

**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 investigated an effective approached 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 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 of~~ under both ~~f~~acclimation salinities 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 ~~is~~has a capable of ~~willingness to~~ physically moving to a more optimal salinity. This enhances their competitiveness as an invader, particularly ~~in~~surviving~~at~~ ~~of~~ lower salinities that

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

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11 ~~The invasive Asian shore crab, *Hemigrapsus sanguineus*, is a~~ particularly successful  
12 ~~invasive decapod crustacean species is the invasive Asian shore crab, *Hemigrapsus sanguineus*,~~  
13 ~~which is now found in estuaries and open coasts in areas along the western North Atlantic coast~~  
14 ~~(Lohrer et al., 2000; Brousseau et al., 2002)~~ and western Europe, ~~displacing resident species~~  
15 ~~(Lohrer et al., 2000; Brousseau et al., 2002; van den Brink, Wijnhoven & McLay, 2012;~~  
16 ~~Landschoff et al., 2013; Gothland et al., 2013; Gothland et al., 2014).~~ The species has become the  
17 most abundant crab in the ~~rocky~~ intertidal in New England ~~(McDermott, 1998; Lohrer &~~  
18 ~~Whitlatch, 2002; Kraemer et al., 2007)~~ since it was first found in New Jersey in 1988 ~~(McDermott,~~  
19 ~~1998; Williams & McDermott, 1990; Lohrer & Whitlatch, 2002; Kraemer et al., 2007; O'Connor,~~  
20 ~~2014)Williams & McDermott, 1990).~~ Previous work ~~in this lab and by others~~ investigated the  
21 behavioral response of the intertidal and subtidal community to this species' presence ~~(Hudson et~~  
22 ~~al., 2016) to test the effect of this new member of the community on its neighbors(Epifanio, 2013;~~  
23 ~~Hudson et al., 2016).~~ Conspecific tolerance also enhances its success in overcoming resistance to

1 invasion (Hobbs et al., 2017). However, beyond community interactions, this species' broad  
2 salinity tolerance ~~in this species~~ could be contributing to its success ~~(Tsai & Lin, 2007)~~ as an  
3 invasive species and for the invasiveness of the genus more broadly (Tsai & Lin, 2007; Urzúa  
4 & Urbina, 2017), so this work aimed to evaluate its survival and behavior ~~of this invasive species~~  
5 with respect to salinity.

6 Salinity is of particular importance in the marine environment to delineate biotic zones in  
7 estuaries. Stable zones of physicochemical parameters in estuaries, particularly salinity, do not  
8 occur (Wolf et al., 2009). O and organisms differ along a broad spectrum in their abilities to handle  
9 salinity changes, with fishes and macroinvertebrates (including crabs) proposed to inhabit five or  
10 six biotic salinity class ~~zones for macroinvertebrates in estuaries (Bulger et al., 1993; Wolf et al.,~~  
11 2009). Utilization of these six classes to classify community makeups as one moves up an estuary  
12 combined with abiotic factors can better inform salinity regimes in marshland streams and  
13 estuaries, and thus have use in determining invasibility (i.e., vulnerability to invasion) of the  
14 system by a particular species by incorporating a community component. This is similar to the  
15 biologically based system with five overlapping zones defined for fishes and invertebrates by  
16 Bulger et al. (1993), in which zones include: 1) freshwater to 4 PSU; 2) 2 PSU to 14 PSU; 3) 11  
17 PSU to 18 PSU; 4) 16 PSU to 27 PSU; and 5) 24 PSU to fully marine. Of these, *H. sanguineus*  
18 would likely reside in zones 4 and 5. This idea has been visited before ~~S~~ (Gunter, 1961; Kneib,  
19 1984), so this paradigm ~~salinity tolerance may benefit~~ could therefore be used in the management of  
20 resources in the context of locational risk for invasion by a particular species.

21 Invasive crab species from estuarine systems often have broader salinity tolerances to  
22 withstand rapid changes in salinity common in their native locales that are predictive for their  
23 ~~prime them for sue~~ success in new systems (McGaw & Naylor, 1992b; Colnar & Landis, 2007;

1 Fowler, Gerner & Sewell, 2011). Salinity tolerance and preference is clearly important in  
2 determining invasiveness to intertidal zones in estuaries, particularly in decapods, and sheds light  
3 on potential areas they can invade successfully. Other notable worldwide invasive decapod  
4 crustacean species that draw attention to this particular salinity tolerance character include the  
5 Chinese mitten crab, *Eriocheir sinensis*, which spends much of its adult life in freshwater, but its  
6 larvae require full-strength seawater to survive (Rudnick et al., 2005). ~~In addition, and~~ the Harris  
7 mud crab, *Rhithropanopeus harrisi*, native to eastern North America ~~and is~~ found in salinities  
8 down to 0.1 PSU ~~that is and establishing itself in new areas~~ (Reisser & Forward, 1991; Roche et  
9 al., 2009; Kotta & Ojaveer, 2012; Fowler et al., 2013). ~~My~~ In fact, much of the work to determine  
10 osmoregulation in crabs was initially performed on a –potent worldwide intertidal/estuarine  
11 invader, the European green crab, *Carcinus maenas* ~~the invasive European green crab, *Carcinus*~~  
12 ~~*maenas*~~ (Towle & Kays, 1986; Cieluch et al., 2004). Crabs osmoregulate utilizing the posterior  
13 gill filaments (Koch, 1954; Burnett & Towle, 1990; Lucu & Towle, 2003), with far greater Na<sup>+</sup>/K<sup>+</sup>  
14 ATPase transport proteins expressed in the posterior gill than in the anterior gill (Burnett & Towle,  
15 1990; Koch, 1954). This transporter's ~~role is implicated~~ in osmoregulation in crabs and other  
16 crustacean species ~~is well-established as well~~ (Reviewed in ~~*Callinectes sapidus*, Neufeld et al.,~~  
17 ~~1980; *Uca pugnator*, D'Orazio & Holliday, 1985; *Uca pugnax*, Holliday, 1985; *Pacifastacus*~~  
18 ~~*leniuseculus*, Henry & Wheatly, 1988; *Artemia salina*, Holliday et al., 1990; *Callinectes sapidus*~~  
19 ~~and *Callinectes similis*, Piller et al., 1995; *Hemigrapsus nudus*, Corotto & Holliday, 1996;~~  
20 ~~*Carcinus maenas*, Henry et al., 2002 [Reviewed in Lucu & Towle 2003]; *Squilla paramamosain*,~~  
21 ~~*Macrophthalmus banzai*, *Macrophthalmus abbreviatus*, *Uca lactea*, *Uca formosensis*, *Ocypode*~~  
22 ~~*stimpsoni*, *Chasmagnathus convexus*, *Helice formosensis*, *Eriocheir sinensis*, *Hemigrapsus*~~  
23 ~~*sanguineus*, *Hemigrapsus penicillatus*, *Perisesarma bidens*, *Chiromantes dehaani*,~~ Tsai & Lin,

2007). Utilizing the changes 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 salinities below 15 PSU~~y during a freshwater event (i.e., rain, snow) in the intertidal zone. Tsai & Lin (2007) noticed little decrease in  $\text{Na}^+/\text{K}^+$  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  $\text{Na}^+/\text{K}^+$  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-observe~~ 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.

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

pas.obs.) Freshwater events are common in spring and fall along the eastern coast of the United States, but estuaries are 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 eChange in salinity is one of the most common forms of stress in the intertidal zone in estuaries, and several invasive crab species are known to beuryhaline in response (and therefore have the ability to osmoregulate well (Reiser & Forward, 1991; Henry et al., 2006; Rocha et al., 2009; Fowler et al., 2011)). This underscores the value here of needing to investigate how broad salinity tolerances, understanding how physiological capacity is related to behavioral choice or avoidance, since both contribute to tare implicated in the invasiveness of a species, i.e., how well a species reproduces and extends its range from its introduction point and starts populations in new places (-(as defined by Rejmánek, 2011).

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 *H. sanguineus* as an indicator of the risk of exposure how well they can tolerate sublethal salinities. By testing the ability of *H. sanguineus* to tolerate sublethal salinities, we can begin to understand how well they can tolerate sublethal salinities. Salinity tolerance is a key 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 salinity tolerance character is clearly important in determining invasiveness, particularly in decapods, and sheds light on potential areas they can invade and environments they can alter. Particularly, this research area provides information on the effectiveness of ballast exchange methods, as this is an a major vector for invasive species introduction (Hulme, 2009).

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## Methods

~~Crabs~~ Adult crabs with carapace widths between 15 mm and 34 mm 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. ~~Crabs~~ Crabs were acclimated for at least 14 days in holding tanks at 35 PSU before use.

### Survival

A lab-based holding study was performed to evaluate the survival of *H. sanguineus* immersed into a broad range of salinity treatments typical for euryhaline species. ~~Specifically,~~ 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



1 satiety. Crab survival was monitored daily over the course of the 14-day experimental trial where  
2 dead specimens were removed ~~upon confirmation~~.

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

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

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

3 The Peto & Peto modification of the Gehan-Wilcoxon was then used to accept or reject the  
4 null hypothesis that there was no statistical difference between survival functions (Harrington &  
5 Fleming, 1982). Multiple pairwise comparisons using the Peto & Peto test with Benjamini-  
6 Hochberg corrections to adjust for significance value inflation were subsequently applied to  
7 determine if and which salinity-dependent survival functions were statistically distinct from one  
8 another. Salinity-dependent survival functions that failed to reject the null hypothesis were  
9 subsequently combined. Preliminary results indicated survival was only significantly different  
10 between the 1 PSU and the 10 PSU, 15 PSU, and 35 PSU treatments ( $p < 0.01$ ), and also between  
11 5 PSU and 35 PSU ( $p < 0.05$ ) (Table S2). However, due to inconsistent significance values between  
12 salinity treatments (Table S2, Figure S1), no objective procedure could be performed to combine  
13 the survival functions with confidence given inconsistencies between pairwise comparison  
14 significance values (Table S2). For instance, while survival was not statistically different between  
15 the 1 PSU and 5 PSU as well as the 5 PSU and 10 PSU groups ( $p > 0.05$ ), they could not be  
16 combined since survival between the 1 PSU and 10 PSU groups was statistically significant ( $p <$   
17 0.01). Coarser salinity categories were therefore examined and presented for easier interpretation  
18 of results, specifically fresh (1 PSU), estuarine (5 – 15 PSU), and seawater (35 PSU) salinity  
19 groups.

20 All survival-related analyses were performed using the statistical computing software R  
21 (version 3.4.2; R Core Team, 2017) with added functionality from the associated package  
22 “survival” (version 2.38; Therneau, 2015) and “survminer” (version 0.4.0; Kassambara &  
23 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 for specific~~ 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~~Temperature 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~~Extended acclimation times ~~up to of two weeks~~ 14 days ~~wasere~~ used to account for longer exposure to lower salinities further up an estuary- and during freshwater influx events ~~or in~~

1 ~~a ballast tank~~, unlike the rapid changes (i.e., ~6 hours) that occur in the littoral zone. Given acclimation  
2 conditions have been shown to modify preference behavior in other crustacean species  
3 (Hernández, 2006; Gross, 1957), we investigated whether acclimation conditions (temperature and  
4 salinity) affected the level of impact this original acclimation period had upon salinity preference.  
5 ~~We determined~~ Subsequent tests were performed to determine whether specimens had any  
6 preference for a lower or higher salinity based upon the salinity and temperature during their  
7 acclimation period. ~~Individual crabs were presented with~~ Salinity comparisons were varied  
8 ~~between pairwise choices between~~ 5, 15, ~~and~~ 35 PSU for a period of 12 hours, with final location  
9 ~~at 12 hours~~ data recorded analyzed, for 25 replicates for each sex and acclimation at two acclimation  
10 temperatures (total of ~100 per salinity comparison). Due to the initial high activity of this crab  
11 species, final location at 12 hours was considered the “chosen” condition.

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

#### 20 Hemolymph Response to Salinity Change:

21 To quantify hemolymph osmolality response to salinity shock, crabs were acclimated to  
22 full-strength seawater salinity (32 PSU) for ~~14 days~~ two weeks to normalize gene expression (Tsai  
23 & Lin, 2007), then 40 specimens were exposed for seven days (168 hours) to each of the following

1 salinity treatments: 32 PSU (control), 17.5 PSU, 10 PSU, and 5 PSU. Five animals were taken out  
2 of the 32 PSU seawater after the 14-day acclimation period and used as the initial time point. A  
3 shared “time zero” was used for all treatments from five animals collected after the two-week  
4 acclimation. A salinity of 32 PSU was used for the full-strength seawater treatment and salinity of  
5 17.5 PSU for the middle salinity treatment, along with low salinity exposures at 10 PSU and 5  
6 PSU. Due to high mortality in the survival study for some of the lowest salinities (Figs. 1, S1), the  
7 experiment was only run for 7 days. Crab hemolymph was sampled from 5 new animals at each  
8 post-exposure time points of 1, 2, 4, 8, 24, 48 ~~and~~, 72, and 168 hours, ~~and at 7 days (168 hours)~~  
9 and frozen at -80 °C. The early time points were chosen to compare with results for other crabs,  
10 as work in another euryhaline crab, *Callinectes sapidus*, supports little change in observed  
11 hemolymph osmolality values within 12 hours (Sommer & Mantel, 1988; Towle, 1997; Henry et  
12 al., 2002) of salinity shock. Hemolymph samples were taken with the use of a 21 gauge syringe  
13 inserted into the crab’s branchial cavity, and stored at -80 °C in 1.5 mL centrifuge tubes. Samples °  
14 ~~were quantified for~~ hemolymph osmolality was measured after removal from thaw, centrifuged  
15 for 1 minute at 10,000 rpm, and run in duplicate on a Wescor 5100C vapor pressure osmometer.  
16 Samples were run in duplicate, with the average of the two taken as the value for that sample.  
17 These results were then analyzed by two-way ANOVA for effects of exposure time and treatment,  
18 along with interactive effects between the two. A repeated measures ANOVA would be  
19 inappropriate to analyze hemolymph data, as the individuals were sacrificed at each time point for  
20 a separate study of the upregulation of proteins in posterior gill tissue. Each time point was  
21 analyzed for differences between the four salinity treatments by a one-way ANOVA with Tukey  
22 post-hoc analysis. Time zero was left out of analysis since it was the same for all four treatments.

1 All statistics were completed in ~~in~~ R statistical computing software (version 3.4.2; R Core Team,  
2 2017).

## 3 4 5 **Results**

### 6 7 Survival

8 The semi-parametric Cox proportional hazards regression model indicated that the *H.*  
9 *sanguineus* survival function was only dependent on salinity treatment with no effect from the sex  
10 covariate (Table S1). When survival data were grouped into broader salinity designations for ease  
11 of interpretation and applicability to representative scenarios, (The non-parametric Kaplan-Meier  
12 estimator ~~suggested~~ indicated that *H. sanguineus* survival functions for ~~differed between~~ the fresh,  
13 estuarine, and seawater salinity groups were distinct (Fig. 1), which was reaffirmed by the Peto &  
14 Peto test against all three survival functions ( $\chi^2 = 26.8$ , d.f. = 2,  $p < 0.001$ ). ~~No effect of sex was~~  
15 seen on survival as previously mentioned (Table S1). Moreover, multiple pairwise comparisons  
16 between all broader salinity survival functions were statistically different, thereby confirming  
17 survival was distinct between groupings (Table S3). For instance, ~~All multiple pairwise~~  
18 comparisons between broad salinity survival functions failed to reject the null hypothesis and  
19 consequently remained separate from one another (Table S3). ~~he~~ difference in survival between  
20 the 35 PSU (highest survival) and pooled 5 PSU/10 PSU/15 PSU treatments (high survival but  
21 some mortality) was significant ( $p < 0.05$ ). There were also significant survival differences  
22 between the 35 PSU treatment and the 1 PSU treatment (lowest survival rate) ( $p < 0.001$ ), and  
23 between 1 PSU (lowest survival) and the pooled 5 PSU/10 PSU/ 15 PSU treatments (high survival

**Commented [CC5]:** Dave; not entirely sure this is necessary since it was mentioned as preliminary information in the methods.

1 but some mortality) ( $p < 0.001$ ) (Table S3). So, it seems that while there is clearly a difference  
2 between the highest and lowest salinity treatments, the middle three salinity treatments have a  
3 moderate survival rate that is significantly different from both the upper and lower salinity  
4 treatments. Interestingly, survival did not differ significantly among the three salinity groups over  
5 the first 7 days of observation ( $p = 0.421$ ).

#### 6 7 Salinity Preference

8 Behavioral preference experiments indicated a significant preference ( $\chi^2 = 5.88$ , d.f. = 1,  $p$   
9  $< 0.05$ ,  $n = 75$ ) of *H. sanguineus* for 35 PSU over 5 PSU seawater at 20 °C regardless of acclimation  
10 (Fig. 2D), but no significant preference was exhibited when individuals were given a choice  
11 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  
12 PSU ( $\chi^2 = 1.174$ , d.f. = 1,  $p > 0.05$ ,  $n = 69$ ). This significance appears to come from two sources.  
13 Males at 20 °C (Fig. 2B) showed a significant preference for 35 PSU over 5 PSU ( $\chi^2 = 9.52$ , d.f. =  
14 1,  $p < 0.01$ ,  $n = 42$ ), and also ~~have had~~ a significant difference in preference towards 35 PSU when  
15 first acclimated to 35 PSU ( $\chi^2 = 7.2$ , d.f. = 1,  $p < 0.01$ ,  $n = 20$ ). Crabs that were acclimated to 5  
16 PSU prior to the experiment ~~significantly~~ chose 35 PSU over 5 PSU ( $\chi^2 = 4.8$ , d.f. = 1,  $p < 0.05$ ,  $n$   
17 = 30). Aside from these, there are no other ~~no~~ significant effects of acclimation on final salinity  
18 choice ~~in this instance~~.

19 These data were also analyzed by whether the crab left the starting salinity in any  
20 experiments (5 PSU, 15 PSU, or 35 PSU). There was a significant effect of starting salinity on  
21 whether crabs ~~w~~ere more or less likely to leave (one-way ANOVA,  $F = 32.55$ , d.f. = 2,  $p < <$   
22 0.001,  $n = 635$ ). Interactions between factors were not significant in the two-way ANOVAs used  
23 to determine interactive effects between acclimation salinity and starting salinity nor between

1 acclimation salinity and sex (Tables S4 and S5, respectively), but the interaction between starting  
2 salinity and temperature was significant (Table S6), and a trend exists for an interaction between  
3 sex and temperature (Table S7). Crabs that started in 5 PSU (at both temperatures), whether for 5  
4 PSU x 35 PSU or 5 PSU x 15 PSU experiments, were more likely to leave that salinity (move to  
5 the other salinity, escape, or move onto the ramp) ~~that salinity~~ (Tukey's post-hoc test,  $\alpha = 0.05$ ,  $p$   
6  $< 0.001$ ) than those which started in 15 PSU or 35 PSU (Fig. 3A). Additionally, and crabs that  
7 started in 15 PSU were more likely to leave than those in 35 PSU (Tukey's post-hoc test,  $\alpha = 0.05$ ,  
8  $p < 0.01$ ). As the experiment was completed in both 10 °C and 20 °C (Fig. 3B), animals were  
9 37.68% likely to leave a salinity at 20 °C, whereas at 10 °C it was 27.49% (one-way ANOVA,  
10  $F = 7.475$ , d.f. = 1,  $p < 0.01$ ,  $n = 635$ ). Acclimation had a significant effect (Fig. 3C), with animals  
11 more likely to leave the starting salinity if they were acclimated to 35 PSU (one-way ANOVA,  $F$   
12  $= 7.585$ , d.f. = 1,  $p < 0.01$ ,  $n = 635$ ), ~~but no interaction effect was found between acclimation~~  
13 ~~salinity and starting salinity nor between acclimation salinity and the sex of the animal.~~ There was  
14 an effect of sex ~~of the animal on its own~~ on the likelihood that an animal would leave the starting  
15 salinity (Fig. 3D), with males more likely to leave at 39.88% and females leaving 25.42% of the  
16 time (one-way ANOVA,  $F = 15.26$ , d.f. = 1,  $p < 0.001$ ,  $n = 635$ ).

#### 18 Hemolymph Response to Salinity Change

19 There was a significant effect of salinity exposure ~~to 32 PSU, 17.5 PSU, 10 PSU and 5~~  
20 ~~PSU seawater~~ ( $n = 160$  total) on hemolymph osmolality for *H. sanguineus* (Fig. 4) over the course  
21 of seven days, ~~by two-way ANOVA, for time of exposure~~ ( $F = 4.6371$ , d.f. = 7,  $p < 0.001$ ),  
22 depending on salinity treatment ( $F = 12.0486$ , d.f. = 3,  $p < 0.001$ ), and interaction between time  
23 of exposure and treatment ( $F = 2.9242$ , d.f. = 21,  $p < 0.001$ ). ~~Time zero was left out of analysis~~



1 ~~since it was the same for all four treatments.~~ Salinity treatments were quite variable in hemolymph osmolality under 8 hours of exposure, but  
2 ~~this significantly varied from mean of 18 m, 24 m, and 8 m, and 1 m, analyzed by Tukey's post-hoc test, one-way ANOVA, and t-test.~~ At  
3 72 hours, hemolymph osmolality was significantly higher (one-way ANOVA,  $F = 7.055$ , d.f. = 3,  
4  $p < 0.01$ ,  $n = 20$ ) in the 32 PSU treatment than both the 5 PSU (Tukey's post-hoc test,  $\alpha = 0.05$ ,  $p$   
5  $< 0.01$ ) and the 17.5 PSU (Tukey's post-hoc test,  $\alpha = 0.05$ ,  $p < 0.05$ ) treatments. At 168 hours (7  
6 days), hemolymph osmolality was significantly different across the four treatments (one-way  
7 ANOVA,  $F = 9.383$ , d.f. = 3,  $p < 0.001$ ,  $n = 20$ ) and the 5 PSU treatment was significantly lower  
8 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  $p < 0.01$ .~~)

## 11 Discussion

12 That survival declines for *H. sanguineus* over time for ~~the 1 PSU treatment (Fig. 1)~~ ~~5 PSU~~  
13 ~~and 1 PSU treatments (Fig. 1, Fig. S1)~~ is noteworthy, but even prolonged periods of changeover  
14 ~~of ballast water to purely~~ freshwater influx may not be effective in keeping ~~out~~ *H. sanguineus* from  
15 surviving to establish a population, since the lowest survival rate after two weeks ~~(ocean crossing~~  
16 ~~time)~~ for these animals is still 65% at 1 PSU. Maintenance of internal hemolymph osmolality over  
17 7 days (Fig. 4) by this species is consistent with its ability to survive ~~during the survival trials~~. The  
18 point at which ~~deaths-mortality~~ began to ~~occur more often~~ increase in the 1 PSU treatment, ~~(~about~~  
19 ~~day 5)~~, is consistent with the significantly lower internal hemolymph osmolality for the 5 PSU  
20 treatment of the hemolymph data only after 7 days of exposure. Work with other euryhaline crabs,  
21 like *Callinectes sapidus*, supports that this ability to maintain hemolymph osmolality ~~values~~ within  
22 12 hours (Sommer & Mantel, 1988; Towle, 1997; Henry et al., 2002) helps the animal deal with  
23 estuarine osmotic stress. ~~Since the work with~~ This study of *H. sanguineus* ~~here sees~~ observed no

**Commented [CC6]:** 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.

1 change in hemolymph osmolality for ~~48 hours~~ 72 hours (three days), this ~~underscores~~ underscores the survival ability of this crab and  
2 therefore its ability to invade new ~~environments~~ areas. This finding adds to earlier work ~~which that~~ which merely indicated  
3 that stress is induced at 15 PSU seawater for *H. sanguineus* (Depledge, 1984).

4 However, survival ~~of in~~ in a particular salinity is likely different from avoidance of suboptimal  
5 salinities. *H. sanguineus* ~~it~~ individuals maintains a functional amount of Na<sup>+</sup>/K<sup>+</sup> ATPase (Tsai & Lin,  
6 2007; Hudson, 2011) to help ~~them~~ them navigate this constantly changing environment, and likely uses  
7 behavioral strategies to avoid suboptimal salinities. There could be a major difference with one of  
8 these physiological characters and the physiological characters of the previously dominant  
9 intertidal crab, *Carcinus maenas*, which still has a depressed hemolymph osmolality at 7 days in  
10 low salinity (Siebers et al., 1982; Henry et al., 2006), that may have impacted its competitive  
11 interaction with *H. sanguineus* and facilitated the latter's ~~invasion~~ invasion. As *H. sanguineus* maintains  
12 internal osmolality regardless of salinity treatment over short exposures, it may be more suited  
13 than competitors to the varying conditions of the intertidal zone. Therefore, it ~~and~~ may be able to  
14 behaviorally maintain its shelter against competitors that leave under suboptimal salinity  
15 conditions, much like its congener *Hemigrapsus nudus* (McGaw, 2001). In the littoral zone, a  
16 change in salinity can occur during each tidal cycle and during a period of prolonged precipitation  
17 or spring melting, allowing these species to maintain territory if they are not behaviorally affected.

18 In the behavioral salinity choice data, a true choice of salinity was a far lesser signal (Fig.  
19 2), and less informative, than the analysis of crabs leaving the starting level of salinity (Fig. 3).  
20 The decrease in likelihood of leaving as salinity increased is expected for optimal behavioral  
21 moderation of osmotic stress, but even the level of 51.3% of crabs leaving 5 PSU after 12 hours  
22 ~~still~~ is far less than the tidal cycle. This means that a large portion of crabs would remain in  
23 intertidal areas affected by regular salinity changes. This is an important finding with respect to

1 metabolic stressors, as crustaceans have to switch to other physiological mechanisms, notably  
2 ammonia excretion (Shinji et al., 2009; Weihrauch et al., 2004), in order to maintain hemolymph  
3 osmolality at low salinities. The implication of males being more likely to leave the original  
4 salinity than females, likely due to differences in overall activity level between the sexes (Fig. 3),  
5 is that males are more likely to relocate into areas that are more suitable when salinity changes,  
6 whereas females will experience greater osmotic stress. Additionally, increased frequency of crabs  
7 leaving the starting salinity with increases in temperature means that *H. sanguineus* will be more  
8 likely to behaviorally respond to stressful salinities at higher temperatures than at those present  
9 during winter months, perhaps resulting in some seasonal differences in osmotic stress and  
10 mortality. Although the species can strongly osmoregulate at other salinities, those individuals  
11 relocating to full-strength salinity are likely to have an energetic advantage since there is less of a  
12 need for the excretion of ammonia (Weihrauch, Morris & Towle, 2004). This is also evident by  
13 the lower frequency of crabs leaving the starting salinity if they are starting in 35 PSU. This  
14 energetics argument is clear from recent work done in the congener *H. crenulatus*, which showed  
15 decreasing oxygen consumption and decreasing ammonia excretion as salinity increased (Urzúa  
16 & Urbina, 2017).

17 ~~It is likely that the~~ A large proportion of *H. sanguineus* individuals stayed in stressful  
18 starting salinities of 5 PSU (48.7% of the time did not leave) and 15 PSU (69.1% of the time did  
19 not leave), indicating that behavioral tolerance qualities of staying put the same amount at both 15  
20 PSU and 35 PSU, allow *H. sanguineus* should maintain territory by to-withstanding fluctuations  
21 in salinity that happen with rain events that compared to other species. Such an inter-species  
22 comparison cannot, which merits further study (Lucu & Towle, 2003; Tsai & Lin, 2007). This  
23 The unlikelyhood-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 meaning that other *Hemigrapsus* genus by decreasing species could be successful invaders as well as this probably allows for these crabs to maintain higher quality shelters at low tide while other species move vertically with the changing tides. When these other species migrate into the intertidal to feed at high tide, there are fewer shelters available, and thus more exposure to predators (Jones & Shulman, 2008) and, at low tide, avian and terrestrial predators. These 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 estuarine 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 using 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 invasion<sup>2</sup> 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 the local~~ 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 ~~its~~some of its ~~particularly adept osmotic-regulatory~~ characters. However, as the energetic demands of this animal ~~do vary~~become more variable as temperature increases (Jungblut, 2017), it is important to investigate the interactive effect of seasonal salinity change on likely distribution.

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

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