986$). There was a significant effect of salinity on haemolymph [Cl^-] ($F_{[1,95]} = 4.77$, $P = 0.031$), with [Cl^-] trending higher in the higher salinity, but there was no significant effect of pH ($F_{[2,95]} = 0.91$, $P = 0.408$) or the interaction ($F_{[2,95]} = 0.73$, $P = 0.485$) (Fig. 1). Haemolymph osmolality was significantly increased by salinity ($F_{[1,40]} = 24.7$, $P < 0.001$) (Fig. 1). Osmolality trended higher at higher pH levels, but no significant effect on osmolality was observed due to differences in pH ($F_{[2,40]} = 1.23$, $P = 0.303$) or the interaction of pH and salinity ($F_{[2,40]} = 0.07$, $P = 0.933$).

Gill enzyme activity

Gill NKA activity remained relatively stable across experimental conditions, though it was significantly affected by the interaction of salinity and pH stressors ($F_{[2,98]} = 3.59$, $P = 0.031$) (Fig. 2). At pH 8.6, NKA activity was higher in the 30‰ salinity condition than

Fig. 2. Gill Na^+,K^+-ATPase activity (white bars; NKA; n = 16–18) and vacuolar-type H^+-ATPase activity (gray bars; VHA; n = 6–8) in the purple marsh crab (*Sesarma reticulatum*) after a 48 h exposure to varying combinations of pH and salinity conditions. Salinity is indicated by hatched (5‰) or solid (30‰) patterns. Data are presented as mean ± SE. There were no significant differences in NKA or VHA between experimental conditions (Tukey's post hoc analysis).



in the 5‰ salinity condition; at pH 6.6, NKA activity was higher in the 5‰ salinity condition than in the 30‰ salinity condition. There was no significant effect on NKA activity by pH ($F_{[2,98]}$ = 0.73, P = 0.484) or salinity ($F_{[1,98]}$ = 0.01, P = 0.928) alone. Gill VHA activity appeared to increase in the 5‰ salinity condition at each pH, but there was no significant effect of either pH ($F_{[2,19]}$ = 1.09, P = 0.355), salinity, ($F_{[2,19]}$ = 1.92, P = 0.182), or the interaction ($F_{[1,19]}$ = 0.94, P = 0.409).

Discussion

High survival rates and normal osmoregulatory function observed in this study indicate *S. reticulatum* is well adapted to tolerate simultaneous stressors of pH and salinity. The relatively small changes in haemolymph ion concentrations upon exposure to dilute seawater agree with haemolymph ion data obtained from single stressor salinity studies on *S. reticulatum* and other osmoregulating salt marsh crab species (Foskett 1977; Staton and Felder 1992; Tsai and Lin 2007). Had the additional challenge of changes in pH limited the ability of the crab to hyper-osmoregulate, then there may have been greater ion loss in the dilute salinity.

Gill NKA and VHA activity appeared relatively unchanged across salinity and pH conditions. Greater up- or down-regulation of enzyme activity may have been observed if the exposures to these experimental conditions were longer, but a 48 h exposure is already longer than expected from the ~12 h tidal cycle seen in the ACE River Basin. The significant interaction of salinity and pH on gill NKA activity is apparent by the opposite relationship between NKA activity in the 5‰ and 30‰ salinity conditions to decreasing ambient pH — as pH decreases, gill NKA activity increases in the 5‰ group and decreases in the 30‰ group. That the persistence of NKA activity was, however, within a normal range of regulatory response for estuarine crabs (Tsai and Lin 2007) across these environmental conditions, coupled with the relatively stable haemolymph ion concentrations, indicates uninterrupted osmoregulatory function and demonstrates adaptation to these simultaneous environmental stressors. Likewise, the increase in VHA activity upon exposure to 5‰ salinity is consistent with the established role of this enzyme in hyper-osmoregulation (i.e., Na^+ uptake) in crabs in dilute seawater (Tsai and Lin 2007). These ion and enzyme activity data suggest that even at the limits of hypothetical simultaneous salinity and pH challenges within an estuary, *S. reticulatum* appears to maintain normal osmoregulatory function.

Historically, biologists thought that major anthropogenic disturbances to estuaries only had a bottom-up origin (Teal 1962; Bertness and Ellison 1987; Bertness et al. 2008), wherein human-induced shifts in abiotic factors (increases in pollutants or nutrients, rising water levels, acidification due to elevated atmospheric CO_2, etc.) have been the drivers for the widespread loss of primary producers. However, such loss may also (or even more so) be attributed to a top-down imbalance, wherein the overharvesting of secondary consumers reduces the predation on primary consumers (Strong 1992; Silliman and Zieman 2001; Silliman and Bertness 2002; Bertness et al. 2014). This appears to be the case with the overharvesting of natural predators to the marsh crab *S. reticulatum*, resulting in increasingly uncontrolled primary consumption by these crabs (Bertness and Silliman 2008).

Up to this point, osmoregulatory studies on estuarine-adapted crabs have looked mainly at the effects of salinity alone. However, there is clear difference in ambient pH across salinities in an estuary. The natural salinity and pH of the water in the upper regions of an estuary (fresh water; pH 6.5–7.5) are both lower than in the lower regions of the estuary (highly buffered seawater; pH 7.5–8.5). If differences in pH limit the osmoregulatory ability of *S. reticulatum* as it moves throughout an estuary, then the range of *S. reticulatum* in the ACE River Basin as defined by its salinity tolerance may be currently overestimated. If so, then the potential geographical range of cordgrass consumption by *S. reticulatum* may also be overestimated. The results of this study, however, do not indicate that physiological interaction of salinity and pH on *S. reticulatum* osmoregulation is sufficient to limit survival, salinity tolerance, or pH tolerance. Although further ecological study of this system is necessary, these results indicate the possibility of an expansion, based on physiological tolerances, of the realized niche of *S. reticulatum*. An expansion of *S. reticulatum* could have potential negative consequences on cordgrass and subsequent shoreline erosion through increased consumption within this river basin.

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