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Population Genetic Analysis of Atlantic Horseshoe Crabs (*Limulus polyphemus*) in
Coastal Massachusetts.

A Thesis Presented

by

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ABSTRACT

POPULATION GENETIC ANALYSIS OF ATLANTIC HORSESHOE CRABS (*LIMULUS POLYPHEMUS*) IN COASTAL MASSACHUSETTS

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Atlantic horseshoe crabs (*Limulus polyphemus*) have endured decades of intense harvest pressure. Genetics studies have shown evidence of distinct sub-groups spanning the coast, although few fine-scale studies have been done to delineate these groups on a local level. Massachusetts lies directly between two of these sub-groups. With documented differences in prosomal widths of horseshoe crabs from either side of Cape Cod, it is possible that Cape Cod is a barrier to gene flow and that there are two distinct genetic groups within Massachusetts. Regulations currently consider all horseshoe crabs to be of one stock. I examined 6 microsatellite loci from 193 horseshoe crabs collected from 7 locations across Massachusetts between 5 May and 24 June 2010. I also analyzed the prosomal widths of 324 horseshoe crabs from 8 locations across Massachusetts. Data analysis revealed low divergence with a G'_{ST} of 0.005 (95% CI -0.004–0.013) and a G''_{ST} of 0.015 (95% CI -0.014–0.045). Wellfleet Bay showed evidence of divergence from all other sites except Buzzards Bay. Isolation by distance is apparent via the Atlantic Ocean. Phenotypic variation in the prosomal widths of horseshoe crabs shows greater divergence among sites than neutral markers and indicates the presence of additive genetic effects. Low divergence and high heterozygosity indicate that although documented population declines have occurred, effective population size (N_e) is still large enough to maintain allele frequencies. With isolation by distance, divergence is likely to increase over time if populations remain low. Phenotypic divergence shows the possibility of local adaptation and that the implementation of management units (MUs) to the north and south of Cape Cod would be recommended as a conservative measure.

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LIST OF ABBREVIATIONS

ASMFC: Atlantic States Marine Fisheries Commission

CMR: Code of Massachusetts Regulations

DNA: Deoxyribonucleic Acid

FDA: Food and Drug Administration

FMP: Fishery Management Plan

GLMM: Generalized Linear Mixed Model

HW: Hardy-Weinberg

IBD: Isolation By Distance

LAL: *Limulus* Amebocyte Lysate

LD: Linkage Disequilibrium

MADMF: Massachusetts Division of Marine Fisheries

MCMC: Markov Chain Monte Carlo

MU: Management Unit

NOAA: National Oceanic and Atmospheric Administration

NPS: National Park Service

PC: Principle Component

PCA: Principle Component Analysis

PCR: Polymerase Chain Reaction

USFWS: United States Fish and Wildlife Service

CHAPTER 1

GENERAL INTRODUCTION

The Atlantic horseshoe crab (*Limulus polyphemus*) is a marine chelicerate arthropod that ranges from the Gulf of Maine to the Gulf of Mexico. It is one of only four extant species in the family Limulidae, which is the sole extant family in the order Xiphosura and class Merostomata. The Delaware Bay region, which lies in the middle of their range, has the highest density of horseshoe crabs. Horseshoe crabs spawn along sandy beaches during the mid to late spring, but spend most of the year just offshore (Botton and Ropes 1987). Trawl surveys have noted that they occur mostly in shallow (< 30 m) waters and that their numbers decrease with increased distance from shore (Botton and Ropes 1987).

Horseshoe crab spawning occurs from April through August, the bulk of which takes place in May and June on Cape Cod (James-Pirri et al. 2005; Rudloe 1980; Schuster and Botton 1985). Most studies of spawning behavior have been performed in Delaware Bay and have shown that horseshoe crabs come in with the extreme high tides of the new and full moon cycles (Smith et al. 2002); however, horseshoe crabs in Massachusetts have been known to spawn subtidally and seem to be less influenced by the lunar cycle (James-Pirri et al. 2005). Most Delaware beaches have a steeper, seaward slope (6.4 degrees, Botton and Loveland 1987) than many Massachusetts beaches and therefore do not present as much optimum spawning habitat during lower tides. Some beaches on Cape Cod have sand flats exposed for almost 2 km from shore at mean low water and remain shallow (< 3 m) for nearly 4 km from shore (NOAA Atlantic Nautical Chart no. 13246). With a tidal cycle that ranges up to 3.8 m on new and full moons, horseshoe

crabs have access to sandy subtidal spawning areas even during neap tides. Spawning activity can be more strongly influenced by water temperature, wave action, weather, and wind when access to optimum habitat is not restricted by the lunar cycle (Ehlinger et al. 2003).

During the spawning season, males arrive inshore and attach themselves to incoming females (Rudloe 1980). The females then dig nests in the sand, deposit their eggs, and drag the trailing males over the nests to fertilize the eggs externally (Brockmann and Penn 1992; Rudloe 1980; Schuster and Botton 1985). Some females can have multiple males clustered around them in a nest (Brockmann and Penn 1992; Rudloe 1980; Schuster and Botton 1985). These satellite males can fertilize up to 74% of the eggs in a clutch and have approximately equal fertilization success as primary males do (Brockmann et al. 2000). Females have been observed to lay between 50 and 7,750 eggs in one clutch, returning to spawn multiple times in one season (Brockmann 1990, Leschen et al. 2006).

Horseshoe crab eggs are deposited around the mean high water line where temperature, moisture and oxygen levels are optimal for development (Penn and Brockmann 1994), although sediment cores from sites on Cape Cod have found eggs in the 10 m range between the low and high tide lines, indicating the occurrence of subtidal spawning (James-Pirri et al. 2005). Eggs develop best when exposed to high oxygen, but are prone to desiccation if left too high above the water line (Penn and Brockmann 1994). Larvae emerge approximately three to five weeks later (Ehlinger et al. 2003). Studies suggest that despite being planktonic as larvae, juveniles remain close to the beaches where they were spawned and that long-range dispersal is limited (Botton and Loveland

2003; Botton et al. 2010). Horseshoe crabs go through an estimated 17 to 19 instars over 8 to 10 years before reaching adulthood and ceasing to grow (Sekiguchi et al. 1988). While no direct observations of how long lived horseshoe crabs are have been made, it can be estimated that they may live at least 17 to 19 years by measuring the size of slipper snails (*Crepidula fornicata*) that settle on the carapaces of adults (Grady et al. 2001; Shuster and Sekiguchi 2003). Horseshoe crabs are benthic feeders, using their walking legs to crush food and push it into their mouths (Botton and Shuster 2003). Their diet consists of various bivalve species and, to a lesser extent, polychaete and nemertean worms, gastropods, and crustaceans (Botton and Ropes 1989; Walls et al. 2002).

Horseshoe crabs are integral to the coastal estuarine ecosystem. Adults are an important part of the diet of some finfish, including leopard sharks (*Triakis semifasciata*), and federally listed loggerhead sea turtles (*Caretta caretta*) (ASMFC 1998). Horseshoe crab eggs and larvae provide a source of food for many fish, arthropods, and birds in the estuarine system (Walls et al. 2002). Migratory shorebirds like the sanderling (*Calidris alba*), dunlin (*Calidris alpina*), semipalmated sandpiper (*Calidris pusilla*), ruddy turnstone (*Arenaria interpres*) and red knot (*Calidris canutus rufa*) use horseshoe crab eggs as a major source of food. These birds need to as much as double their body weight during their migration stopover in the mid-Atlantic, as their journey can range from as far as Tierra del Fuego in Chile and Argentina to the Arctic Circle (Castro and Myers 1993). Without horseshoe crabs (and their eggs), these birds may not be able to accumulate enough fat reserve from other sources to make it to their breeding grounds and breed successfully (Castro and Myers 1993).

Horseshoe crabs have a strong economic importance as well and are harvested for multiple uses (Shuster and Botton 1985). In the 1870s as many as 4.3 million horseshoe crabs per year were used as fertilizer and animal feed (Shuster and Botton 1985). This particular use stopped around 1960, but other harvests continue to this day (Shuster and Botton 1985). Horseshoe crabs are presently used heavily as bait for the conch (*Busycon spp.*) and, to a lesser extent, the eel (*Anguilla rostrata*) fisheries. The use of horseshoe crab bait for eels is declining (ASMFC 2009a). The Atlantic States Marine Fisheries Commission (ASMFC) stock assessment has determined the eel population to be depleted and that U.S. landings have declined by approximately 1200 metric tons (approximately 72%) from 1979 through 2012 (ASMFC 2012). However the conch fishery has increased since 1990, showing a positive correlation with increased horseshoe crab landings (peaking around 2.5 million crabs in 1998) over the same period (ASMFC 2009a).

Besides being used as bait, horseshoe crabs are in high demand due to a unique and useful property of their blood. The white blood cells contain a compound called *Limulus* Amebocyte Lysate (LAL). This compound coagulates in the presence of endotoxins that occur on the cell membranes of Gram-negative bacteria. These bacteria can be deadly when introduced into the human bloodstream so any medical devices or intravenous drugs must be tested to be sure the bacteria are not present. There is as yet no other way to detect this type of bacteria at the same level of sensitivity as LAL (Walls and Berkson 2003). The use of LAL for testing sterility of medical equipment and vaccines is required by the FDA (ASMFC 1998).

While the process of bleeding horseshoe crabs to extract LAL is not immediately fatal and those harvested in Massachusetts under a biomedical use permit must be returned to the same location from which they were taken (322 CMR 6.34 (6)(b)), there are questions regarding the recovery of horseshoe crabs after they have been bled. Mortality has been reported to occur in up to 30% of bled horseshoe crabs, even under ideal conditions (Hurton and Berkson 2006; Leschen and Correia 2010). Studies have examined blood protein levels, mortality, and disoriented behavior after bleeding. The observed mortality rates in these studies ranged from 8% to 20% (Anderson et al. 2013; Kurz and James-Pirri 2002; Walls and Berkson 2003).

Horseshoe crabs contribute to a few smaller industries as well. The spectacle of birds eating horseshoe crab eggs attracts a substantial number of visitors to Delaware Bay beaches each year, providing an important source of tourist revenue to the area (Walls et al. 2002). Visitors often come to see the horseshoe crabs themselves, as their prehistoric-looking bodies and spawning aggregations can be somewhat of a novelty. The horseshoe crab is also an important study animal for scientists. A large amount of our understanding of the neurophysiology of vision has arisen from studies featuring the horseshoe crab due to its having a large optic nerve and demonstrated visually-based behavior (Barlow et al. 1977; Barlow et al. 2001; Passaglia et al. 1997; Passaglia et al. 1998).

Over one hundred fifty years of unregulated fishing have led to concern from regulatory agencies and environmental organizations regarding the sustainability of a large commercial harvest on horseshoe crabs (ASMFC 1998). Regulations on the horseshoe crab fishery have only recently been developed. Trawl data suggesting a decline in the population led Delaware to become one of the first states to begin

regulating the fishery in 1997 (ASMFC 1998). Massachusetts enacted its first harvest quota of 330,377 horseshoe crabs three years later (ASMFC 2001). However, this was largely ineffective as the fishery landings were less than half that allowed (Figure 1), so in 2009 the quota was reduced to 165,000 horseshoe crabs (Glenn 2009). Delaware data through 2007 provide evidence for an increase in juvenile horseshoe crabs, suggesting that populations are now starting to recover, but in New York and New England, populations are showing evidence of continued decline (ASMFC 2009b, 2013).

The horseshoe crab fishery spans the entire Atlantic Coast of the United States, and is therefore difficult to manage. The Atlantic States Marine Fisheries Commission (ASMFC) is a regulatory body comprised of members from each of the 15 states that border the Atlantic Ocean. Representatives of the ASMFC include the director of each state's marine fisheries management agency. They work together to regulate species of interest whose ranges cross state lines (like horseshoe crabs). Horseshoe crab stock assessments are based mostly on trawl survey data, fishery landings, and to a much smaller degree, spawning surveys (ASMFC 2009a). These data are then used to build interstate fishery management plans (FMPs), which make suggestions of how the horseshoe crab fishery should be managed most efficiently. The ASMFC charges each state to manage their horseshoe crab populations following guidelines laid out in the FMP. The populations in each state are arbitrarily considered separate management units (MUs), defined as populations that have low connectivity to adjacent populations that should be managed independently of each other (Palsbøll et al. 2006). Without determining the degree of connectivity between and within different states, horseshoe crabs may be subject to overly generalized regulations within one state, or different (and

sometimes conflicting) regulations between different states (ASMFC 1998). For example, Delaware has sanctuaries in which no harvest is allowed and male-only harvests everywhere else (ASMFC 1998). Rhode Island bans harvest around peak spawning times, but both males and females may be taken (RI Marine Fisheries Statutes and Regulations 15.22.4). South Carolina has a biomedical-only harvest (ASMFC 1998).

As of 2010 in Massachusetts (322 CMR 6.34), harvest is banned during peak spawning times (the five days around new and full moons from April 16 to June 30). No horseshoe crabs under 7" (178 mm) may be taken. There is a daily possession limit of 400 animals per day, per person (1000 for biomedical permits; as of 2014, 300 for mobile gear), and a seasonal limit of 165,000 total horseshoe crabs. Biomedical harvest is not counted towards this quota. Pleasant Bay, MA is open only to biomedical harvest. The Cape Cod National Seashore (National Park Service) and Monomoy National Wildlife Refuge (Fish and Wildlife Service) are federally owned areas that are closed to harvest completely.

Local depletion remains a serious concern under current Massachusetts regulations. If populations are demographically independent (defined as having no correlation in population vital rates such as births and deaths), then harvesting too many individuals from one area could lead to local extirpation. Migrant horseshoe crabs from neighboring sites may be moving between populations, but they may not be moving at a rate that can sufficiently re-colonize a depleted site. Further, if migrant crabs are genetically distinct, this can lead to problems such as "genetic swamping" in which local adaptations are lost as new individuals inundate the site (Allendorf et al. 2008). The degree to which neighboring groups of horseshoe crabs are demographically correlated is

not well understood and current harvesting practices could lead to a loss of local adaptations, reducing the stock's ability to withstand and recover from harvest pressure.

Little is currently known about horseshoe crab population dynamics. Many researchers have done spawning surveys (James-Pirri et al. 2005; Widener and Barlow 1999) and capture-mark-recapture programs (Baptist et al. 1957; Smith et al. 2006) in efforts to estimate the total population size. Some work has been done on habitat preference (Ehlinger et al. 2003), age and stage structure (Sweka et al. 2007; Grady and Valiela 2006; Carmichael et al. 2003), and movement patterns (Brousseau et al. 2004; James-Pirri et al. 2005; James-Pirri 2010; Martinez 2012; Watson et al. 2009), each of which provide some insight into overall population structure. Many of these studies suggest some level of localized populations (Baptist et al. 1957; Botton and Loveland 2003; James-Pirri et al. 2005; James-Pirri 2010; Widener and Barlow 1999). Genetic analyses provide another tool with which we can further our understanding of population structure and dynamics.

The genetic structure of horseshoe crab populations needs to be better understood in order to determine whether the populations are demographically independent and to better define the scale of MUs. Atlantic horseshoe crabs range from Maine to the Yucatan Peninsula, but tend to exhibit small-scale spatial movements, which would suggest a certain amount of geographic isolation (James-Pirri et al. 2005; James-Pirri 2010; Saunders et al. 1986). Mitochondrial DNA has been used to distinguish two genetically distinct Atlantic groups of populations (Saunders et al. 1986). King et al. (2005) used microsatellites to distinguish five distinct Atlantic groups of populations on a range-wide scale. F_{ST} is a measure of the divergence of allele frequencies among population groups,

ranging from zero (all groups have equal allele frequencies) to one (all groups are fixed for different alleles and are completely divergent). Many of the samples taken by King et al. (2005) showed F_{ST} values significantly different from zero, suggesting that even finer scale genetic structure within the five population groups they described might exist. Pierce et al. (2000) found significant differences in mitochondrial DNA between horseshoe crabs from the Chesapeake Bay and Delaware Bay. Pierce et al. (2000) estimated gene flow to be 1.07 migrants per generation, which is consistent with the occurrence of demographically independent populations within the mid-Atlantic genetic group described by King et al. (2005). King et al. (2005) also performed assignment tests and found females had a higher rate of assignment to their population of origin than did males, suggesting that males may move between localities more readily than females and may account for much of the gene flow between adjacent populations. Julian and Bartron (2005) also used microsatellites to look for variation on a smaller scale (across Delaware Bay), but found no significant differences.

In my research, I tested for genetic subdivision of horseshoe crab populations within Massachusetts. King et al. (2005) observed a genetic break between populations in the Gulf of Maine and the mid-Atlantic. The only population from Massachusetts included in the study came from Pleasant Bay, which they found to be more closely related to mid-Atlantic populations than to the Gulf of Maine group. Pleasant Bay is somewhat isolated from the rest of Massachusetts spawning habitats and may not be representative of all populations. High turbidity in the Atlantic Ocean and strong currents in the Cape Cod Canal would appear to make Cape Cod a potential barrier to movement between the Gulf of Maine and mid-Atlantic regions. Horseshoe crabs from different

regions also exhibit morphological divergence (Riska 1981). Horseshoe crabs north of Cape Cod have been observed to have smaller prosomal widths than those found south of Cape Cod (Perry 2014). It is unknown whether these differences in body size are a product of genetic subdivision or different environmental conditions. I sampled microsatellite data from horseshoe crabs across multiple Massachusetts populations to help identify potential genetic divergence on a local scale. I also tested for evidence of additive genetic effects that may influence variation in body size among populations. In the next chapter, I present my results in the context of current regulations to help inform and improve future management strategies.

CHAPTER 2

POPULATION GENETIC ANALYSIS OF ATLANTIC HORSESHOE CRABS (*LIMULUS POLYPHEMUS*) IN COASTAL MASSACHUSETTS

Introduction

The Atlantic horseshoe crab (*Limulus polyphemus*) holds an intrinsic value to the ecosystem, but is also an important resource for commercial fishermen, scientific researchers and the medical community (ASMFC 1998, 2009a; Barlow et al. 1977; Barlow et al. 2001; Castro and Myers 1993; Passaglia et al. 1997; Passaglia et al. 1998; Shuster and Botton 1985; Walls et al. 2002). With so many stakeholders, thoughtfully designed management is necessary to maintain healthy populations and a sustainable fishery. Evidence of sharp declines emphasizes the importance of effective management (ASMFC 2009b, 2013). Regulations have been in place since 1997 and are frequently being rewritten to better meet the needs of the fishery (ASMFC 1998, 2001; Glenn 2009). The Atlantic States Marine Fisheries Commission (ASMFC) laid out the interstate fishery management plan (FMP) in 1998, requiring each state to monitor its horseshoe crab populations and habitats and to begin limiting harvest levels (ASMFC 1998). Limited information about stock structure in this species represents an information gap that might prevent effective management. Massachusetts currently regulates all horseshoe crabs as one management unit. Management units (MUs) are populations that have low connectivity to adjacent populations that should be managed independently of each other (Palsbøll et al. 2006). It is important for fisheries managers to properly identify MUs within their jurisdictions so they can maintain a healthy fishery by protecting independent populations without unnecessarily allocating resources to differently manage populations that are not independent (Palsbøll et al. 2006; Waples 1998).

An increasing number of studies have revealed evidence that horseshoe crabs exhibit little movement between embayments and therefore could become increasingly isolated as populations decline (Allendorf et al. 2008; James-Pirri et al. 2005; James-Pirri 2010; Saunders et al. 1986). Genetic studies have found evidence of divergence across the species range, including four distinct sub-groups along the east coast of North America (King et al. 2005; Saunders et al. 1986). Only a small number of fine-scale local population studies have been performed and some provide evidence for divergent populations while others do not (Julian and Bartron 2005; Pierce et al. 2000). More localized studies are needed to reveal if genetic divergence occurs in states managed as one MU and also whether any divergence detected is indeed great enough to warrant creating separate MUs.

King et al. (2005) found a distinction between populations in the Gulf of Maine and the mid-Atlantic regions. Massachusetts lies directly between those two areas and could potentially have populations of horseshoe crabs that belong to either group. No fine-scale genetic studies have been performed in Massachusetts waters. Studies have shown Cape Cod to be a bio- and phylogeographic barrier to many species, including other benthic marine invertebrates (Jennings et al. 2009; Palumbi 1994; Wares 2002). Furthermore, the outer, eastern edge of Cape Cod facing the Atlantic Ocean has only two estuarine systems, both of which are physically isolated from neighboring embayments. Phenotypic divergence in body size on opposite sides of Cape Cod (Perry 2014) could also be consistent with genetic divergence, although environmental differences could also be responsible.

The goals of my research were to determine whether there is evidence of genetically divergent groups of horseshoe crabs within Massachusetts and whether there is evidence of a genetic component of previously observed phenotypic divergence in body size. This work will help managers in Massachusetts determine whether they should continue to apply the same harvest regulations to all horseshoe crab populations or if there is a need for population- or region-specific strategies.

Methods

Sample Collection

I traveled to nine different embayments in Massachusetts (Figure 2) to collect horseshoe crabs at spawning beaches. I tried to collect tissue only from breeding pairs to ensure my samples were representative of the breeding population. Horseshoe crabs were removed from the water for only a few minutes. I removed a small portion of tissue from one of their walking legs using scissors sterilized in bleach to prevent cross contamination (per King et al. 2005). Each sample was stored in 95% ethanol in an individually labeled tube and kept on ice while in the field and during transport. Samples were then stored in the freezer to prevent ethanol evaporation. Each horseshoe crab was marked with a tag provided by the US Fish and Wildlife Service (USFWS) before it was released. Each tag was labeled with a unique identification number and the USFWS horseshoe crab capture-mark-recapture program phone number so that researchers, harvesters, or beach-goers may report when they see a tagged horseshoe crab. The identification number ensures that I sampled each individual only once. I also recorded the sex and width of the prosoma (measured across the widest point) of each horseshoe crab I sampled. The prosoma, or cephalothorax, is the anterior body segment consisting

of a fused head and thorax. I collected tissue samples from a total of 327 individuals from nine different embayments between 5 May and 24 June 2010.

Laboratory Analysis

I examined variation at 16 of the 22 microsatellite loci outlined in King and Eackles (2004). Those loci were: *LpoA5*, *LpoA37*, *LpoA38*, *LpoA40*, *LpoA52*, *LpoA58*, *LpoA64*, *LpoA67*, *LpoA68*, *LpoA73*, *LpoA74*, *LpoA211*, *LpoA315*, *LpoD3*, *LpoD6*, and *LpoD60*. I extracted DNA from my samples using a standard salt precipitation extraction protocol. Once the DNA was extracted, I amplified it by polymerase chain reaction (PCR) using the primers outlined in King and Eackles (2004) and following manufacturer's recommendations for QIAGEN multiplex mastermix (QIAGEN, Germantown, MD). The primers were grouped into five mixes for PCR. The thermocycler profile was as follows: 95°C at 15 min, then 32 cycles of 94°C for 30 sec, 57°C for 90 sec and 72°C for 60 sec, followed by a final step of 60°C for 30 min. After the DNA was amplified, I used 0.6 µl of the PCR product and an internal size standard to perform capillary electrophoresis using an ABI Prism 3130-xl genetic analyzer (Applied Biosystems Inc., Foster City, CA).

Data Analysis

Towards the end of the laboratory analysis, many of the electrophoresis plates failed to yield any signal, regardless of which primer mix or sample was used. These plates could not be used for analysis. I also had to exclude the samples from Plum Island Estuary, as the number of individuals ($N = 2$) was too low for a powerful statistical analysis. I used Peak Scanner version 1.0 software (Applied Biosystems Inc., Foster City, CA) to determine the size of alleles in base pairs. I excluded any samples that had data

for fewer than 8 of the 16 loci. I then excluded loci for which more than 10% of the data were missing. Only 6 loci fit the criteria (*LpoA58*, *LpoA64*, *LpoD60*, *LpoA38*, *LpoA52*, and *LpoD3*). I removed any other individuals that had missing data at these loci. The final data set consisted of 193 individuals from 7 populations (Table 1).

Statistical Analysis

I used GENEPOP version 4.2 (Rousset 2008) to test for Hardy-Weinberg (HW) proportions, linkage disequilibrium (LD), genic differentiation, and to estimate allele frequencies. I used exact tests to test for deviation from HW proportions. To test for LD, I performed a test that calculates the log likelihood ratio (G-test) using a Markov chain algorithm (Raymond and Rousset 1995) that genotypes occur independently of each other.

Tests of genic differentiation were conducted to examine allele frequency divergence among populations. I used GENEPOP to calculate Fisher's exact test for each pair of populations across all loci. F_{ST} is the measure of how divergent populations are from each other by measuring the allele frequency divergence among populations as compared to what would be expected in a panmictic population under Hardy-Weinberg equilibrium. G_{ST} is the equivalent to F_{ST} when more than two alleles exist in a population. G'_{ST} (Nei) is an unbiased value for G_{ST} , which can be an underestimate when the sampled number of populations is small (in my case, $k = 7$; Meirmans and Hedrick 2011). G'_{ST} can never reach a value of 1 even when no alleles are shared among populations, so G''_{ST} standardizes that value by the maximum possible value for G'_{ST} based on the within population variation (Meirmans and Hedrick 2011). I used GenoDive version 2.0b27 (Meirmans and Van Tienderen 2004) to estimate G'_{ST} (Nei) and G''_{ST} , as well as to

perform a principal component analysis (PCA). PCA uses an eigenanalysis on a covariance matrix of pairs of allele frequencies by population. The data are re-arranged into principal components (PCs) according to the maximum amount of variance that can be explained by each and plotted along PC axes according to the eigenvectors of the matrix. The resulting plots can elucidate unseen patterns in the data.

I used STRUCTURE version 2.3.4 (Pritchard et al. 2000) as a model-based test of population subdivision. I conducted 10 replicate runs for each estimated value of K from one to seven populations, both with and without sampling site as a location prior. The results were analyzed using STRUCTURE HARVESTER web version 0.6.94 (Earl and vonHoldt 2012).

I measured isolation by distance (IBD) using Isolation By Distance, webservice version 3.23 (Jensen et al. 2005). I performed the analysis using both G'_{ST} and G''_{ST} for the genetic distances, but got the same slope and distribution of points for both. I chose to only include the results from using G''_{ST} for my genetic distances, following Meirmans and Hedrick (2011). Cape Cod's general shape and the waterways that pass through it make IBD analyses slightly more complicated than if there were only one simple straight line for travel between populations. There are three different paths to get from Barnstable Harbor and Wellfleet Bay to sites on the other side of Cape Cod (see Figure 2). The first is the ocean route, which goes north towards Provincetown, then along the outside of the Cape to the east and heading south along the coast until reaching Nauset Estuary, Pleasant Bay, Stage Harbor, and on to Nantucket then Buzzards Bay. To measure the distance from Barnstable Harbor, I could either hug the coastline along the towns of Brewster, Eastham, Wellfleet and Truro, or take a straight-line route directly north across

Cape Cod Bay to the tip of Provincetown. Both approaches yielded very similar results, so I chose to include only the straight-line route for this analysis. The second route is via a canal that used to exist connecting Cape Cod Bay on the Eastham/Orleans town line with Nauset Estuary (Kelley 2006). This canal, called Jeromiah's Gutter, was built over a marsh that would have connected the two embayments during high tides, which would have allowed horseshoe crabs to easily pass between the two. The canal was filled in during the latter part of 1800 and a road was constructed there that permanently blocked passage (Kelley 2006). I considered this canal route in my analysis and found it was no different in structure from the ocean route that travels around the outside of Cape Cod via Provincetown, so I chose not to include it. The third passageway is the Cape Cod Canal, which connects Cape Cod Bay with Buzzards Bay. The Cape Cod Canal would only have allowed passage since it was built in the early 1900s (Parkman 1978), which would still be enough time (~110–120 generations) to show changes in F_{ST} (Bradbury and Bentzen 2007).

To estimate the power of my data set to detect significant genetic differentiation, I tested the power that different sample sizes using 6 microsatellite loci had to detect changes in F_{ST} based on an $N_e = 2000$ for $t = 20$ generations (expected $F_{ST} = 0.005$), $t = 40$ generations (expected $F_{ST} = 0.010$), or $t = 60$ generations (expected $F_{ST} = 0.015$). I ran 200 simulations per combination of conditions using POWERSIM version 4.1 (Ryman and Palm 2006). Two populations were used in each simulation. The first population was always $N = 50$ and the second was $N = 5, 10, 20, 30, 40, \text{ or } 50$. Allele frequency data from Wellfleet Bay (largest empirical sample, $N = 45$) were used to parameterize simulations. $N_e = 2000$ was chosen to maintain alleles in the Wright-Fisher simulations.

All alleles were maintained in each case. Power was calculated from the proportion of significant results of F_{ST} at each expected level of divergence based on genic exact tests and Fisher's method of combining p -values (following the programming implemented by GENEPOP version 3.4, Raymond and Rousset 1995; Ryman and Palm 2006).

Observing differences in phenotypic traits can also be used to test for adaptive divergence among populations. Q_{ST} is the additive genetic divergence of a quantitative trait, analogous to F_{ST} . P_{ST} is the measure of variance of a phenotypic trait across populations and can approximate Q_{ST} using the equation:

$$P_{ST} = \frac{\frac{c}{h^2} \sigma_B^2}{\frac{c}{h^2} \sigma_B^2 + 2\sigma_W^2},$$

where σ_B^2 and σ_W^2 represent the phenotypic variance both between and within populations, respectively, h^2 is the heritability or proportion of phenotypic variance due to additive genetic effects within the population, and c is the proportion of total variance that is due to additive genetic effects across all populations (Brommer 2011). If values for both h^2 and c are known, the above equation can be used to directly calculate Q_{ST} from phenotypic data collected from the wild (Brommer 2011). Unfortunately it is impossible to measure those parameters without rearing individuals from different populations in a common environment to isolate the phenotypic divergence due to additive genetic effects from those due to environmental conditions (Brommer 2011).

We can determine whether the calculated P_{ST} exceeds that expected from drift alone, which we can conservatively assume occurs at any point when $c > h^2$ (Brommer 2011). We can estimate the robustness of this determination by plotting the observed P_{ST} as a function of varying values of c/h^2 and seeing where the lower 95% confidence

interval crosses the upper 95% confidence interval of F_{ST} , when the null hypothesis is assumed that F_{ST} is not different from zero (Brommer 2011). The lower the value for c/h^2 is at the point where P_{ST} and F_{ST} diverge, the more robust the difference between P_{ST} and the null F_{ST} is.

I used this approach to test the hypothesis that adaptive phenotypic divergence in body size occurs to the north and south of Cape Cod. I calculated the P_{ST} of the prosomal widths I measured while collecting tissue samples and compared the phenotypic divergence to the G''_{ST} values calculated from the microsatellite data. To estimate P_{ST} of horseshoe crab prosomal widths, I used a generalized linear mixed model (GLMM) with sex as a fixed effect and sampling site as the random effect, as I wanted to see how much size variation is explained by site while accounting for size differences between males and females. The GLMM was performed in R version 3.0.3 (R Core Team 2014) using the MCMCglmm package (Hadfield 2010). I excluded the size data from Plum Island Estuary because I only had two individuals, however I did include the data from Duxbury Bay ($N = 31$), even though I had no genetic data from that site.

Results

Genetic Variation within Populations

I identified a total of 71 different alleles (between 8 and 17 per locus). Each population had between 2 and 12 different alleles per locus present (Table 2). Allelic richness and heterozygosity across all loci were similar in each population (Table 3). Heterozygosity for each locus was consistent with that expected under Hardy-Weinberg equilibrium. Only three F_{IS} values for each locus were significantly divergent from 0 at $\alpha = 0.05$. Out of 42 tests (6 loci for 7 populations), only 2.1 were expected to be divergent

by chance. Of the three significant F_{IS} values, each occurred in a different population, but two occurred at the same locus, *LpoD60*. Following a Bonferroni correction for all 42 tests ($\alpha = 0.00119$), only the *LpoD60* locus in the Pleasant Bay population had a significant deficit of heterozygotes ($p = 0.0005$). If I only corrected for the number of loci ($\alpha = 0.05/6 = 0.00833$) or the number of populations ($\alpha = 0.05/7 = 0.00712$), again, only the *LpoD60* locus at Pleasant Bay was significant. Only three linkage disequilibrium tests were significant at $\alpha = 0.05$ (5.25 tests were expected to be significant by chance) and after a Bonferroni correction for all 105 tests ($\alpha = 0.00048$), none were significant. After a Bonferroni correction for 15 tests per population, there were still no significant results ($\alpha = 0.00333$).

Genetic Variation among Populations

Allele frequency divergence was modest (frequencies listed in Table 4). Wellfleet Bay was the only population to show significant ($\alpha = 0.05$) genic differentiation from all other populations except Buzzards Bay. After applying the Benjamini-Yekutieli False Discovery Rate correction for 21 tests, Wellfleet Bay was still significantly different from all other populations besides Buzzards Bay.

Overall G'_{ST} was 0.005 (95% CI -0.004 – 0.013). Overall G''_{ST} was 0.015 (95% CI -0.014 – 0.045). Pairwise G'_{ST} ranged from -0.008 to 0.016 and G''_{ST} ranged from -0.025 to 0.051 (Table 5). Power analysis showed that for 6 loci, at least 20 individuals were needed to have 80% power to detect a true F_{ST} of 0.015, and at least 50 individuals were needed to have 80% power to detect an F_{ST} of 0.01 (Figure 3). With the exception of Buzzards Bay, I had data for more than 20 individuals at each site; therefore sample sizes were generally sufficient to detect significant divergence had it occurred.

PCA revealed subtle geographic patterns of genetic differentiation (Figure 4). Buzzards Bay was divergent from all other sites along PC 1. Nantucket and Stage Harbor diverged positively along PC 2 while Wellfleet Bay diverged negatively. Barnstable Harbor, Nauset Estuary, and Pleasant Bay remained close to the center. Nauset Estuary and Pleasant Bay diverged positively along PC 3 while Barnstable Harbor diverged negatively. Wellfleet Bay, Stage Harbor, and Nantucket were closest to the center.

Results from STRUCTURE and STRUCTURE HARVESTER were consistent with $K = 1$, both with and without the use of a location prior. There was a significant isolation by distance relationship via the ocean (Mantel $R = 0.4553$, $p = 0.0170$; Figure 5a) but not via the Cape Cod Canal (Mantel $R = 0.2210$, $p = 0.1580$; Figure 5b).

Phenotypic Variation among Populations

Phenotypic divergence was pronounced among populations (Figure 6). Comparison of P_{ST} to G''_{ST} revealed that the pronounced phenotypic divergence observed among populations might be consistent with local adaptation. The lower 95% CI of the P_{ST} GLMM with evenly weighted priors (Figure 7a) overlapped the upper 95% CI for overall empirical G''_{ST} at a c/h^2 near 0.25 (Figure 8a). Under highly conservative conditions (priors weighted to give more influence to within site variation; Figure 7b), the lower 95% CI for P_{ST} overlapped the upper 95% CI of G''_{ST} near a c/h^2 of 0.5 (Figure 8b).

Individuals from north of Cape Cod were on average 7–15 mm smaller across the prosoma than those to the south of Cape Cod (Figure 6). While I was able to only measure two individuals from Plum Island Estuary, historical data of horseshoe crabs measured by Baptist et al. (1957) between 1952–54, showed them to exhibit even smaller widths (Figure 9). Male prosomal widths averaged 118.1 mm (SD 8.95; $N = 1,467$) and

female prosomal widths averaged 155.5 mm (SD 11.04; $N = 1,387$) for these historical data, as compared with average prosomal widths of horseshoe crabs I sampled from north of Cape Cod of 175.68 mm for males and 230.78 mm for females.

Discussion

There were some emerging patterns in my data that were apparent after analysis. Genetic variation within populations was slight. There was little to suggest that the populations I sampled had a deficit of heterozygotes or were not in linkage disequilibrium. Genetic variation among populations did show evidence of divergence, especially with increased geographic distance. There was also evidence of adaptive phenotypic divergence among populations.

Genetic Variation within Populations

Allelic richness and heterozygosity across all loci were at similar levels (Table 3). This was consistent with high gene flow. Each region or local embayment population therefore likely contained sufficient variation to respond to environmental change. We lack historical data, but my results suggest that population declines have not yet caused a drastic decline in genetic diversity. Theoretically, heterozygosity is lost at rate of $-1/(2N_e)$ per generation, so even if N and N_e have declined by orders of magnitude (ASMFC 2009b, 2013), this suggested that the contemporary N_e has remained relatively high.

Genetic Variation among Populations

The results of my study showed some evidence of genetic divergence among populations. There was signal from Wellfleet Bay, indicating it may receive few migrants, even from nearby sites like Barnstable Harbor. Buzzards Bay was also genetically divergent, although the sample size was too low to be conclusive. Analyses of

patterns of genetic differentiation through PCA revealed some spatial components of genetic structure, as geographically close sites Stage Harbor and Nantucket grouped with each other, as did Nauset Estuary and Pleasant Bay. Wellfleet Bay was divergent from all other populations except Buzzards Bay, which might have been due to low sample size (8 individuals from Buzzards Bay). Buzzards Bay was also divergent from other sites, but again, care must be taken with this interpretation due to small sample size. Results from STRUCTURE did not provide evidence for any divergence among sites, but this was not surprising given the generally recognized low sensitivity of this approach when genetic differentiation is low (below $F_{ST} = 0.02$; Latch et al. 2006). The isolation by distance analysis provided one of the most compelling sources of evidence for genetic structure. This analysis suggested that gene flow occurs more often among geographically proximate populations and that it is more likely to occur via the Atlantic Ocean than via the Cape Cod Canal. This pattern of genetic structure might reflect historical conditions. However, given the recent population declines, this pattern might also have developed more recently. If so, it is possible that IBD will become more apparent in future generations under recent effective population sizes and migration rates. It is possible that gene flow may be more restricted now than it was in past; however, it is more likely that N_e has declined, making the isolation begin to be apparent. Historical catch rates indicate that declines have already depressed the population and that if numbers do not recover, F_{ST} can increase over time. This study provides a baseline for recent declines, but after declines have occurred.

It is interesting to note that there was evidence of Wellfleet Bay being more genetically divergent from other populations than Barnstable Harbor is, even though

Wellfleet Bay was only divergent along PC 2 (Figure 4a) and IBD via the Atlantic Ocean was apparent. There is a possibility of unknown effects on partial harvest closures of certain water bodies. Half of Wellfleet Bay is within the limits of the National Seashore and the other half is under the jurisdiction of the state. Harvest is only permitted in state waters. Barnstable Harbor is not protected from harvest at all. Neither are Nantucket and Buzzards Bay. Stage Harbor is not protected either and is in fact heavily fished, but it is also directly adjacent to Monomoy National Wildlife Refuge where harvest is prohibited. Horseshoe crabs do exhibit movement between those two embayments and have been observed to spawn in both locations (Martinez 2012). Pleasant Bay has a biomedical-only harvest, where horseshoe crabs taken from this area must be returned to the place where they were captured after they have been bled. Nauset Estuary is completely inside the National Seashore and is entirely closed to harvest. These varying harvest pressures could affect the demographic structure of the breeding populations differently and create unknown patterns in how demographically correlated neighboring systems are under similar rates of migration.

Estimating the degree of demographic independence based on genetic differentiation at neutral markers is already challenging (Allendorf and Luikart 2007). Putting genetic divergence into an ecological context helps remove some of the bias created as an artifact of statistical models, namely Wright's Island Model, which assumes constant population sizes, non-overlapping generations, a common pool of migrants each generation, and the lack of natural selection (Palsbøll et al. 2006). Larger populations will show less divergence from one another than smaller populations with the same amount of gene flow (m , defined as the proportion of individuals that immigrated from another

population). Simply comparing genetic divergence will not tell you whether or not populations are demographically independent. A threshold rate of migration above which populations would likely be demographically correlated should be chosen *a priori* based on population size and generation length (Palsbøll et al. 2006). For horseshoe crabs, the lack of population abundance estimates further complicates this problem. Capture-mark-recapture studies have led to estimates ranging from 2.3–20 million horseshoe crabs in Delaware Bay (Botton and Ropes 1987; Hata and Berkson 2003; Smith et al. 2006). This is an enormous range, which leads to highly variable migration rate estimates. My results showed a small level of divergence and indicated that populations are isolating. The degree to which this was happening was too slight to be well defined. If I assume horseshoe crabs are more likely to follow a stepping-stone model than Wright's island model, as would be consistent with the IBD results, then F_{ST} will be greater for the same m (Allendorf and Luikart 2007). When N_e is large and m is small, it can take much more time for populations to reach equilibrium, so changes in F_{ST} may take longer to become apparent, even when there is little or no gene flow (Allendorf and Luikart 2007). Given that horseshoe crabs have an estimated ten-year generation length, it follows that even low estimates of F_{ST} can be indications of increasing demographic isolation, especially among geographically isolated sites.

Power analysis suggested that the lack of genetic differentiation observed among some of the sites might have been due to low power. All but one of my sample sizes were above 20 individuals and all were below 50 (ranging from $N = 21$ to $N = 45$, with Buzzards Bay having $N = 8$; see Table 1). In the power simulations, a sample size of 20 individuals had 80% power to detect a simulated genetic differentiation of $F_{ST} = 0.015$.

My estimated G'_{ST} of 0.005 is the best value to compare to the simulated F_{ST} . Thus, the significant results I observed occurred in the face of relatively low power (< 80%).

Phenotypic Variation among Populations

Horseshoe crab body size also showed evidence of divergence among populations. Horseshoe crabs at both the northernmost and southernmost extremes of their range have the smallest prosomal widths and horseshoe crabs in mid-range (around Georgia) have the largest (Sekiguchi and Shuster 2009). The reason for this particular pattern is still unknown, although it is hypothesized that horseshoe crabs in the Atlantic dispersed from the Florida/Georgia region towards the north and south and that the smaller body sizes in the extremes of their range is an adaptive response to less optimal temperatures (Sekiguchi and Shuster 2009). While ocean currents, wave action and food type, quality and availability are all environmental factors that influence plasticity in growth rates, temperature and salinity are especially well documented to have an effect. Laboratory growth rates were fastest around 20 ppt salinity and increased with temperatures as high as 40°C (Shuster and Sekiguchi 2003), suggesting warm water horseshoe crabs would grow larger. However, there could also be genetic components because body sizes are smaller with both increasing as well as decreasing temperature, suggesting adaptation. My data as well as other studies (Carmichael et al. 2003; James-Pirri et al. 2005; Riska 1981; Smith et al. 2009) showed divergence between horseshoe crabs from the north and south sides of Cape Cod.

My models of the phenotypic variance suggested that divergence was greater than that which we would expect from drift alone, indicating there may be an adaptive genetic component to the variance among sample sites. The P_{ST} models revealed that even with

the assumption of very conservative priors, the threshold value of c/h^2 where the lower 95% CI of the model overlaps the upper 95% CI of G''_{ST} is quite low, indicating the analysis to be fairly robust (Brommer 2011). This suggested there was a fair amount of phenotypic variation that likely resulted from additive genetic differences among sites, as prosomal widths appeared to be more divergent than we would expect to see from drift alone. A common garden experiment would be necessary to estimate the proportion of that variation which was due to additive genetic effects versus environmental effects. Common garden experiments can be extremely difficult to perform with certain organisms, especially horseshoe crabs, which take 8 to 11 years to reach their final adult body size (Sekiguchi et al. 1988). Furthermore, no one has yet been able to successfully raise a horseshoe crab in captivity. The oldest recorded *Limulus* specimens reared in captivity died after 6–8 years, before reaching maturity (Sekiguchi et al. 1988). These factors make common garden experiments impractical. It is important to note that since the actual values of c and h^2 are unknown and likely unequal when different populations are subject to different environmental conditions, we cannot use P_{ST} estimates alone to determine the proportion of phenotypic variation due to additive genetic effects (Brommer 2011). We can only use these estimates to support the conclusions drawn from other observations, such as allele frequency divergence and environmental measurements. This analysis provided an initial indication that additive genetic effects influencing differences in observed prosomal widths of horseshoe crabs occur across Massachusetts. Given the possibility that these differences are indeed adaptive, I recommend as a conservative measure that two separate MUs be created in Massachusetts: one to the north of Cape Cod and one to the south.

Conclusions

While there was indication that some population differentiation occurs across Massachusetts, the differentiation was very slight. Some of the observed lack of genetic differentiation might have been due to low power. Larger sample sizes and more genetic markers might reveal more genetic divergence. Similar future studies will be necessary to show if any significant divergence is occurring and to monitor its progression if populations remain depressed and gene flow becomes more limited with time.

Unfortunately my statistical power was low to detect any significant changes in G'_{ST} greater than 0.015. I had originally planned on sampling 50 individuals from 11 sites and examining 16 microsatellites. I had a lot of difficulty even finding 50 individuals from each site, which may have been indicative of already depressed populations. Historical accounts of spawning activities showed horseshoe crab numbers to be in the hundreds at any given spawning beach and the spawning season lasted well into July and even into August (Baptist et al. 1957; Barlow et al. 1986; Shuster and Botton 1985). Now, numbers are few and the time in which to find them is much shorter (James-Pirri 2005, 2012; Landi et al. 2015). I travelled to three sites along Plum Island Estuary on 26 May 2010. Baptist et al. (1957) collected horseshoe crabs in some of the same and some nearby sites as I did during the same time period. Despite the historical presence of spawning horseshoe crabs, I was only able to locate two individuals. I purposefully visited Mashnee Dike in Buzzards Bay because of its history of being surveyed (Widener and Barlow 1999). Widener and Barlow (1999) reported seeing 3,171 horseshoe crabs between 13 May and 7 July in 1984, but only 148 individuals between 28 May and 7 June

in 1999. I visited on 18 June 2010 and was only able to locate 6 horseshoe crabs from this beach (along with an additional 6 from nearby sites within Buzzards Bay).

It was also my intention to sample only breeding pairs of horseshoe crabs in order to sample an equal number of males and females that I could confirm were contributing to the breeding population. I was unable to achieve this. Many of the horseshoe crabs I sampled on Nantucket were lone females (17 lone females out of 40 total samples; 13 were lone males and only 10 were in pairs). I travelled to Nantucket on 15 and 16 June 2010, which was slightly after the peak spawning period. Lone males are more commonly found late in the season, as most females return to deeper water after completing their spawning (James-Pirri 2012). Lone females have been known to bury themselves in a nest without a male present, presumably expending energy to lay eggs that will remain unfertilized (James-Pirri 2012; K. Johnson, personal observation). It is unknown why these females are alone, especially in the light of sex ratios skewed towards more males (James-Pirri 2005, 2012). The lack of breeding pairs available later in the season meant I was forced to sample lone individuals, both male and female, that may not have been breeding successfully.

Incomplete genetic data for the samples I was able to collect also limited my sample size and statistical power. The three final electrophoresis plates I ran in January 2011 failed to yield any signal whatsoever, and I was unable to collect data from any more samples beyond that time. It is unknown why the samples failed. There was no apparent reason for the failures. It is possible that the samples became contaminated during laboratory handling. Perhaps there was an unknown PCR inhibitor or DNA degrading enzyme present in the tissue samples. It is also possible that the DNA degraded

due to exposure to water during multiple freeze thaw cycles during laboratory handling, although this is unlikely.

Future Research Directions

More fine-scale genetics research will certainly be useful. My study provides a baseline, but future studies should aim to get larger sample sizes from more sites. King et al. (2005) defined two distinct genetic groups in the Gulf of Maine and mid-Atlantic regions. It would be largely beneficial to figure out exactly where the break between those groups is, since the fishery crosses several state lines where it is subject to vastly different harvest regulations and pressures. Analyses like this will help managers in New Hampshire, Massachusetts, Rhode Island, Connecticut and New York make better decisions if they know their stock belongs to a particular genetic group or groups. Other types of genetic research could also be useful for fisheries managers. New advances in genomics will make more complex genetic analyses easier and less expensive in the future, helping to address shortcomings inherent in current techniques. Perhaps as new technologies evolve, we will someday be able to directly study regions of the genome that influence selective adaptations and be able to measure divergence more accurately.

Research efforts should focus on getting more concrete population estimates. Having accurate population estimates is essential to interpreting the results from all other types of studies. Trawl surveys, spawning surveys, and catch reports all help to give us a picture of the population size. Acoustic and radio telemetry and capture-mark-recapture studies are also great tools to gauge stock size as well as to study how horseshoe crabs move between embayments. Data from these types of studies particularly have increasingly provided evidence of localized populations (Brousseau et al. 2004; James-

Pirri 2010; Martinez 2012; Smith et al. 2010) in addition to local population estimates (Smith et al. 2006). Along with genetic studies, these will give us a more detailed picture of population dynamics. Spawning surveys and investigations of juvenile dispersal help identify critical habitat areas that may need extra protection. Future research could also focus on understanding the environmental drivers of the possibly adaptive phenotypic divergence observed here and how this phenotypic divergence might respond to changing climate conditions. Continued emphasis on population modeling, movement patterns, genetic relatedness, and locating sensitive nursery and spawning habitats will be positive steps towards knowing how to best help recruitment and to identify potentially divergent populations.

Implications for Management

If horseshoe crab populations remain at low levels or continue to decline, they might become more genetically divergent and isolated over time (Allendorf et al. 2008). The more fragmented breeding groups become, the less they will be able to withstand and recover from intense fishing pressure (Allendorf et al. 2008). Managers need to focus on a strategy to return populations to healthy levels and to create a sustainable level of fishing. Because the population is already depressed, it is imperative to be aware of the effects of harvest and be sensitive to potential areas of regulation that can be improved. The patterns of isolation by distance and divergence of some populations, as well as the evidence of adaptive phenotypic divergence suggest that delineating separate MUs on either side of Cape Cod may be warranted as a conservative approach to management.

Regulations must be tailored to suit populations in different MUs. Larger female horseshoe crabs tend to be more desirable to harvesters (Rutecki et al. 2004). As larger

females are more gravid (Leschen et al. 2006), the fishery is disproportionately removing large quantities of eggs from the system. Protecting spawning females should be a priority of managers. Limitations such as a size window (an upper and lower size limit) would be beneficial to protect large females in addition to smaller, sub-adults. This would best be applied separately to populations north and south of Cape Cod, as body size is divergent. Ideally, size limits should be sex-specific. Unfortunately, more complicated regulations tend to be met with pushback from harvesters. Following Delaware and implementing a male-only harvest would be the easiest regulation change to protect large females and allow fishermen to continue using the resource. A biomedical-only harvest, such as the one South Carolina has, would likely be the best way to allow population levels to rebound. However, this is unlikely to be implemented except as a last resort, due to the number of bait harvesters who rely on the fishery for income.

Fisheries managers could also consider the strategic temporal closures of certain embayments. Sites could be chosen to close temporarily for a given length of time, which should be determined as biologically significant. Typically a minimum of one generation interval would be required, although a ten-year closure is likely to be met with resistance from harvesters. Managers would need to closely monitor spawning population size and juvenile recruitment in an area to determine if a shorter length of time would achieve positive results. For example, one harbor could be closed for five years and fishing pressure would shift to other locations across the state. Then if after five years the site has shown enough signs of recovery, it could be reopened and a different site would close for the next five years. If the site has not recovered to a satisfactory level, then the closure could be extended. Staggering closures in this fashion would allow some overfished

populations to recover a little while not putting too much pressure on any one other population. Managers could also institute some permanent closures if some populations are determined to be more damaged or sensitive.

Managers should also be aware that fishing is not the only threat faced by horseshoe crabs (Berkson 2009). Sea walls, jetties, and other types of beach revetments prevent the natural accretion of sand, effectively washing away the sandy beach habitat horseshoe crabs need for spawning (Jackson and Nordstrom 2009). Dredging harbors and channels can kill horseshoe crabs and should be scheduled for times outside of the breeding season. Pollutants may also do harm to horseshoe crab populations. Some insecticides are designed to inhibit the molting process and are known to adversely affect marine invertebrates when introduced to the estuarine system (Zulkosky et al. 2005). Other unknown effects of chemical runoff can be damaging to horseshoe crab recruitment. Cooperation among fisheries managers, environmental groups and the entire community is essential to combating the many threats to horseshoe crab propagation and survival.

TABLES

Table 1. Number of individuals used in final statistical analyses and their site number for locating on corresponding map in Figure 2.

Population	Number of Individuals	Site Number (Figure 2)
Barnstable Harbor	25	3
Wellfleet Bay	45	4
Nauset Estuary	30	5
Pleasant Bay	21	6
Stage Harbor	37	7
Nantucket	27	8
Buzzards Bay	8	9
Total	193	

Table 2. Number of different alleles identified at each locus in each population. Number of individual alleles sampled at each locus ($2N$) in each population is listed beside population name.

Locus	Number of Different Alleles	Barnstable Harbor (50)	Wellfleet Bay (90)	Nauset Estuary (60)	Pleasant Bay (42)	Stage Harbor (74)	Nantucket (54)	Buzzards Bay (16)
<i>LpoA58</i>	11	6	8	8	6	9	9	5
<i>LpoA64</i>	8	3	3	5	2	6	3	2
<i>LpoD60</i>	8	4	7	6	4	6	5	4
<i>LpoA38</i>	15	9	8	9	9	11	11	8
<i>LpoA52</i>	12	5	7	6	5	9	6	3
<i>LpoD3</i>	17	12	9	11	11	11	10	5
Total across all Loci	71	39	42	45	37	52	44	27

Table 3. Allelic richness and heterozygosity averaged across all loci. Allelic richness is weighted by the Buzzards Bay sample size ($N = 8$).

Site	N	Average number of alleles	Allelic Richness	Heterozygosity	F_{IS}
Barnstable Harbor	25	6.5	4.4	0.682	-0.046
Wellfleet Bay	45	7.0	4.3	0.691	0.03
Nauset Estuary	30	7.5	4.8	0.702	0.027
Pleasant Bay	21	6.2	4.3	0.667	0.084
Stage Harbor	37	8.7	5.1	0.708	0.001
Nantucket	27	7.3	4.9	0.699	0.063
Buzzards Bay	8	4.5	4.5	0.674	0.073

Table 4. Allele frequencies for each site by locus. Alleles are listed by their size in base pairs. The number of alleles sampled at each locus ($2N$) in each population is listed in parenthesis beside each population name.

Locus	Alleles (bp)	Barnstable Harbor (50)	Wellfleet Bay (90)	Nauset Estuary (60)	Pleasant Bay (42)	Stage Harbor (74)	Nantucket (54)	Buzzards Bay (16)
<i>LpoA58</i>	91	0.44	0.422	0.283	0.357	0.473	0.259	0.5
	93	0	0	0	0	0.014	0	0
	95	0.02	0.067	0.033	0.048	0.027	0.056	0
	97	0.02	0	0	0	0	0	0
	101	0.46	0.4	0.5	0.452	0.27	0.407	0.25
	103	0	0.022	0.05	0	0.014	0.056	0
	105	0.04	0.011	0	0	0.027	0.093	0.125
	107	0	0.011	0.017	0.024	0.027	0.056	0.062
	109	0.02	0.011	0.05	0.071	0.027	0.019	0
	111	0	0.056	0.05	0.048	0.122	0.037	0.062
	113	0	0	0.017	0	0	0.019	0
<i>LpoA64</i>	124	0	0	0.017	0	0	0	0
	132	0	0	0	0	0.014	0	0
	134	0.52	0.556	0.55	0.548	0.419	0.352	0.375
	135	0	0	0	0	0.014	0	0
	136	0	0	0.017	0	0.027	0	0
	144	0.42	0.422	0.4	0.452	0.514	0.611	0.625
	146	0.06	0.022	0.017	0	0	0.037	0
	148	0	0	0	0	0.014	0	0
<i>LpoD60</i>	167	0	0.011	0	0	0	0.019	0
	171	0.02	0.044	0.067	0.024	0.041	0.074	0.062
	175	0.5	0.422	0.4	0.429	0.486	0.389	0.312
	179	0.16	0.256	0.217	0.262	0.216	0.185	0.25
	183	0.32	0.244	0.283	0.286	0.216	0.333	0.375
	187	0	0.011	0.017	0	0.014	0	0
	191	0	0	0.017	0	0.027	0	0
	217	0	0.011	0	0	0	0	0
<i>LpoA38</i>	125	0	0	0	0.024	0	0	0
	129	0.02	0	0.033	0	0.014	0.019	0
	131	0	0	0	0.048	0.027	0	0.062
	132	0	0	0	0	0	0.019	0
	133	0.04	0.189	0.083	0.024	0.041	0.019	0.125
	135	0.28	0.322	0.283	0.452	0.284	0.222	0.25
	137	0.3	0.2	0.3	0.333	0.419	0.407	0.188
	139	0.02	0.022	0.017	0.024	0.041	0.056	0.062
	140	0	0	0	0	0.014	0	0
	141	0	0.011	0	0.024	0	0	0
	143	0.04	0.033	0.067	0	0.041	0.074	0.062
	145	0.08	0.211	0	0	0.027	0.13	0.188
	147	0.2	0	0.083	0.048	0.054	0.019	0.062
	149	0	0	0.083	0.024	0.041	0.019	0
153	0.02	0.011	0.05	0	0	0.019	0	
<i>LpoA52</i>	143	0	0.011	0	0	0	0	0
	153	0	0	0	0	0.014	0	0
	155	0	0.011	0	0	0.014	0	0
	157	0.22	0.178	0.217	0.095	0.122	0.13	0
	159	0	0	0	0	0	0.037	0
	161	0	0	0	0	0.041	0	0
	163	0.28	0.256	0.217	0.238	0.162	0.222	0.125
	166	0	0	0	0	0.014	0	0
	167	0.38	0.422	0.467	0.571	0.446	0.519	0.688
	169	0.04	0.078	0.05	0.071	0.095	0.037	0.188
	171	0	0	0.017	0	0	0	0
173	0.08	0.044	0.033	0.024	0.095	0.056	0	
<i>LpoD3</i>	134	0.06	0.178	0.133	0.167	0.122	0.185	0.125
	138	0.1	0.089	0.067	0.119	0.095	0.093	0.062
	140	0	0	0	0	0.027	0	0
	142	0.46	0.411	0.433	0.357	0.378	0.444	0.562
	146	0.02	0.011	0.033	0.024	0.027	0.037	0
	147	0	0	0	0.024	0	0	0
	150	0.08	0.022	0.033	0.024	0.095	0.056	0
	154	0	0	0	0	0.041	0.019	0
	156	0.04	0.044	0.033	0.024	0.041	0.056	0
	160	0.02	0	0.033	0.048	0.041	0	0.125
	162	0	0	0.017	0	0	0	0
	163	0	0	0	0	0	0.019	0
	164	0.12	0.2	0.183	0.143	0.108	0.074	0.125
	168	0.04	0.022	0	0	0	0	0
	172	0.02	0.022	0.017	0.048	0.027	0	0
176	0.02	0	0	0	0	0.019	0	
180	0.02	0	0.017	0.024	0	0	0	

Table 5. Pairwise values for G'_{ST} (above diagonal) and G''_{ST} (below diagonal). Population pairs with significant results from Fisher's exact test for genic differentiation are highlighted in blue.

	Barnstable Harbor	Wellfleet Bay	Nauset Estuary	Pleasant Bay	Stage Harbor	Nantucket	Buzzards Bay
Barnstable Harbor	—	0.004	-0.004	0.002	0.006	0.009	0.016
Wellfleet Bay	0.014	—	0.002	0.003	0.013	0.015	0.005
Nauset Estuary	-0.013	0.007	—	-0.008	0.009	0.004	0.014
Pleasant Bay	0.005	0.008	-0.025	—	0.002	0.004	0.002
Stage Harbor	0.019	0.043	0.029	0.005	—	0.004	0.001
Nantucket	0.030	0.051	0.014	0.014	0.014	—	-0.006
Buzzards Bay	0.049	0.016	0.044	0.006	0.003	-0.02	—

FIGURES

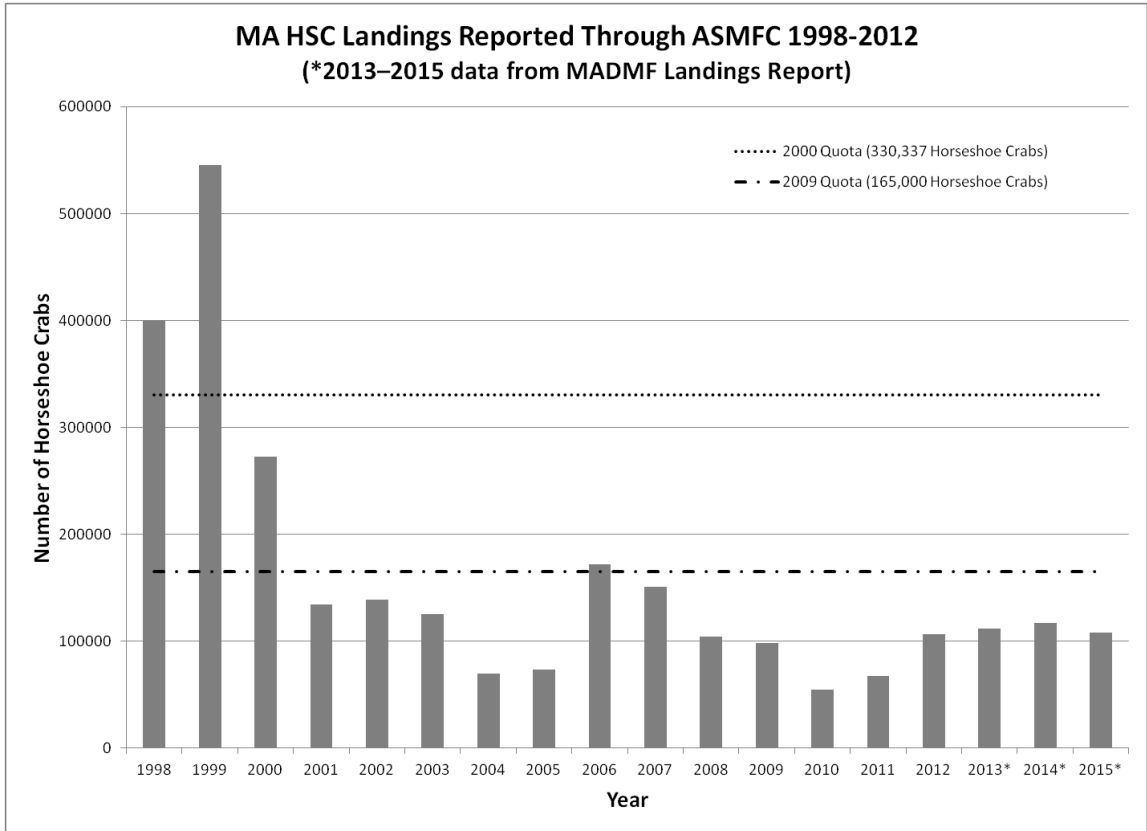
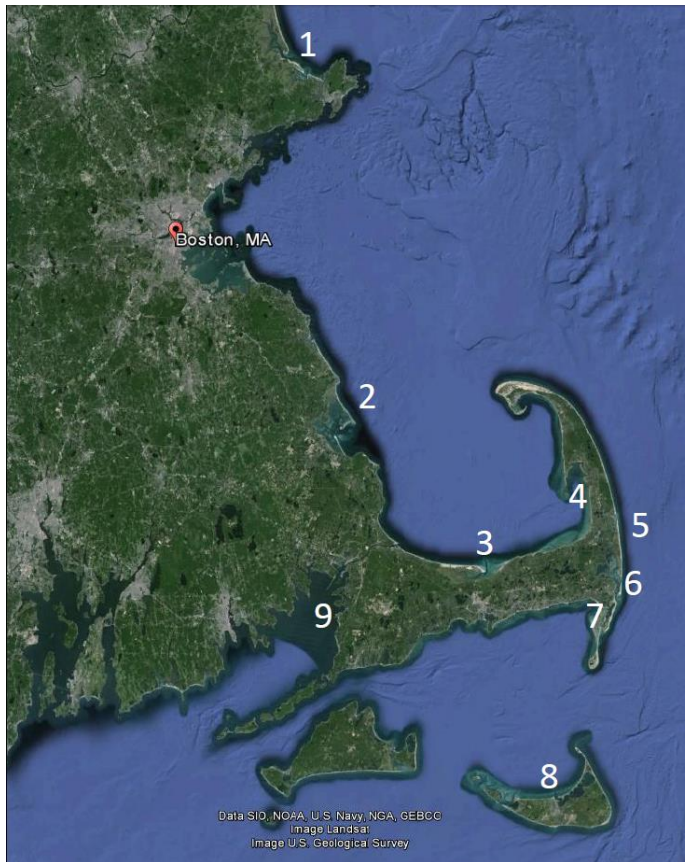


Figure 1. MA horseshoe crab fishery landings 1998–2015. Massachusetts landings for horseshoe crabs as reported by the Atlantic States Marine Fisheries Commission from 1998 through 2012 (ASMFC 2013). Data for 2013, 2014, and 2015 (as of 28 Nov 2015) reported by the Massachusetts Division of Marine Fisheries (<http://www.mass.gov/eea/agencies/dfg/dmf/commercial-fishing/quotas-and-landings/>).



Sampling Sites

- 1 - Plum Island Estuary (2)
- 2 - Duxbury Bay (31)
- 3 - Barnstable Harbor (42)
- 4 - Wellfleet Bay (50)
- 5 - Nauset Estuary (50)
- 6 - Pleasant Bay (50)
- 7 - Stage Harbor (50)
- 8 - Nantucket (40)
- 9 - Buzzards Bay (12)

Figure 2. Sampling locations. The number of tissue samples collected at each site is listed in parentheses.

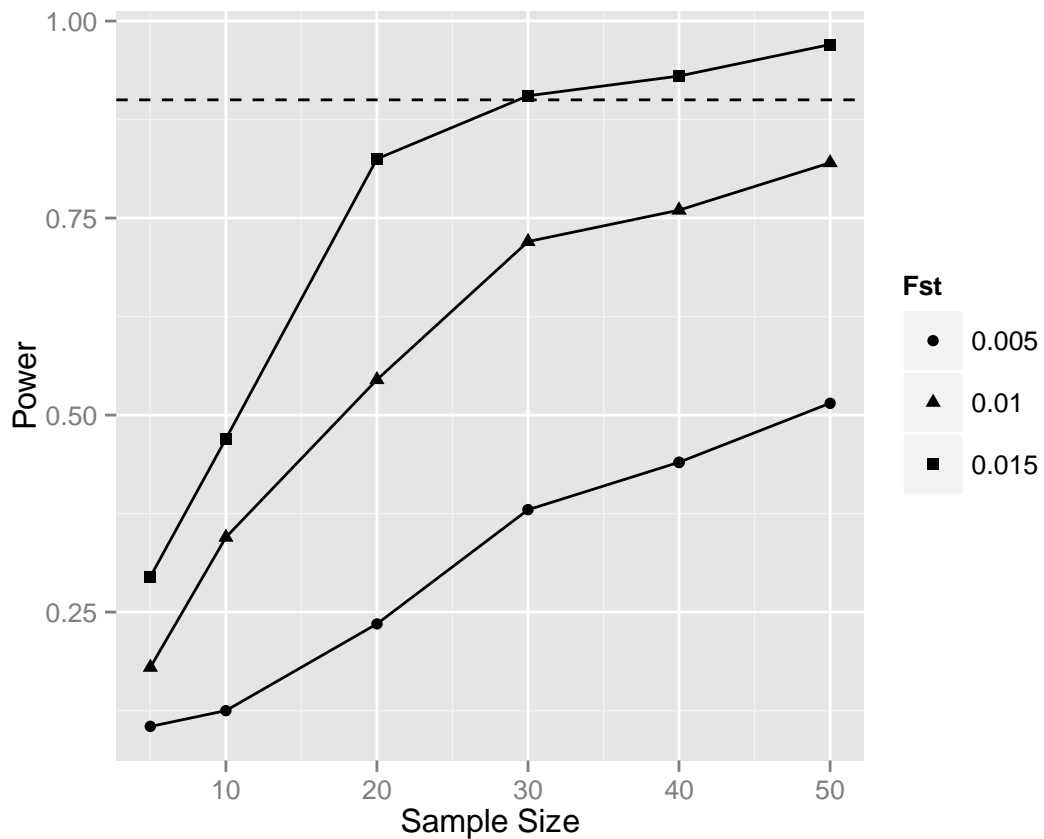
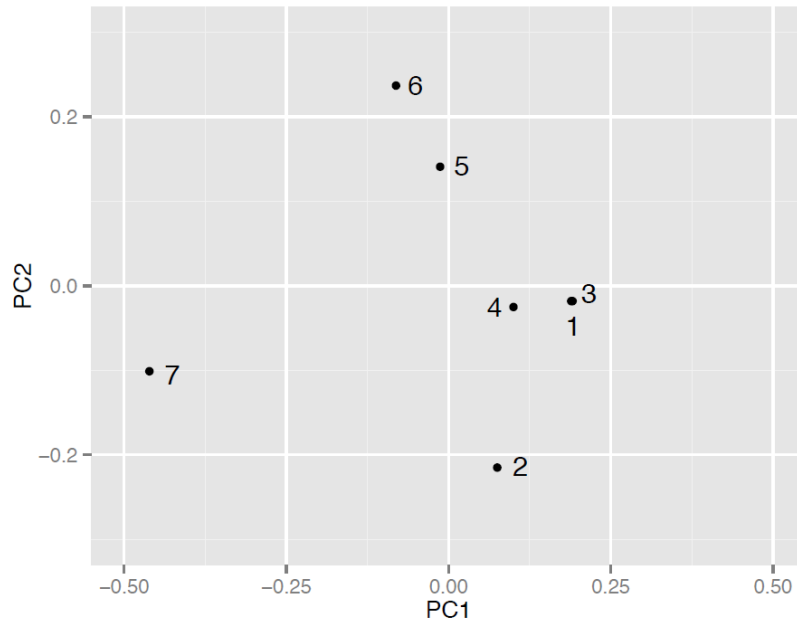


Figure 3. Power analysis. Simulations to test the power of 6 loci to detect genetic differentiation in horseshoe crabs with $N_e = 2000$ for $t = 20$ (expected $F_{ST} = 0.005$, circles), $t = 40$ (expected $F_{ST} = 0.010$, triangles), or $t = 60$ (expected $F_{ST} = 0.015$, squares) generations. Power (y-axis) represents the proportion of simulations where genic exact tests were significant ($p < 0.05$). The dashed line shows power = 90%.

(a)



(b)

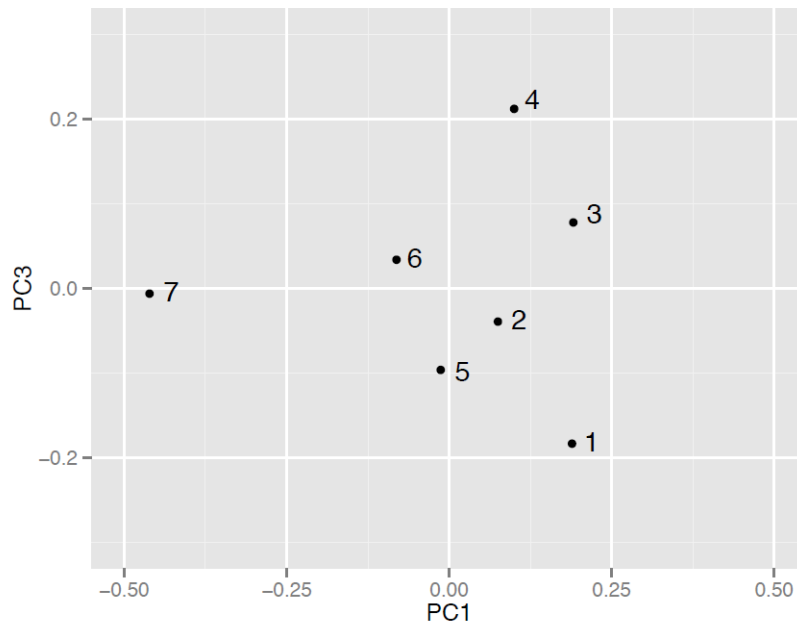
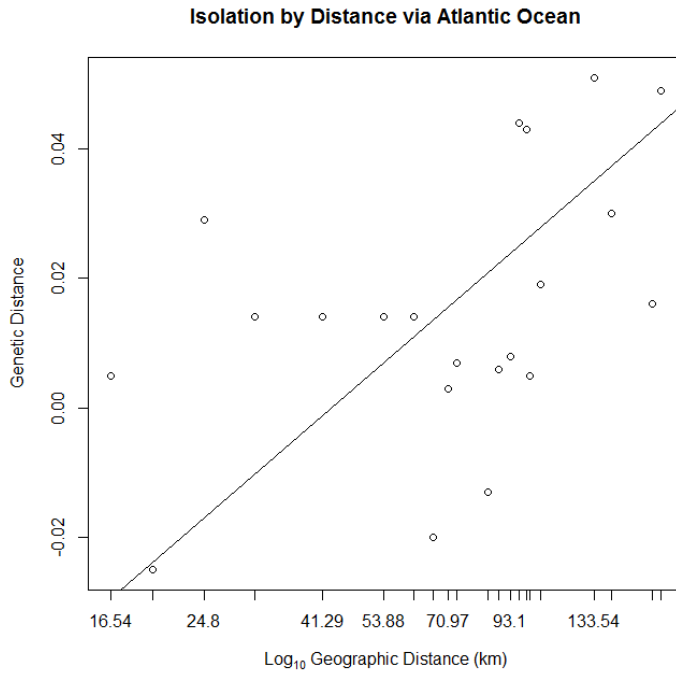


Figure 4. Principal component analysis. Populations are: 1. Barnstable Harbor, 2. Wellfleet Bay, 3. Nauset Estuary, 4. Pleasant Bay, 5. Stage Harbor, 6. Nantucket, and 7. Buzzards Bay. PC 1 accounts for 42.33% of the variance, PC 2 accounts for 18.49% (60.82% cumulative), and PC 3 accounts for 13.30% (74.12% cumulative). Buzzards Bay diverges from all other sites along PC 1. (a) PC 1 versus PC 2. Stage Harbor and Nantucket diverge together along PC 2, while Wellfleet Bay diverges the other way. (b) PC 1 versus PC 3. Pleasant Bay diverges along PC 3, as does Barnstable Harbor in the opposite direction.

(a)



(b)

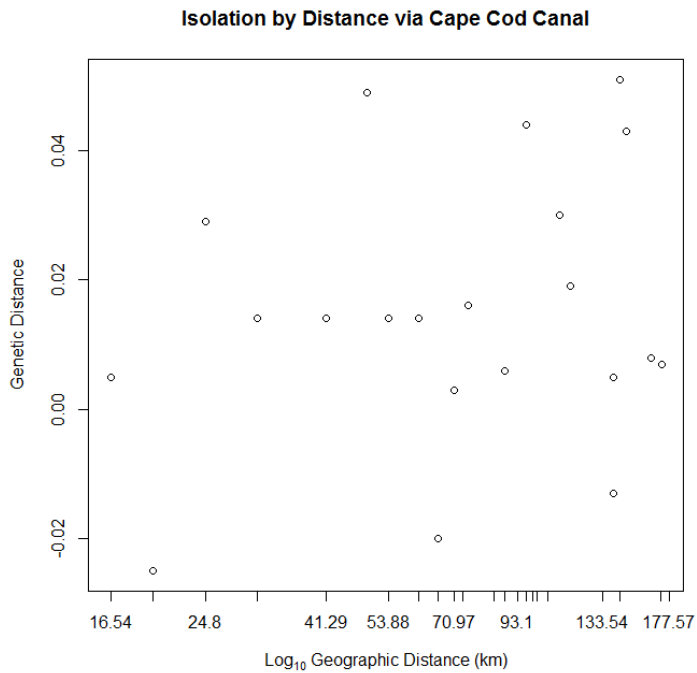


Figure 5. Isolation by distance. (a) Travel via the Atlantic Ocean shows a better fit (Mantel $R = 0.4553$, $p = 0.0170$) to the data than (b) travel via the Cape Cod Canal (Mantel $R = 0.2210$, $p = 0.1580$).

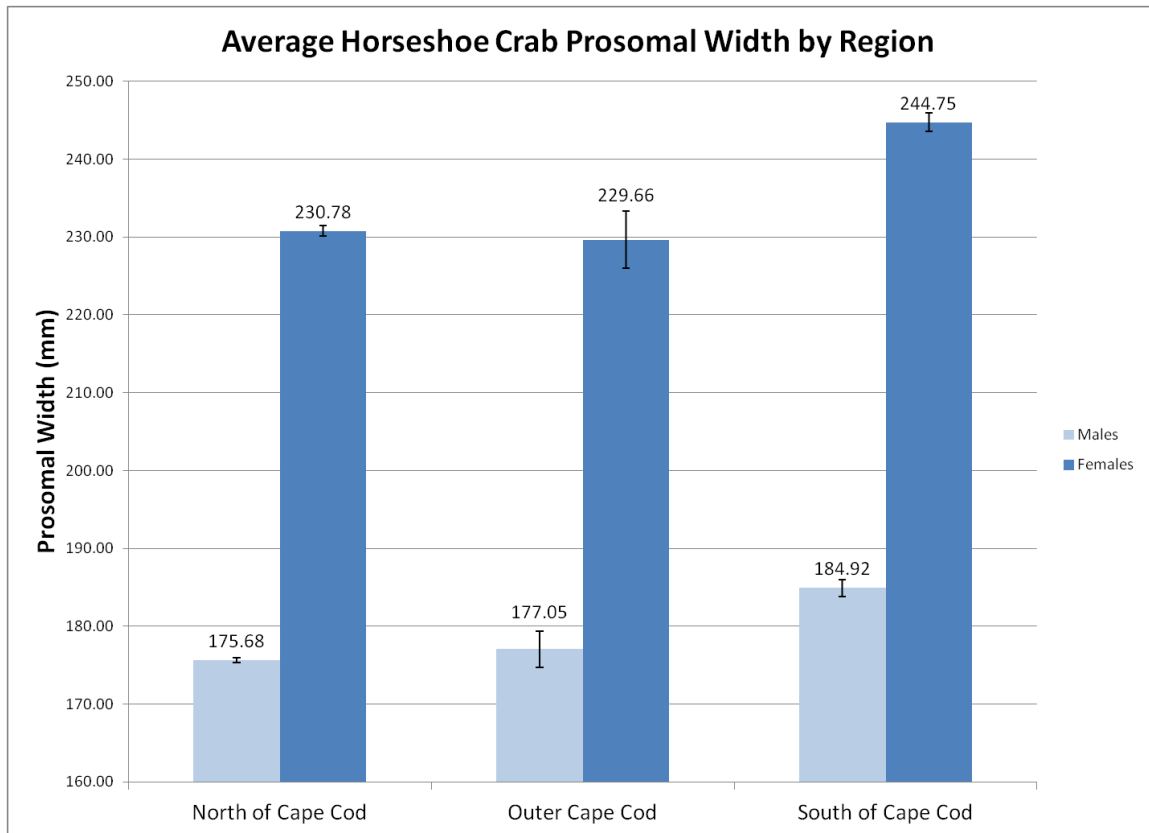


Figure 6. Prosomal widths by region. Average prosomal width (with standard error) of horseshoe crabs from across Massachusetts. North of Cape Cod sites: Plum Island Estuary, Duxbury Bay, Barnstable Harbor and Wellfleet Bay. Outer Cape Cod sites: Nauset Estuary and Pleasant Bay. South of Cape Cod sites: Buzzards Bay, Stage Harbor and Nantucket. Only the 2010 data collected specifically for this study were used in the P_{ST} analysis. This chart includes additional data collected between 2007 and 2010 (K. Johnson and S. Martinez, Massachusetts Audubon Society, unpublished data).

(a)

```
> p.var <- var(dat$Width, na.rm = TRUE)

> prior1.1 <- list(G = list(
+   G1 = list(V = matrix(p.var/2), n = 1)),
+   R = list(V = matrix(p.var/2), n = 1))

> model1.1 <- MCMCglmm(Width~Sex, random = ~Site,
+   data = dat, nitt = 65000, thin = 50,
+   burnin = 15000, prior = prior1.1)

> posterior.mode(model1.1$VCV)
  Site      units
194.3135  289.1808

> HPDinterval(model1.1$VCV)
      lower      upper
Site    84.49808  838.5560
units  258.29808  352.2714
attr(,"Probability")
[1] 0.95
```

(b)

```
> p.var <- var(dat$Width, na.rm = TRUE)

> prior1.3 <- list(G = list(
+   G1 = list(V = matrix(p.var*0.05), n = 1)),
+   R = list(V = matrix(p.var*0.95), n = 1))

> model1.3 <- MCMCglmm(Width~Sex, random = ~Site,
+   data = dat, nitt = 65000, thin = 50,
+   burnin = 15000, prior = prior1.3)

> posterior.mode(model1.3$VCV)
  Site      units
175.9346  292.5059

> HPDinterval(model1.3$VCV)
      lower      upper
Site    56.25056  653.3732
units  255.83073  353.3972
attr(,"Probability")
[1] 0.95
```

Figure 7. P_{ST} GLMM R code. GLMM inputs and outputs in R for P_{ST} models with (a) evenly weighted priors and (b) priors weighted towards within site variation. Each shows the priors used in the model, the sum of squares (posterior.mode) for among (Site) and within (units) sampling sites, and the 95% confidence interval (HPDinterval). Note that the specific outputs will change with multiple runs of the code due to Monte Carlo sampling.

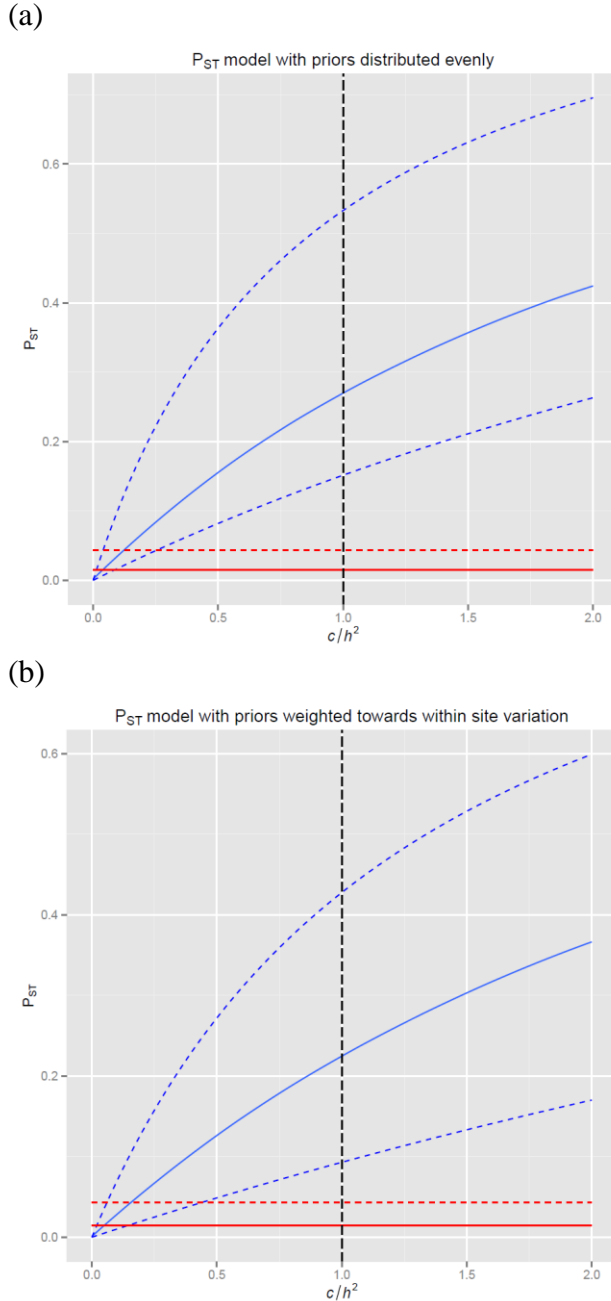


Figure 8. P_{ST} models. P_{ST} mixed effects model (blue line) plotted over a range of values for c/h^2 as compared to G''_{ST} (red line). The blue dashed lines are the 95% confidence intervals (CIs) for P_{ST} . The red dashed line is the upper 95% CI for G''_{ST} . The dashed black line represents the point at which $c = h^2$, the threshold at which P_{ST} exceeds that which is expected by drift alone (Brommer 2011). (a) When location priors are given equal weight, the lower CI for P_{ST} overlaps the upper CI of G''_{ST} at an approximate c/h^2 of 0.25. This is well below the $c = h^2$ threshold, indicating the comparison between P_{ST} and G''_{ST} to be relatively robust. (b) The lower CI for P_{ST} overlaps the upper CI of G''_{ST} around a c/h^2 of 0.05, still below the $c = h^2$ threshold, despite very large confidence intervals calculated using the most conservative priors (weighted towards variation within sites instead of among sites).

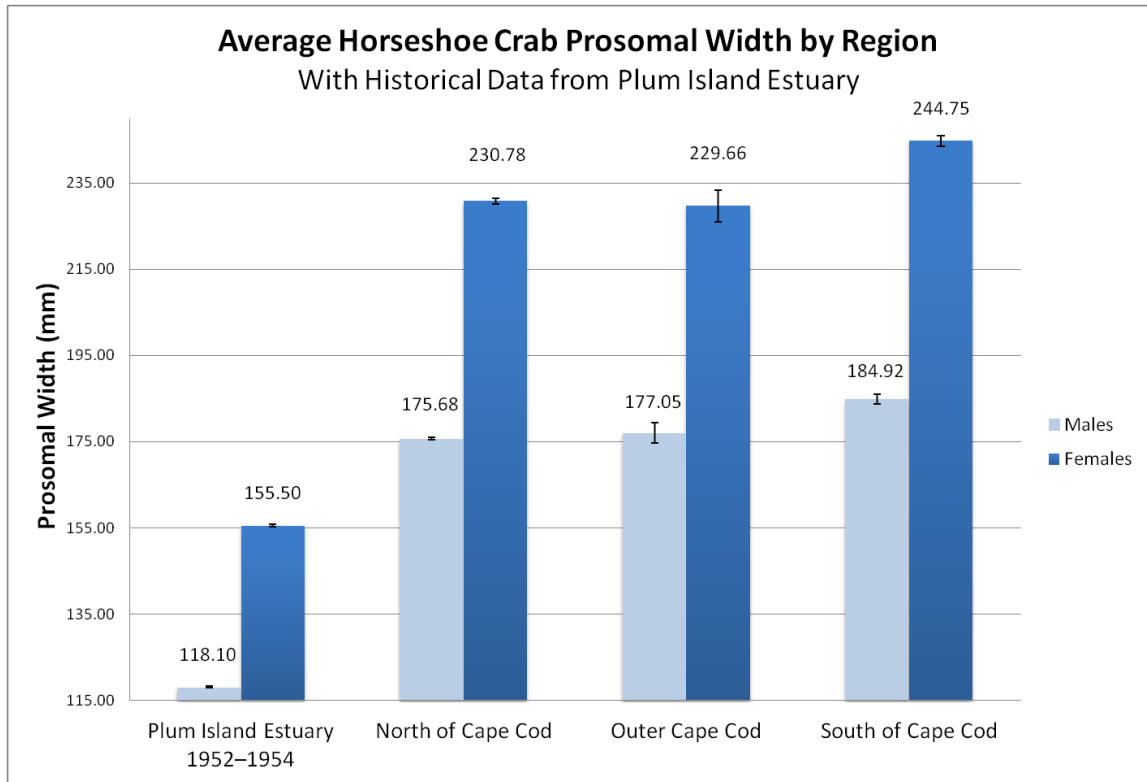


Figure 9. Prosomal widths by region with Plum Island Estuary historical data. Historical size data (with standard error) from Plum Island Estuary 1952–1954 (Baptist et al. 1957) as contrasted with average prosomal width of horseshoe crabs from across Massachusetts as presented in Figure 6. Historical data were not used in P_{ST} analysis.

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