Physiological and Behavioral Response of the Asian Shore Crab, *Hemigrapsus sanguineus*, to Salinity: Implications for Estuarine Distribution and Invasion

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Abstract

The invasive Asian shore crab, *Hemigrapsus sanguineus*, is ubiquitous in the rocky intertidal zone of the western ~~north~~ North Atlantic. A likely contributor to this colonization is that *H. sanguineus* is able to handle a wide range of salinities, and is thus more likely to spread through a greater geographic area of estuaries~~adequately defend shelter from competitors within the mid and upper intertidal zone~~. This study~~We~~ investigat~~used an effective approach~~ed the salinity effects on this animal ~~question~~ by observing survival across a range of salinities, ~~and also running trials to determine~~ the maintenance of hemolymph osmolality under different salinities, and behavioral preference for and avoidance of salinities. ~~We find that~~ *H. sanguineus* showed~~s~~ high survival across a broad range of salinities, ~~has~~ had little change in hemolymph osmolality over a short-term salinity shock, and behaviorally ~~distinguishes~~ distinguished between salinities when presented with a choice, ~~regardless o~~under both ~~f~~ acclimation salinities~~y~~ of 5 PSU or 35 PSU. Such results suggest *H. sanguineus* has a hardiness for the rapid changes in salinity that happen in the intertidal zone, yet has a capability ~~willingness~~ to physically move to a more optimal salinity. This enhances their competitiveness as an invader, particularly in survival of lower salinities that present ~~PSU~~

~~water~~ ~~strategies in ballast water~~challenges during high-precipitation events in rocky intertidal areas, and partially explains this species' dominance in this habitat type.

The invasive Asian shore crab, *Hemigrapsus sanguineus*, is a~~A~~ particularly successful invasive decapod crustacean species~~ is the invasive Asian shore crab, *Hemigrapsus sanguineus*,~~ which is now found in estuaries and open coasts in areas along the western N~~n~~orth Atlantic~~ coast~~ ~~(Lohrer et al., 2000; Brousseau et al., 2002)~~ and western Europe, displacing resident species (Lohrer et al., 2000; Brousseau et al., 2002; van den Brink, Wijnhoven & McLay, 2012; Landschoff et al., 2013; Gothland et al., 2013; Gothland et al., 2014). The species has become the most abundant crab in the rocky intertidal in New England ~~(McDermott, 1998; Lohrer & Whitlatch, 2002; Kraemer et al., 2007)~~ since it was first found in New Jersey in 1988 ~~(~~(McDermott, 1998; Williams & McDermott, 1990; Lohrer & Whitlatch, 2002; Kraemer et al., 2007; O'Connor, 2014)~~Williams & McDermott, 1990)~~. Previous work in this lab and by others investigated the behavioral response of the intertidal and subtidal community to this species' presence~~ (Hudson et al., 2016) to test the effect of this new member of the community on its neighbors~~(Epifanio, 2013; Hudson et al., 2016). Conspecific tolerance also enhances its success in overcoming resistance to invasion (Hobbs et al., 2017). However, beyond community interactions, this species’ broad salinity tolerance ~~in this species~~ could be contributing to its success ~~(Tsai & Lin, 2007)~~ as an invasive species and for the invasiveness of the genus more broadly (Tsai & Lin, 2007; Urzúa & Urbina, 2017), so this work aimed to evaluate survival and behavior of this invasive species with respect to salinity.

Salinity is of particular importance in the marine environment to delineate biotic zones in estuaries. Stable zones of physicochemical parameters in estuaries, particularly salinity, do not occur (Wolf et al., 2009), and organisms differ along a broad spectrum in their abilities to handle salinity changes, proposed to inhabit six biotic salinity classes for macroinvertebrates in estuaries (Wolf et al., 2009). Utilization of these six classes to classify community makeups ~~as~~ one moves up an estuary, combined with abiotic factors, can better inform salinity regimes in marshland streams and estuaries, and thus have use in determining invasibility (i.e., vulnerability to invasion) of the system by a particular species by incorporating a community component. This is similar to the biologically-based system with five overlapping zones defined for fishes and invertebrates by Bulger et al. (1993), in which zones include: 1) freshwater to 4 PSU; 2) 2 PSU to 14 PSU; 3) 11 PSU to 18 PSU; 4) 16 PSU to 27 PSU; and 5) 24 PSU to fully marine. Of these, *H. sanguineus* would likely reside in zones 4 and 5. This idea has been visited before (Kneib, 1984; Gunter, 1961~~;~~ ~~Kneib, 1984~~), so this paradigm may benefit management of resources in the context of risk for invasion by a particular species.

Invasive crab species from estuarine systems often have broader salinity tolerances to withstand rapid changes in salinity common in their native locales that are predictive for their ~~prime them for suc~~success in new systems (McGaw & Naylor, 1992b; Colnar & Landis, 2007; Fowler, Gerner & Sewell, 2011). Salinity tolerance and preference is clearly important in

Commented [KC1]: If not used/referred to later in the paper, could pare down or remove this information about different proposed “zones” of salinity tolerance determining invasiveness to intertidal areas, particularly in decapods, and sheds light on potential areas they can invade successfully. Other notable worldwide invasive decapod crustacean species that draw attention to this particular salinity tolerance character include the Chinese mitten crab, *Eriocheir sinensis*, which spends much of its adult life in freshwater, but its larvae require full-strength seawater to survive (Rudnick et al., 2005) and the Harris mud crab, *Rhithropanopeus harrisii*, native to eastern North America and found in salinities down to 0.1 PSU that is establishing itself in new areas (Reisser & Forward, 1991; Roche et al., 2009; Kotta & Ojaveer, 2012; Fowler et al., 2013). A potent worldwide intertidal/estuarine invader, the European green crab, *Carcinus maenas*, is a useful comparison for understanding this salinity tolerance concept, particularly since~~In fact,~~ much of the work to determine osmoregulation in crabs was initially performed on ~~the invasive European green crab, *Carcinus maenas*~~ that species (Towle & Kays, 1986; Cieluch et al., 2004). Crabs osmoregulate utilizing the posterior gill filaments (Koch, 1954; Burnett & Towle, 1990; Lucu & Towle, 2003), with far greater Na$^+$/K$^+$ ATPase transport proteins expressed in the posterior gill than in the anterior gill (Burnett & Towle, 1990; Koch, 1954). This transporter is well-established as implicated in osmoregulation in crabs and other crustacean species as well (Reviewed in ~~*Callinectes sapidus*, Neufeld et al., 1980; *Uca pugilator*, D'Orazio & Holliday, 1985; *Uca pugnax*, Holliday, 1985; *Pacifasticus leniusculus*, Henry & Wheatly, 1988; *Artemia salina*, Holliday et al., 1990; *Callinectes sapidus* and *Callinectes similis*, Piller et al., 1995; *Hemigrapsus nudus*, Corotto & Holliday, 1996; *Carcinus maenas*, Henry et al., 2002 [*Reviewed in*~~ Lucu & Towle 2003~~]~~; ~~*Scylla paramamosain, Macrophthalmus banzai, Macrophthalmus abbreviates, Uca lactea, Uca formosensis, Ocypode stimpsoni, Chasmagnathus convexus, Helice formosensis, Eriocheir sinensis, Hemigrapsus sanguineus, Hemigrapsus penicillatus, Perisesarma bidens, Chiromantes dehaani,*~~ Tsai & Lin, 2007). Utilizing the changes

Commented [KC2]: Since C. maenas is not referred to/used as a comparison later in the MS, I suggest changing or removing this wording

Commented [KC3]: Can this be re-worded for clarity?

in hemolymph osmolality as a result of this transporter's activity over time of exposure, along with behavior, can therefore be a useful determinant of the implications of salinity change in the whole animal.

~~Salinity stress for~~ *H. sanguineus* experiences salinity stress below~~seems to begin at~~ 15 PSU regardless of acclimation ~~seawater~~, indicated by increased heart rate and activity level (Depledge, 1984). The congener *Hemigrapsus crenulatus* shows increased oxygen consumption as salinity stress increases (as salinity decreases), strong hyper regulation at low salinities, with increases in regulatory capacity as crab size increases (Urzúa & Urbina, 2017). However, this species is easily exposed to this level of salinity during a freshwater event (i.e., rain, snow) in the intertidal zone. Tsai & Lin (2007) noticed little decrease in Na^+/K^+ ATPase activity in *H. sanguineus* between 5 PSU and 35 PSU treatments, while optima studies of congeners ~~*Hemigrapsus*~~ *H. crenulatus* (Urbina et al., 2010) and *Hemigrapsus takanoi* (Shinji et al., 2009) determined a 21 PSU optimum and 24.4 PSU optimum, respectively. Similarly, our previous initial gill work found no significant change in Na^+/K^+ ATPase activity in posterior gill of *H. sanguineus* when exposed to 35 PSU, 15 PSU, or 5 PSU seawater for 7 days, but did see a short-term increase in activity at 2 and 4 hours post-treatment for 15 PSU treatments (Hudson, 2011). Therefore, *H. sanguineus* has similar osmoregulatory ability with this transporter regardless of treatment, but may be able to increase its activity in the short term.

Commented [KC4]: Not clear what level of salinity this refers to (no particular levels are specified above)

Species' responses to gradients are particularly important in determining where they will fall within a physical range (Case & Taper, 2000), so a wider tolerance will mean a wider geographic footprint is possible. Specifically, salinity tolerance levels can greatly alter distribution of species along a coast (Teal, 1958; Barnes, 1967; Engel, 1977; Felder, 1978; Young, 1978; Young, 1979; Rabalais & Cameron, 1985; Hulathduwa et al., 2007; Fowler et al., 2011; DMH pers. obs.). Freshwater events are common in ~~spring and fall along the eastern coast of the United States, bu~~estuarine areas, and salinity can also change on an hourly timescale with the tides, meaning that organisms living there must rapidly manage these challenges behaviorally and/or physiologically. As change in salinity is one of the most common forms of stress in the intertidal zone, and several invasive crab species are known to be euryhaline (~~and therefore have the ability to osmoregulate well (~~Reisser & Forward, 1991; Henry et al., 2006; Roche et al., 2009; Fowler et al., 2011), there is value in ~~a need to~~ investigatinge the role of ~~how b~~broad salinity tolerances in understanding how physiological capacity is related to behavioral choice or avoidance in contributing to t~~ are implicated in t~~he invasiveness of a species, “the degree to which a species is able to reproduce, spread from its place of introduction, and establish in new locations” (as defined by Rejmánek, 201).

Commented [KC5]: Can this sentence be broken into 2 sentences?

To investigate this interaction between physiology and behavior, the work reported here includes physiological tolerance (i.e., maintenance of hemolymph ion concentration) and survival, but also incorporates the behavioral preference of the animal as an indicator of ~~true risk of exposur~~how well they can avoid risk~~e, mainly utilizing three methods: survival, maintenance of internal ion concentration, and behavioral choice of salinity~~. ~~With the apparent advantage of *H. sanguineus* in surviving stressful changes in salinity during ocean crossings, it may arrive with more founding members and be more likely to establish in new areas.~~ Since little change was detectable in gill physiology in previous work, ~~we took~~ a behavioral approach for this work ~~to look at~~investigated sublethal effects by quantifying behavioral avoidance and hemolymph osmolality change, along with investigating differences in overall survivorship over time. This tests the idea that *H. sanguineus* has an ability to tolerate wide salinity changes for a significant amount of time, and can also behaviorally avoid ~~maladaptive~~ stressful salinities at small spatial scales, as have other species (Teal, 1958; Lagerspetz & Mattila, 1961; Jansson, 1962; Thomas et al., 1981; Ameyaw-Akumfi & Naylor, 1987; McGaw & Naylor, 1992a; McGaw & Naylor, 1992b). ~~This~~

Commented [KC6]: Specify H. sanguineus

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Methods

Crabs were collected by hand off Avery Point in Groton, Connecticut, USA under Connecticut Department of Environmental Protection Scientific Collector's Permits # SC-06040 and # SC-09015. Adult c~~C~~rabs with carapace widths between 15 mm and 34 mm were acclimated for at least 14 days in holding tanks at 35 PSU before use.

Commented [KC8]: "Adult crabs with carapace widths between 15mm-34mm…"

Survival

A lab-based holding study was performed to evaluate the survival of *H. sanguineus* to a broad range of salinity treatments typical for euryhaline species. ~~Specifically, c~~Crabs were exposed to salinity treatments of 1 PSU, 5 PSU, 10 PSU, 15 PSU, ~~and~~ or 35 PSU ~~over~~ for 14 days, given the observed ability of ~~members of~~ the genus *Hemigrapsus* ~~genus~~ to tolerate low salinities for extended periods of time (McGaw, 2001; Tsai & Lin, 2007). ~~In addition to this typical range of salinities, a~~The ~~treatment of~~ 1 PSU treatment, in particular, was included to simulate the ~~use of~~ nearly freshwater surface conditions during precipitation events in estuaries and tide pools~~ballast in transoceanic shipping as an invasive species control measure~~. Specimens were kept in tanks at ~~room temperature (~~~20°C ~~)~~ that corresponded with spring and fall environmental conditions from the original capture location, Long Island Sound, including a 12-hour light/dark cycle. Crabs were held in groups, and cannibalism was accounted for as a cause of mortality if it occurred upon observation of mortality events, as were molt failures. Each salinity treatment consisted of 20 males and 20 females, which were fed with shrimp pellets every day to satiety. Crab survival was

Commented [KC9]: Immersed in/exposed to monitored daily over the course of the 14-day experimental trial where dead specimens were removed~~ upon confirmation~~.

H. sanguineus survivorship over time (i.e., the survival function) was evaluated using methods traditionally used in the context of longitudinal survival analyses. ~~In contrast to “cross-sectional” data, which yield numbers of alive and dead specimens at a specific point in time,~~L~~“l~~ongitudinal~~”~~ data provide information on the time animals either died or were last observed alive due to ongoing monitoring of survival (Cox & Oakes, 1984; Benoit et al., 2015). Such longitudinal data for *H. sanguineus* consist of records for each crab specimen, which include information about the occurrence and timing of an event as well as salinity treatment values and sex that might affect survival (i.e., covariates). Crabs that were still alive when last observed or at the end of the experiment were treated as “right-censored” observations, for which ~~where~~ their time of death wa~~i~~s unknown either because mortality did not occur or was not observed during the holding period ~~the event was not achieved or data collection was too short~~ (Singer & Willett, 2003).

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A set of non- and semi-parametric longitudinal analyses were first employed to ~~select and~~ evaluate the effect of salinity treatment and sex on ~~a parsimonious set of covariates that best describe~~ the *H. sanguineus* survival function. The semi-parametric Cox proportional-hazards regression model was initially used given its ability to simultaneously evaluate the additive effect of multiple covariates (Cox, 1972). Preliminary regression model results suggested that the survival function was only dependent upon salinity (Table S1). Consequently, the non-parametric Kaplan-Meier estimator of survival was used to preliminarily identify if each salinity treatment produced distinct survival functions (Kaplan & Meier, 1958; Cox & Oakes, 1984; Fig. S1). The Kaplan-Meier estimator follows the proportion of individuals alive as a function of time in the

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absence of censored observations and is well-suited for univariate analyses with multiple factor levels.

The Peto & Peto modification of the Gehan-Wilcoxon was then used to accept or reject the null hypothesis that there was no statistical difference between survival functions (Harrington & Fleming, 1982). Multiple pairwise comparisons using the Peto & Peto test with Benjamini-Hochberg corrections to adjust for significance value inflation were subsequently applied to determine if and which salinity-dependent survival functions were statistically distinct from one another. Salinity-dependent survival functions that failed to reject the null hypothesis were subsequently combined. Preliminary results indicated survival was only significantly different between the 1 PSU and the 10 PSU, 15 PSU, and 35 PSU treatments ($p < 0.01$), and also between 5 PSU and 35 PSU ($p < 0.05$) (Table S2). However, ~~due to inconsistent significance values between salinity treatments (Table S2, Figure S1),~~ no objective procedure could be performed to combine the survival functions with confidence given inconsistencies between pairwise comparison significance values (Table S2). For instance, while survival was not statistically different between the 1 PSU and 5 PSU as well as the 5 PSU and 10 PSU groups ($p>0.05$), they could not be combined since survival between the 1 PSU and 10 PSU groups was statistically significant ($p < 0.01$). Coarser salinity categories were therefore examined and presented for easier interpretation of results, specifically fresh (1 PSU), estuarine (5 – 15 PSU), and seawater (35 PSU) salinity groups.

All survival-related analyses were performed using the statistical computing software R (version 3.4.2; R Core Team, 2017) with added functionality from the associated package “survival” (version 2.38; Therneau, 2015) and “survminer” (version 0.4.0; Kassambara & Kosinski, 2017). Statistical significance was accepted at a level of $p < 0.05$.

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Salinity Preference

The behavioral preference of *H. sanguineus* to varying salinities was evaluated through a separate lab-based experimental trial with new specimens. This study utilized~~Since past literature primarily offered choice between only two salinities, (Teal, 1958; Lagerspetz & Mattila, 1961; Jansson, 1962; Thomas et al., 1981; Ameyaw-Akumfi & Naylor, 1987; McGaw & Naylor, 1992a, McGaw & Naylor, 1992b), we designed~~ an arena that contained two 10 cm x 10 cm chambers, each with a different salinity and bubbled with an airstone, connected by an above-water bridge to offer a binary choice, consistent with past studies (Teal, 1958; Lagerspetz & Mattila, 1961; Jansson, 1962; Thomas et al., 1981; Ameyaw-Akumfi & Naylor, 1987; McGaw & Naylor, 1992a, McGaw & Naylor, 1992b). *H. sanguineus* is a highly mobile crab that in initial trials actively ran back and forth between the chambers over the bridge, meaning that it was able to effectively sample the conditions of both chambers. Therefore, individual crabs could chose to either 1) stay in the initial chamber, 2) relocate to the second chamber by using the connecting bridge, or 3) remain on the bridge since these are intertidal crabs. Because this species exists in estuaries in the field and therefore along a broad salinity gradient, individual *H. sanguineus* were acclimated to either 5 PSU or 35 PSU for a period of ~~at least~~ 14 days prior to the experiment to test the effects of acclimation. Since these are poikilothermic animals, activity increases with temperature. As such, t~~T~~emperature effects on preference were quantified by acclimating specimens at either 10°C or 20°C at those same salinities to simulate seasonal water temperature differences and gauge the general capacity of the animals to behaviorally regulate during different seasons. An e~~E~~xtended acclimation time~~s~~ ~~up to~~of ~~two weeks~~ 14 days was~~ere~~ used to account for longer exposure to lower salinities further up an estuary~~-~~ and during freshwater influx events~~or in a ballast tank~~, unlike the

Commented [KC13]: Should probably say "preference for salinities" rather than "to" salinities.

Also in this sentence - the salinities of the chambers are specific/various but not "varying" rapid changes (i.e., ~6 hours) that occur in the littoral zone. Given acclimation conditions have been shown to modify preference behavior in other crustacean species (Hernández, 2006; Gross, 1957), we investigated the level of impact this original acclimation period had upon salinity preference. Subsequent tests were performed to determine whether specimens had any preference for a lower or higher salinity based upon the salinity and temperature during their acclimation period. Salinity comparisons were varied between pairwise choices between 5, 15, or~~and~~ 35 PSU for a period of 12 hours, with final location at 12 hours~~data~~ recorded~~analyzed~~, for 25 replicates for each sex and acclimation at two acclimation temperatures (total of ~100 per salinity comparison). Due to the initial high activity of this crab species, final location at 12 hours was considered the "chosen" condition.

Commented [KC14]: Thank you for clarifying methods above.

This highlighted sentence suggests that the behavioral trials will assay different acclimation periods, or that there will be different levels of behavior -

Can this be reworded to more simply state "we investigated whether acclimation conditions (temperature and salinity) affected salinity preference" or perhaps removed? The next sentence could be modified to just state "We determined whether specimens had any preference…"

Commented [KC15]: "Individual crabs were presented with…"

Behavioral choice of salinity data were analyzed for binary choice by Chi-Square test, and then the probability of leaving starting salinity was analyzed by one-way ANOVA for each of starting salinity, sex, acclimation salinity, and temperature. In order to test interactive effects ~~Interaction~~ between those four factors, multiple two-way ~~was tested by a multi-way~~ ANOVAs were completed ~~with the addition of multiple pairwise comparisons,~~ in the statistical computing software R (version 3.4.2; R Core Team, 2017).

Hemolymph Response to Salinity Change:

To quantify hemolymph osmolality response to salinity shock, crabs were acclimated to full-strength seawater salinity (32 PSU) for two weeks to normalize gene expression (Tsai & Lin, 2007), then 40 specimens were exposed for seven days (168 hours) to each of the following salinity treatments: 32 PSU (control), 17.5 PSU, 10 PSU, and 5 PSU. Five animals were collected after the

Commented [KC16]: 14 days (to be consistent with other subsections of the methods reporting

14-day acclimation period and used as the initial time point~~A shared "time zero" was used~~ for all treatments~~ from five animals collected after the two week acclimation~~. A salinity of 32 PSU was used for the full-strength seawater treatment and salinity of 17.5 PSU for the middle salinity treatment, along with low salinity exposures at 10 PSU and 5 PSU. Due to high mortality in the survival study for some of the lowest salinities (Fig~~s~~. 1, S1), the experiment was only run for 7 days. Crab hemolymph was sampled from 5 new animals at each post-exposure time points of 1, 2, 4, 8, 24, 48~~ and,~~ 72, and 168 hours~~, and at~~ (7 days~~ (168 hours~~) and frozen at -80°C. The early time points were chosen to compare with results for other crabs, as work in another euryhaline crab, *Callinectes sapidus*, supports little change in observed hemolymph osmolality values within 12 hours (Sommer & Mantel, 1988; Towle, 1997; Henry et al., 2002) of salinity shock. Hemolymph samples were taken with the use of a 21 gauge syringe inserted into the crab's branchial cavity, and stored at -80°C in 1.5 mL centrifuge tubes. Samples'~~ were quantified for~~ hemolymph osmolality was measured after removal from thaw, centrifuged for 1 minute at 10000 rpm, and run in duplicate on a Wescor 5100C vapor pressure osmometer. Samples were run in duplicate, with the average of the two taken as the value for that sample. These results were then analyzed by two-way ANOVA for effects of exposure time and treatment, along with interactive effects between the two. A repeated measures ANOVA would be inappropriate to analyze hemolymph data, as the individuals were sacrificed at each time point for a separate study of the upregulation of proteins in posterior gill tissue. Each time point was analyzed for differences between the four salinity treatments by a one-way ANOVA with Tukey post-hoc analysis. All statistics were completed in in R statistical computing software (version 3.4.2; R Core Team, 2017).

Commented [KC17]: Were these newly collected from the field to assess the background/field osmolality? How is this value included in analyses? If it is not analyzed, this can probably be omitted since all comparisons are made between treatments..

Commented [KC18]: These treatment groups are described already, so this sentence can be omitted

Results

Survival

The semi-parametric Cox proportional hazards regression model previously indicated that the *H. sanguineus* survival function was only dependent on salinity treatment with no effect from the sex covariate (Table S1). When survival data were grouped into broader salinity designations for ease of interpretation and applicability to representative scenarios, t~~T~~he non-parametric Kaplan-Meier estimator ~~suggested~~ indicated that *H. sanguineus* survival functions for the ~~differed between~~ the fresh, estuarine, and seawater salinity groups were distinct (Fig. 1), which was reaffirmed by the Peto & Peto test against all three survival functions (χ^2 = 26.8, d.f. = 2, p <<0.001). No effect of sex was seen on survival as previously mentioned (Table S1). Moreover, multiple pairwise comparisons between all broader salinity survival functions were statistically different, thereby confirming survival was distinct between groupings (Table S3). For instance, t~~All multiple pairwise comparisons between broad salinity survival functions failed to reject the null hypothesis and consequently remained separate from one another (Table S3).~~ here were significant survival differences between the 35 PSU and pooled 5 PSU/10 PSU/ 15 PSU treatments ($p < 0.05$), between 35 PSU and 1 PSU treatments ($p < 0.001$), and between 1 PSU and the pooled 5 PSU/10 PSU/ 15 PSU treatments ($p < 0.001$) (Table S3). So, it seems that while there is clearly a difference between the highest and lowest salinity treatments, the middle three salinity treatments have a moderate survival rate that is significantly different from both the upper and lower salinity treatments. Interestingly, survival did not differ significantly among the three salinity groups over the first 7 days of observation (p = 0.421).

Commented [CC19]: Dave; not entirely sure this is necessary since it was mentioned as preliminary information in the methods.

Commented [KC20]: Remove extra "the"

Commented [KC21]: This is stated above, so can be excluded here

Commented [KC22]: It would be appropriate (and helpful) to add here the directionality of these differences (i.e. that individuals in the highest salinity had the highest survival, and individuals in near freshwater had the lowest)

Salinity Preference

Behavioral preference experiments indicated a significant preference ($\chi^2 = 5.88$, d.f. = 1, $p < 0.05$, n = 75) of *H. sanguineus* for 35 PSU over 5 PSU seawater at 20°C regardless of acclimation (Fig. 2D), but no significant preference was exhibited when individuals were given a choice between 35 PSU and 15 PSU ($\chi^2 = 0.653$, d.f. = 1, $p > 0.05$, n = 75), nor for 5 PSU and 15 PSU PSU ($\chi^2 = 1.174$, d.f. = 1, $p > 0.05$, n = 69). This significance appears to come from two sources. Males at 20°C (Fig. 2B) showed a significant preference for 35 PSU over 5 PSU ($\chi^2 = 9.52$, d.f. = 1, $p < 0.01$, n = 42), and also have a significant difference in preference towards 35 PSU when first acclimated to 35 PSU ($\chi^2 = 7.2$, d.f. = 1, $p < 0.01$, n = 20). Crabs that were acclimated to 5 PSU prior to the experiment significantly chose 35 PSU over 5 PSU ($\chi^2 = 4.8$, d.f. = 1, $p < 0.05$, n = 30). Aside from these, there are no other ~~no~~ significant effects of acclimation on final salinity choice in this instance.

These data were also analyzed by whether the crab left the starting salinity in any experiments (5 PSU, 15 PSU, or 35 PSU). There was a significant effect of starting salinity on whether crabs where more or less likely to leave (one-way ANOVA, F = 32.55, d.f. = 2, $p << 0.001$, n = 635). Interactions between factors were not significant in the two-way ANOVAs used to determine interactive effects between acclimation salinity and starting salinity nor between acclimation salinity and sex (Tables S4 and S5, respectively), but the interaction between starting salinity and temperature was significant (New Table S6), and a trend exists for an interaction between sex and temperature (New Table S7). Crabs that started in 5 PSU (at both temperatures), whether for 5 PSU x 35 PSU or 5 PSU x 15 PSU experiments, were more likely to leave (move to the other salinity, escape, or move onto the ramp) that salinity (Tukey's post-hoc test, $\alpha = 0.05$, $p < 0.001$) than those which started in 15 PSU or 35 PSU (Fig. 3A), and crabs that started in 15 PSU were more likely to leave than those in 35 PSU (Tukey’s post-hoc test, $\alpha = 0.05$, $p < 0.01$). As the experiment was completed in both 10°C and 20°C (Fig. 3B), animals were 37.68% likely to leave a salinity at 20°C, whereas at 10°C it was 27.49% (one-way ANOVA, $F = 7.475$, d.f. = 1, $p < 0.01$, $n = 635$). Acclimation had a significant effect (Fig. 3C), with animals more likely to leave the starting salinity if they were acclimated to 35 PSU (one-way ANOVA, $F = 7.585$, d.f. = 1, $p < 0.01$, $n = 635$), but no interaction effect was found between acclimation salinity and starting salinity nor between acclimation salinity and the sex of the animal. There was an effect of sex of the animal on its own on the likelihood that an animal would leave the starting salinity (Fig. 3D), with males more likely to leave at 39.88% and females leaving 25.42% of the time (one-way ANOVA, $F = 15.26$, d.f. = 1, $p < 0.001$, $n = 635$).

Commented [KC23]: If I am following the results correctly, I believe this result was already stated at the beginning of this paragraph, so should be removed here.

Hemolymph Response to Salinity Change

There was a significant effect of salinity exposure to 32 PSU, 17.5 PSU, 10 PSU and 5 PSU seawater ($n = 160$ total) on hemolymph osmolality for *H. sanguineus* (Fig. 4) over the course of seven days, by two-way ANOVA, for time of exposure ($F = 4.6371$, d.f. = 7, $p < 0.001$), salinity treatment ($F = 12.0486$, d.f. = 3, $p \ll 0.001$), and interaction between time of exposure and treatment ($F = 2.9242$, d.f. = 21, $p < 0.001$). Time zero was left out of analysis since it was the same for all four treatments. Salinity treatments were quite variable in hemolymph osmolality under 8 hours of exposure, but did not significantly vary from one another for the 8-hour, 24-hour, and 48-hour treatments, as analyzed by Tukey multiple pairwise comparisons after a one-way ANOVA for each time point. At 72 hours, hemolymph osmolality was significantly higher (one-way ANOVA, $F = 7.055$, d.f. = 3, $p < 0.01$, $n = 20$) in the 32 PSU treatment than both the 5 PSU (Tukey’s post-hoc test, $\alpha = 0.05$, $p < 0.01$) and the 17.5 PSU (Tukey’s post-hoc test, $\alpha = 0.05$, $p <$

0.05) treatments. At 168 hours (7 days), hemolymph osmolality was significantly different across the four treatments (one-way ANOVA, F = 9.383, d.f. = 3, $p < 0.001$, n = 20) and the 5 PSU treatment was significantly lower in osmolality than all others (Tukey's post-hoc test, $\alpha = 0.05$, vs. 32 PSU $p < 0.01$, vs. 17.5 PSU $p < 0.01$, vs. 10 PSU $p < 0.01$).

Discussion

That survival declines for *H. sanguineus* over time for the 1 PSU treatment (Fig. 1)~~5 PSU and 1 PSU treatments (Fig. 1, Fig. S1)~~ is noteworthy, but even prolonged periods of ~~changeover of ballast water to purely~~ freshwater influx may not be effective in keeping ~~out~~ *H. sanguineus* from surviving to establish a population, since the lowest survival rate after two weeks ~~(ocean crossing time)~~ for these animals is still 65% at 1 PSU. Maintenance of internal hemolymph osmolality over 7 days (Fig. 4) by this species is consistent with its ability to survive during the survival trials. The point at which deaths began to occur more often, about day 5, is consistent with the significantly lower internal hemolymph osmolality for the 5 PSU treatment of the hemolymph data only after 7 days of exposure. Work with other euryhaline crabs, like *Callinectes sapidus*, supports that this ability to maintain hemolymph osmolality values within 12 hours (Sommer & Mantel, 1988; Towle, 1997; Henry et al., 2002) helps the animal deal with estuarine osmotic stress. Since the work with *H. sanguineus* here sees no change in hemolymph osmolality for 48 hours~~72 hours (three days)~~, this underscores the survival ability of this crab and therefore its ability to invade new continents. This finding adds to earlier work that merely indicated that stress is induced at 15 PSU seawater for *H. sanguineus* (Depledge, 1984).

Commented [CC24]: Dave; attempting to remove confusion by removing the 5 PSU group. At this point, we should be remain consistent with the three groups we used in the main results. This may conflict with some data you have a few sentences later in this paragraph.

If you disagree, feel free to remove.

Commented [KC25]: So that the reader is reminded that the survival trials and osmolality trials have slightly different salinities, you might consider inserting "in the 1 PSU treatment" here

However, survival ~~of~~ in a particular salinity is likely different from avoidance of suboptimal salinities. It maintains a functional amount of Na^+/K^+ ATPase (Tsai & Lin, 2007; Hudson, 2011) to help it navigate this constantly changing environment, and likely uses behavioral strategies to avoid suboptimal salinities. There could be a major difference with one of these physiological characters and the physiological characters of the previously dominant intertidal crab, *Carcinus maenas*, which still has a depressed hemolymph osmolality at 7 days (Siebers et al., 1982; Henry et al., 2006), that may have impacted its competitive interaction with *H. sanguineus* and facilitated the latter's introduction. As *H. sanguineus* maintains internal osmolality regardless of salinity treatment over short exposures, it may be more suited than competitors to the varying conditions of the intertidal zone. Therefore, it ~~and~~ may be able to behaviorally maintain its shelter against competitors that leave under suboptimal salinity conditions, much like its congener *Hemigrapsus nudus* (McGaw, 2001). In the littoral zone, a change in salinity can occur during each tidal cycle and during a period of prolonged precipitation or spring melting, allowing these species to maintain territory if they are not behaviorally affected.

Commented [KC26]: Probably "invasion" or spread should be used here since talking about interactions that are occurring after introduction and establishment.

In the behavioral salinity choice data, a true choice of salinity was a far lesser signal (Fig. 2), and less informative, than the analysis of crabs leaving the starting level of salinity (Fig. 3). The decrease in likelihood of leaving as salinity increased is expected for optimal behavioral moderation of osmotic stress, but even the level of 51.3% of crabs leaving 5 PSU after 12 hours still is far less than the tidal cycle. This means that a large portion of crabs would remain in intertidal areas affected by regular salinity changes. This is an important finding with respect to metabolic stressors, as crustaceans have to switch to other physiological mechanisms, notably ammonia excretion (Shinji et al., 2009; Weihrauch et al., 2004), in order to maintain hemolymph osmolality at low salinities. The implication of the fact that males are more likely to leave the original salinity than females, likely due to differences in overall activity level between the sexes (Fig. 3), is that males are more likely to behaviorally modify their location when salinity changes into areas that are more suitable, whereas females will experience greater osmotic stress. Additionally, increased frequency of crabs leaving the starting salinity with increases in temperature means that *H. sanguineus* will be more likely to behaviorally respond to stressful salinities at higher temperatures than at those present during winter months, perhaps resulting in some seasonal differences in osmotic stress and mortality. Although the species can strongly osmoregulate at other salinities, those individuals physically moving themselves closer to full-strength salinity are likely to have an energetic advantage since there is less of a need for the excretion of ammonia (Weihrauch, Morris & Towle, 2004). When those animals that are currently at this salinity optimum already, as is the case in animals acclimated to 35 PSU (Fig. 3C), are placed in one of the other salinities, they are more likely to attempt to maintain this optimum by leaving that stressful salinity. This is also evident by the lower frequency of crabs leaving the starting salinity if they are starting in 35 PSU. This energetics argument is clear from recent work done in the congener *H. crenulatus*, which showed decreasing oxygen consumption and decreasing ammonia excretion as salinity increased (Urzúa & Urbina, 2017).

~~It is likely that the~~ *H. sanguineus* still stays in stressful starting salinities of 5 PSU (48.7% of the time did not leave) and 15 PSU (69.1% of the time did not leave), indicating that ~~behavioral tolerance qualities of staying put the same amount at both 15 PSU and 35 PSU, allow~~ *H. sanguineus* should maintain territory by ~~to~~ withstanding fluctuations in salinity that happen with rain events that other species cannot, which merits further study (Lucu & Towle, 2003; Tsai & Lin, 2007). This ~~unlikelihood~~ low likelihood of moving under stressful salinities may mean that this is a common trait to the genus, like their congener *H. nudus* (McGaw, 2001), and could result in faster geographic expansion and increased invasiveness of multiple members of the [illegible] behavioral differences ~~is~~ may be part of what is responsible for the more subtidal ~~than intertidal~~ distribution of *C. maenas* ~~we~~ observed in ~~other~~ previous work (Hudson et al., 2016), not seen in the intertidal zone in areas where it does not overlap with *H. sanguineus* (Behrens Yamada & Gillespie, 2008; Amaral et al., 2009). ~~,~~D~~. Additionally, ~~ecreased desiccation of the smaller ~~size of~~ *H. sanguineus* ~~may make it able to~~ usinge ~~of~~ microhabitats in intertidal cobble fields ~~that cause it to experience lesser desiccation~~when compared to ~~than~~ the larger *C. maenas* (Altieri et al., 2010) may also have contributed to this intertidal dominance. This is also~~, as is~~ true for the mud crab *Eurypanopeus depressus* in intertidal oyster reefs (Grant & McDonald, 1979). ~~This is particularly evident in the large quantity of *C. maenas* found in the intertidal zone in areas where it does not overlap with *H. sanguineus* (Behrens Yamada & Gillespie, 2008; Amaral et al., 2009), whereas *H. sanguineus* does not seem to have high densities in the subtidal (Hudson et al., 2016).~~

Physiological responses will continue to be useful in models to predict future invasions and the likely finer-scale distributions and competitive interactions in a new environment (Kneib, 1984; Zacherl et al., 2003; Kimball et al., 2004; Rudnick et al., 2005; Herborg et al., 2007). Biological invasions continue worldwide with increasing human commerce (Pimentel et al., 2005) so predicting a species' probable impact by utilizing behavioral along with physiological characters synthesized with ecological and biogeographical theory will help facilitate our understanding of these processes. Behavior is becoming more prevalent as an explanation for invasives' success (Weis, 2010), and this study adds to our understanding of how this invader's distribution and use pattern arises from its physiology and behavior. Combination of large-scale physical models as done for *H. sanguineus* in the Gulf of Maine (Delaney et al., 2012) with small-scale estuarine behavior will offer a far higher resolution to spatial prediction. As invasions often gain a foothold on a small scale, the overall picture must include how the species in question interacts with these parameters on that scale, in order to more accurately predict invasion success.

Conclusions

As survival is high in this crab under low salinity conditions, ~~the effectiveness of ballast water exchange at mid-ocean or with freshwater would likely be ineffective. Additionally, seasonal~~ freshwater input into an estuary ~~, unless it causes particularly low salinity for a long period of time,~~ will probably not greatly affect survival of populations of this species. The findings here indicate an advantage of *H. sanguineus* in surviving stressful changes in salinity during those periods, so more founding members should survive and therefore be more likely to establish in areas where it is introduced. The level at which *H. sanguineus* maintains its internal hemolymph osmolality, along with its high survival rate in a broad salinity range in this study, highlight some of its particularly adept osmotic regulation characters. However, as the energetic demands of this animal do vary as temperature increases (Jungblut, 2017), it is important to investigate the interactive effect of seasonal salinity change on likely distribution.

The genus *Hemigrapsus* includes two prominent invaders in Europe and North America, as *H. takanoi* demonstrates a wide salinity tolerance (Shinji et al., 2009) and invaded Europe (originally misidentified as *H. penicillatus*) (Gollasch, 1999; Asakura & Watanabe, 2005) shortly followed by *H. sanguineus* (d'Udekem d'Acoz & Fasse, 2002). The combination of the effects of the behavioral dominance of *H. sanguineus* for shelter (Hudson et al., 2016) with its ability to withstand salinity changes give it a unique ability to maintain valuable intertidal shelter from predators and competitors during changes in tides and freshwater events. In the end, *H. sanguineus* is a hardy, effective invasive species with broad salinity tolerances and preferences, which provide it the opportunity to outlast competitors for shelter and food when exposed to maladaptive salinities~~, and allows it to survive ballast water changes~~.

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