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ARTICLE

Mitochondrial Haplotypes suggest Genetic Component for Habitat Preference in Blue Crabs

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ABSTRACT

Atlantic blue crabs (*Callinectes sapidus*) are ecologically and commercially fundamental. Life stages are punctuated with migration. Adults and juveniles live in estuaries and sounds. Larval stages develop in the coastal ocean. Juvenile and adult crabs occupy habitats from high salinities to fresh water. We determined whether maturing juvenile and adult blue crab habitat use is reflected in mitochondrial cytochrome oxidase 1 haplotypes. High salinity crabs had lower haplotype diversity (0.7260 ± .03900) compared to spawning crabs (0.9841 ± .00021) and low salinity crabs (0.94154 ± .00118). Significant pairwise differences in haplotypes were found between high salinity and spawning crabs ($Nm = 0.26018$, $p < 0.001$), and between high salinity and low salinity crabs ($Nm = 0.19482$, $p < 0.001$) indicating a lack of gene flow. Crabs from high salinity had highly significant genetic differentiation compared to spawning crabs ($Fst = 0.11830$, $p < 0.001$) and low salinity crabs ($Fst = 0.09689$, $p < 0.001$). Results support the hypothesis that genetics influence habitat selection. Crab larvae mix in the coastal ocean but occupy specific habitats upon return to sounds and estuaries. These findings have implications for the management of fisheries.

1. Introduction

The Atlantic blue crab (*Callinectes sapidus*) is an ecologically and commercially fundamental species whose life cycle is punctuated with migrations [1]. Juveniles and adults reside throughout estuaries. Mature females store sperm and migrate to higher salinity waters [2-5] to extrude and brood multiple clutches of eggs [6-8]. When eggs hatch around the time of the nocturnal high tide, they release zoea in sounds, mouths of estuaries, and the coastal ocean [9-11]. Zoeal behavior delivers them to the coastal ocean [12] where they spend 4 to 7 weeks [13] or more, while mixing with other larvae, molting multiple times, and metamorphosing to megalopa. Megalopal behavior carries them from the coastal ocean back into sounds and estuaries [14-16].

The presence of odors of vegetation and macroalgae in sounds and estuaries initiates metamorphosis in megalopa. Seagrass and oyster beds serve as critical, structural habitats for juvenile crabs [7,8,17-19]. As they maneuver closer to the mouths of estuaries, environmental cues influence crabs to move upward during nocturnal flood tides and rest near the bottom during other phases allowing for selective tidal-stream transport [8,20,21]. Blue crabs have an expansive range of nursery habitats to select from during their migration inland [1,22]. Most juvenile crabs

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migrate further up estuary [11]. However, some crabs remain throughout sounds and estuaries, including near the mouths of inlets [4,6,,21,23,24].

The physical, chemical, and biological characteristics of estuarine habitats vary greatly. Blue crabs can select from a variety of habitats displaying a wide range of environmental conditions [20]. Crab movement behavior is influenced by environmental cues and ultimately determines where crabs live. Juvenile stage and adult crabs move with the flow [25]. This means that crabs can occupy any habitat where flows go in two directions. In North Carolina, final habitat selection can vary from ~0 to > 35 PSU. Habitat at 0 PSU is ecologically and physiologically different than habitat at 35 PSU. Blue crab juveniles and adults are capable of hyper- and hypo-osmotic regulation [13]. Since genetic diversity within blue crab populations is known to be very high, habitat selection may help explain some of this genetic variation [26,27]. Crabs in our hypothetical scenario would migrate to habitats that suited their genetic make-up. Here we use salinity as a proxy for the differences in habitats.

We chose to use the mitochondrial-encoded cytochrome oxidase I (COI) to compare genetic diversity. Mitochondrial markers are often used to examine population origins and interrelatedness due to their maternal inheritance, rapid rate of evolution, and low rate of recombination [28]. Using COI, Darden reported reduced gene flow along the western Gulf of Mexico [29]. COI was also used to evaluate the variation and genetic structuring of blue crab populations on the east coast of the US [24,30]. These authors reported minimal geographic structuring and provided evidence that there was a dramatic increase in the population after the last ice age.

Blue crabs have a high level of diversity in COI haplotypes [26]. The only concern with using COI is the abnormally high levels of heteroplasmy that have been found in blue crabs [25]. Since there is routinely a dominant mtDNA type, the COI sequences can still be used for studies like the one proposed here [25]. We compared crabs resident in two habitats with differing salinity levels as well as spawning (aka. migratory) crabs representing the entire estuary.

2. Materials and Methods

2.1 Data Collection

Sampling of high salinity crabs was conducted at low tide on Bird Shoal (~29-35 PSU), part of the Rachel Carson Reserve in Beaufort, North Carolina. Only late stage juvenile crabs that were preterminal molt, and mating female-male pairs were used, because they were known to have grown up at high salinity [21,22]. Pressure was applied near the merus-basis joint of the fourth leg on the right side of each crab to trigger limb autotomy. After sampling, crabs were returned to the environment. If a crab was already missing its fourth leg on the right side, it was assumed that it had already been sampled. Each leg was stored individually with 95% ethanol. Tissue was removed from each crab leg for DNA extraction.

We used data collected by the same methods described in this section for samples from 26 female crabs that had grown up in Lake Mattamuskeet (~0-3 PSU) and 28 spawning females from the Rachel Carson Reserve. These crabs were known to be spawning since they carried a visible egg mass. Though they were collected from the same location as the resident crabs, the spawning crabs are representative of the entire watershed (0-35 PSU) and were collected as they were migrating to release clutches of eggs [8,12,21].

2.2 DNA Extraction, PCR, and Sequencing Procedures

DNA was extracted from tissue samples using the Wizard® Genomic DNA Purification Kit (Promega, USA). Broad spectrum primers (LC0I/HCOI) were used to amplify a 710 base pair fragment of the cytochrome c oxidase I (COI) gene encoded in the mitochondrial genome (Folmer et al., 1994). A total volume of 20 µl was used for each polymerase chain reaction (PCR), including 0.2 µl MyTaq™ HS DNA polymerase (Bioline), 2 µl 10x reaction buffer, 1.6ml 25mM $MgCl_2$, 1.6ml dNTPs (2.5mM), 1 µl of each primer (10mM), and 2 µl DNA template. PCR products were analyzed using agarose gel electrophoresis in Tris-acetate-EDTA buffer, stained with GelRed®, and visualized in UV light. Once it was ensured that the COI fragment was successfully amplified, samples were purified using Exonulcease I and Antarctic Phosphatase (New England Biolabs). Further DNA sequencing services were performed by Eurofins Genomics LLC (Kentucky, USA). Processed sequences were edited and aligned using Codon Code Aligner 9.0.1 with MUSCLE algorithms [31].

2.3 Data Analysis

The program DnaSP 5.10.01 was used to calculate the haplotype and nucleotide diversity, variable sites, nucleotide divergence, nucleotide differences, and the net genetic distance between sample groups [32]. Molecular variance was analyzed to examine the population and subdivision structure. Arlequin 3.5.2.2 was used to compute the Fst statistics, conduct neutrality tests, and report shared haplotypes between the sample groups [33,34]. For the Fst statis-

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tic, a pairwise Fst significance test was conducted through nonparametric permutation with 1,000 data permutations. PopArt 1.7 was used to create non-parsimonious (TCS) haplotype networks [35].

3. Results

From the high salinity sequences 26 were female and 69 (73%) were male. Though herteroplasmy was common, levels were low and did not interfere with interpretations. These CO1 sequences were compared to 26 female sequences from low salinity (Lake Mattamuskeet) and 28 spawning female sequences (Bird Shoal). Our data set was comprised of a total of 244 COI sequences. After alignment, there was a full overlap of 552 base pairs from all three locations.

3.1 Haplotype Distribution

For all 149 samples, sequence analysis revealed 56 variable sites, 24 singleton variable sites, and 54 haplotypes. The total haplotype diversity was 84.4% and the total nucleotide diversity was 0.55%. Haplotype diversity was 76.62% for high salinity females, 71.70% for high salinity males, 94.25% for low salinity females, and 98.411% for spawning females. Nucleotide diversity was 0.00211% for high salinity females, 0.00288% for high salinity males, 0.01070% for low salinity females, and 0.00933% for spawning females (Table 1). The greatest number of haplotypes was found in spawning females, while the least number of haplotypes was found in the high salinity females.

Table 1. Haplotype statistics for each group sampled. SD = standard deviation

	No. Individuals	No. of Haplotypes	Haplotype Diversity ± SD	Nucleotide Diversity ± SD	Average No. of Nucleotide Differences (k)
High Salinity Females	26	10	0.7662 ± .06700	0.00211 ± .00038	1.163
High Salinity Males	69	21	0.7170 ± .00228	0.00288 ± .00046	1.590
High Salinity (both sexes)	95	26	0.7260 ± .03900	0.00266 ± 0.00035	1.466
Low Salinity Females	26	18	0.94154 ± .00118	0.01070 ± .00277	5.905
Spawning Females	28	23	0.9841 ± .00021	0.00933 ± .00198	5.148

Visual inspection of haplotypes by location is informative (Table 2). Haplotypes H3 and H8 were very common in high salinity residents. Haplotype H3 had a frequency of 42.3% in the high salinity female group and 47.8% in the high salinity male group. Haplotype H8 had a frequency of 26.9% in the high salinity female group and 24.6% in the high salinity male group. Out of the 10 haplotypes found in high salinity females, 4 were unique and not shared with any other group. The high salinity males also had 14 out of 21 haplotypes that were unique and not shared with any other group. In low salinity females, H3 and H8 were 11.5% and 23.1% of the total respectively. Thirteen of the total 16 haplotypes in low salinity were unique to low salinity. For spawning females the H3 haplotype was 8% and H8 was 13%. Spawning females had 15 out of the 21 remaining haplotypes that were unique to their group. Thus, of the 54 haplotypes, 48 were only represented in one specific group and 3 out of 54 haplotypes were found in all groups.

Table 2. Distribution of haplotypes in crabs of varying salinities

Haplotype	High Salinity Females	High Salinity Males	Spawning Females	Low Salinity Females	Total
H1	0	0	2	0	2
H2	0	0	2	0	2
H3	11	33	2	3	49
H4	0	0	1	0	1
H5	0	0	1	0	1
H6	0	0	1	0	1
H7	0	1	1	0	2
H8	7	17	3	6	33
H9	0	0	1	0	1
H10	0	0	1	0	1
H11	0	0	1	0	1
H12	0	0	1	0	1
H13	0	0	1	0	1
H14	0	0	1	0	1
H15	0	0	1	0	1
H16	0	0	1	0	1
H17	1	0	1	0	2
H18	1	1	1	0	3
H19	0	1	1	1	3
H20	1	1	1	1	4
H21	0	0	1	0	1
H22	0	0	1	0	1
H23	0	1	1	0	2
H24	0	1	0	0	1
H25	0	1	0	0	1
H26	0	1	0	0	1

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H27	1	0	0	0	1
H28	0	1	0	0	1
H29	1	0	0	0	1
H30	1	1	0	1	3
H31	0	1	0	0	1
H32	0	1	0	0	1
H33	0	1	0	0	1
H34	0	1	0	0	1
H35	1	0	0	0	1
H36	0	1	0	0	1
H37	0	1	0	0	1
H38	0	1	0	0	1
H39	0	1	0	0	1
H40	1	0	0	0	1
H41	0	1	0	0	1
H42	0	0	0	1	1
H43	0	0	0	1	1
H44	0	0	0	1	1
H45	0	0	0	1	1
H46	0	0	0	1	1
H47	0	0	0	2	2
H48	0	0	0	1	1
H49	0	0	0	1	1
H50	0	0	0	1	1
H51	0	0	0	1	1
H52	0	0	0	1	1
H53	0	0	0	1	1
H54	0	0	0	1	1
Total	26	69	28	26	149

3.2 Genetic Structure

An AMOVA analysis was conducted to analyze genetic structure. Based on the results, 92.24% of the variation occurred within groups, and 7.76% of the variation occurred among the three separate groups (Fst = 0.07762, $p < 0.001$). Significant, genetic differentiation was observed among the sampled groups. The highest Fst value resulted from the comparison of high salinity males and spawning females (Fst = 0.10037, $p < 0.001$), while the lowest Fst value was found between high salinity males and high salinity females (Fst = -0.01753, $p > 0.05$). The corrected pairwise differences between each group also suggested that there was high genetic differentiation (Table 3). Four of six corrected pairwise tests were significant and were comparisons with high salinity crabs and another group. The highest corrected pairwise difference existed between high salinity males and spawning females (Nm = 0.25207, $p < 0.001$). The two comparisons that were not significant had compared high salinity crabs to each other and low salinity females to spawning females.

Table 3. Fst, genetic variance between the two groups; Nm, corrected average pairwise difference; Dxy, nucleotide divergence; Da, net genetic distance

		Haplotypes Shared	Fst	Nm	Dxy	Da
High Salinity Males	**Spawning Females**	7	0.10037***	0.25207***	0.00656	0.00046
High Salinity Males	**Low Salinity Females**	5	0.07969**	0.18583**	0.00713	0.00034
High Salinity Females	**Spawning Females**	5	0.07531**	0.26884**	0.0062	0.00049
Low Salinity Females	**High Salinity Females**	4	0.05503*	0.20580*	0.00677	0.00037
Low Salinity Females	**Spawning Females**	4	-0.00512	-0.02913	0.00996	-0.00005
High Salinity Males	**High salinity Females**	5	-0.01563	-0.01753	0.00246	-0.00003

Notes: P-values: * < 0.05; ** < 0.01; *** < 0.001.

An overall comparison from each sample type (Table 4) shows high salinity crabs had highly significant genetic differentiation when compared to spawning females (Fst = 0.11830, $p < 0.001$) as well as low salinity females (Fst = 0.09689, $p < 0.001$). Significant, corrected average pairwise differences were found between high salinity and spawning females (Nm = 0.26018, $p < 0.001$), as well as between high salinity and low salinity crabs (Nm = 0.19482, $p < 0.001$). Taken together, these statistics indicate that there is significant genetic differentiation between high salinity and low salinity crabs, as well as between high salinity and spawning females. Low salinity females were not significantly different from spawning females, and high salinity males and females were similar.

Table 4. Statistical summary for spawning females, high salinity crabs (both sexes), and low salinity crabs. Fst, genetic variance between the two groups; Nm, corrected average pairwise differences; Dxy, nucleotide divergence; Da, net genetic distance

		Haplotypes Shared	Fst	Nm	Dxy	Da
Spawning Females	**High Salinity Crabs**	8	0.11830***	0.26018***	0.00646	0.00047
High Salinity Crabs	**Low Salinity Females**	5	0.09689***	0.19482***	0.00703	0.00035
Spawning Females	**Low Salinity Females**	4	-0.00512	-0.02913	0.00996	-0.00005

Notes: P-values: * < 0.05; ** < 0.01; *** < 0.001.

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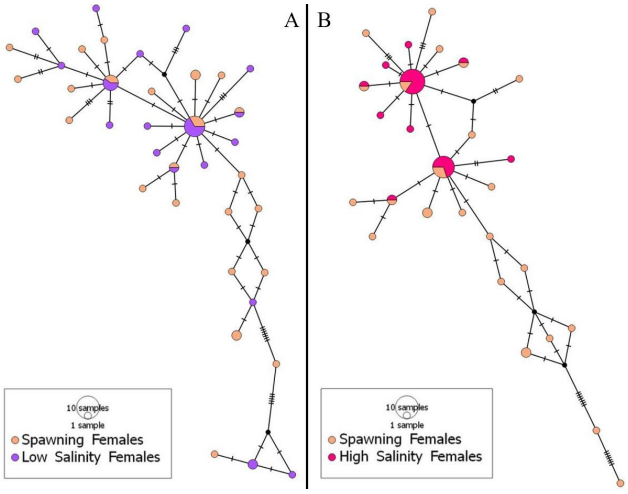

Figure 1. TCS haplotype network demonstrating the relationship between (A) spawning females and low salinity females and (B) spawning females and high salinity females

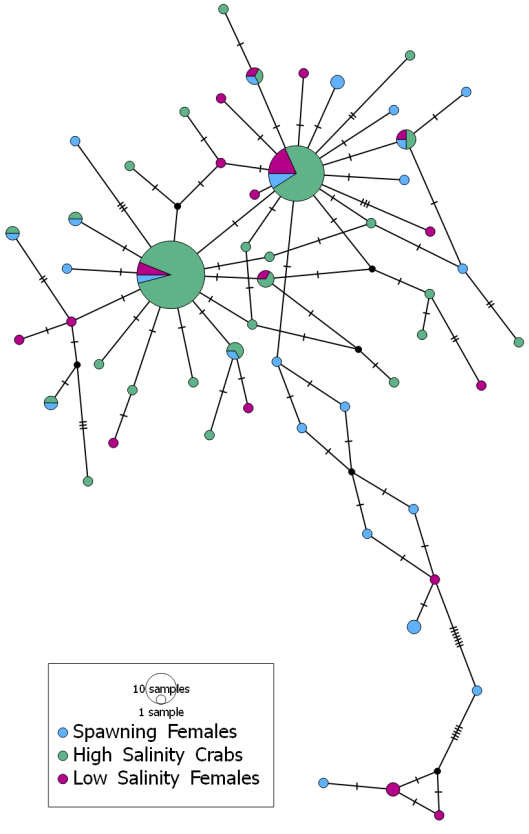

Figure 2. TCS haplotype network demonstrating the relationship between spawning females, high salinity crabs (both sexes), and low salinity females

4. Discussion and Conclusion

This study tested the hypothesis that habitat preference in blue crabs is influenced by genetics. With a knowledge of migration patterns and using salinity as a proxy for habitat, we sampled blue crabs living in different salinities and spawning females that had migrated to high salinity. We sequenced and analyzed a region of the mitochondrial cytochrome c oxidase 1 (COI) gene for genetic differentiation and gene flow. Differences would support our hypothesis.

Spawning females matured in a wide range of habitats and had the highest haplotype and nucleotide diversity. Spawning females arrive at the Rachel Carson Reserve from many watersheds [2]. Many spawning females migrated down the Intracoastal Waterway from the Neuse River. Others are from the Newport River, North River and the southwestern end of the Pamlico Sound. Because of the low number of resident blue crabs where the spawning crabs were sampled, representatives of the local population were rare. Local spawning crabs end up in the coastal ocean when they release their first clutch of eggs because crabs migrate on falling tides with late stage embryos and move offshore through the inlet [1-3]. If our hypothesis is correct, the high haplotype and nucleotide diversity found within the spawning females reflects the large geographic expanse and diversity of habitats. Like the spawning females, the haplotype and nucleotide diversity in the low salinity habitat was high. Juvenile crabs migrate into Lake Mattamuskeet through canals that connect it to the Pamlico Sound and Albemarle Sound [11].

Water control structures on the canals are constructed to only let water out of the lake if it is higher than the water level in the canals. As a management strategy, the refuge slightly opens the gates which enables small organisms such as crabs and elvers to enter the lake in the spring and summer. Once small crabs pass the water control structures into the lake, they are not able to exit whether they prefer the lower salinity or not. The control structures are closed all summer because the lake is used to irrigate crops which keeps the lake significantly lower than the canals. The exception is heavy rains which raise the lake level and cause the structures to open. However, in heavy rains, the crabs become inactive. This setup provides some explanation for why the haplotype and nucleotide diversity is also high within the low salinity lake environment. Though salinity is rarely if ever higher than that 3 PSU, depending upon rainfall, the canal waters can have salinities that range from 0 to over 10 PSU. Once crabs get into the lake they are trapped until fall and are adults by then. In contrast, the haplotype and nucleotide diversity from crabs resident in high salinity were significantly lower. This supports the hypothesis that specific genotypes took up residence less than a mile from the inlet. Thus all the data are consistent.

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Genetic diversity is influenced by population size, immigration rates, and mutation rates. However, the rate of gene flow among populations and the history of ancestral populations directly influence genetic diversity [36]. AMOVA results also support the hypothesis of a pattern of genetic differentiation by habitat origin and significant genetic structuring among the crabs sharing the same habitat location. High genetic differentiation was found between high salinity and spawning crabs, as well as between high salinity and low salinity. Regardless of sex, sequences from high salinity crabs were centered around two major haplotypes. There was a correlation between genetic differentiation and habitat suggesting significant structuring. Structuring is evident within the haplotype maps for the high salinity group. This supports the hypothesis that specific genotypes select high versus low salinity habitats. Significant average pairwise comparisons between high salinity crabs and spawning females, as well as between high salinity crabs and low salinity crabs indicates the lack of strong gene flow among the crabs from those locations. Crabs migrating to these separate locations are acting independently.

This structuring strongly reflects the migration to contrasting environmental conditions since salinity varied greatly per sampling site [11,12]. Thus, a lack of gene flow and high genetic differentiation may be a result of genetic adaptation to different salinity environments. Different salinities pose different kinds of challenges. Previous studies report blue crabs, which are hypersaline regulators [37] expend more energy at lower salinities versus higher salinities. Expending more energy could negatively affect growth rate and result in a decrease in molt increment and/or an increase in intermolt period [38,39]. However, low salinity waters are warmer and nurseries for many prey, offsetting energy losses due to osmotic regulation. Despite the increased energetic costs, crabs from lower salinity waters are routinely larger than those from high salinity [26].

Genetic differentiation by habitat is in stark contrast to the high genetic diversity [24,40] but general lack of strong genetic structuring seen at large regional scales [28] is consistent with our hypothesis. The genetic differentiation found between distinct blue crab habitats supports the hypothesis that there is a genetic component in blue crabs that influences migration and habitat selection and is likely to be reflected in physiological responses. In the future, genome-wide scans should be capable of identifying candidate enzymes responsible for adaptation to the spectrum of habitats found in estuaries and sounds.

5. Implications for Fisheries Management

The high salinity population we sampled was about 73% male, very similar to the low salinity population in Lake Mattamuskeet which was 70% male [25] and very different from the sex ratios in the commercial fishery which is male limited [41-45]. The high salinity population has a minimal recreational fishery as does the low salinity population. Over time, the high salinity crabs should have a reproductive advantage because they are subjected to the least pressure from the fishery. In the long term this habitat isolation could result in sympatric speciation.

Major blue crab fisheries are managed by small and large size cutoffs [42], spawning sanctuaries, migration corridors, restrictions on females brooding eggs and trawling bans [1]. The small size cutoff is accomplished with cull rings which enable small crabs to escape and reduces most the work fishers must do to meet the small size cutoff. Large size cutoffs are ineffective because large crabs are rare and a sufficient percentage of large crabs is allowed. Spawning sanctuaries have become popular and may be effective at increasing the total number of clutches that crabs produce. Deep water migration corridors are an attractive management tool which is hard to implement because of crab foraging behavior. Spawning females migrate in the corridors but stop to forage in shallow water in the midst of the fishery [3,4]. Some states restrict taking of brooding females. Especially in the warm summer months, pot stress causes females to damage or remove their egg masses [3]. Bans on trawling seem effective especially when the trawled crabs are spawning females that have run the gauntlet of the fishery.

If we are correct that blue crabs mix genotypes in the coastal ocean and then occupy different habitats in sounds and estuaries based upon their genotypes, then this could set the stage for sympatric speciation over a long time period. There could be profound impacts due to fisheries pressure over time as well. For example, all spawning crabs must move back to high salinity to hatch eggs and release larvae. Genotypes of crabs migrating to fresh water are at a disadvantage because they have to return through the entire fishery to spawn. Crabs resident in spawning sanctuaries would experience no fishery pressure. This is supported by the 3:1 male to female sex ratio we found in the high salinity crabs which is similar to that of Lake Matamuskeet [25] which also only has a recreational fishery.

Changes in fisheries regulations threaten human sub-cultures and livelihoods. Historically, as fisheries crash due to ineffective management, fishers go out of business. Instead of discarding skilled fishers and eliminating their way of life, it would be an interesting social experiment to support impacted fishers and protect livelihoods until effectiveness of new regulations to the fishery is demonstrated.

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6. Conclusions

On the east and Gulf of Mexico coasts of the United States blue crabs are predators, prey, and important to commercial and recreational fisheries. Crabs mature and mate in sounds and estuaries. Larvae develop in the coastal ocean. The last pre-juvenile stage, the megalopa returns to sounds and estuaries and metamorphoses to a juvenile which migrates variable distances from less than a kilometer from an inlet to hundreds of kilometers to fresh water. We hypothesized there that there is a genetic component to habitat selection. We tested the hypothesis using CO1 haplotypes and high salinity, low salinity and spawning crabs. Analysis supports our hypothesis. This finding has potential for fisheries management as fisheries pressure is most intense on females crabs returning from fresh water through the fishery to spawn in high salinity water, next most intense on crabs that comprise the majority of the fishery in mid salinities and least intense on essentially nonmigratory crabs resident near inlets. If our hypothesis is correct fisheries managers should consider practical ways to level the playing field.

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