FINAL PERFORMANCE REPORT
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Project Title: Determining the relative importance of coastal habitat types to Horseshoe Crab reproduction and migratory shorebird foraging in South Carolina

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Objectives:

1. Document the range of habitat types (*e.g.* sandy beach, shelly beach, marsh) used by spawning Horseshoe Crabs;

2. Identify significant Horseshoe Crab habitat by quantifying Horseshoe Crab egg development (and thus quality of habitat for Horseshoe Crabs) across each habitat type;

3. Spatially compare significant Horseshoe Crab spawning habitat with areas suitable for foraging shorebirds to quantify the proportion of Horseshoe Crab spawning habitats that are used by migratory shorebirds, representing a critical nexus of these species.

Accomplishments

The American Horseshoe Crab (*Limulus polyphemus*) is an ecologically- and economically-important species found in the United States along the Atlantic and Gulf coasts. Horseshoe Crabs are a vital food resource for multiple threatened and endangered species, including the migratory shorebird, *Calidris canutus rufa* (Botton 2009) that eats Horseshoe Crab eggs, and sea turtles that eat juvenile and adult Horseshoe Crabs (Keinath 1987, Seney & Musick 2007). Additionally, Horseshoe Crab hemolymph ("blood") is harvested from adults for sterilization testing in the biomedical industry (ASMFC 2019), and Horseshoe Crabs are harvested in the northeastern U.S. as bait for the eel and whelk fisheries. For these reasons, Horseshoe Crabs are managed across the Atlantic states of the United States (ASMFC 2019).

Management decisions are in part decided based on population health. One of the main contributors to estimations of population health occurs through surveys of Horseshoe Crab abundance. The methods used in these surveys vary but can consist of some combination of active sampling approaches (*e.g.* trawl, trammel net, seine) and spawning surveys. While active

sampling approaches have the potential to encounter and quantify both juvenile and adult Horseshoe Crabs present in the area, spawning surveys provide an index of the number of spawning adult Horseshoe Crabs. These surveys have traditionally taken place exclusively on beaches where Horseshoe Crabs aggregate to spawn. Population health assessments that use spawning surveys in their models assume that these surveys target areas where a high proportion of the Horseshoe Crabs spawn and that interannual variability in survey numbers are indicative of changes in population health. One or both assumptions, however, may not be valid if Horseshoe Crabs frequently spawn in locations that are not surveyed (*i.e.* non-beach areas).

Undisturbed sandy beaches with relatively low wave energy are considered preferred habitats for spawning Horseshoe Crabs and developing eggs, especially in Delaware Bay and other parts of the northeastern U.S. (Jackson *et al.* 2020; Smith *et al.* 2002; Landi *et al.* 2015; Botton *et al.* 2022). On many beaches, spawning occurs at the high tide line, which is thought to provide the optimal conditions for embryonic development (Penn & Brockmann 1994, Vasquez *et al.* 2015a, b). Spawning has been observed in a variety of other habitats throughout their range, including muddy substrates, fringing salt marsh, and loam-clay sediments (Rosales-Raya 1999; Beekey & Mattei 2008; Shuster & Sekiguchi 2009; Castro 2019), but these observations are often considered rare and non-adaptive (Botton *et al.* 1988).

While the majority of South Carolina (SC)'s coastline consists of salt marsh habitat dominated by smooth cordgrass, *Spartina alterniflora* (Tiner 1974, 2013), prior to this study, spawning surveys of Horseshoe Crabs in SC have been conducted exclusively on beaches. Reports to SCDNR, however, suggest that Horseshoe Crabs commonly use salt marsh habitat for spawning. If so, current spawning surveys may significantly underestimate the number of reproductively active animals in the population.

The contribution of salt marsh habitat to population recruitment will depend on the frequency with which the habitat is used for spawning, as well as the differences in the viability of embryos across habitats. While salt marsh habitat has often been considered sub-optimal for development (*e.g.* Botton *et al.* 1988, 2022), this has rarely been empirically tested. Recent research based on laboratory studies, however, has shown that low oxygen content and temperature may slow or cease embryonic development in Horseshoe Crabs (Funch *et al.* 2016, Vasquez *et al.* 2015a, b), both of which might be common in salt marsh habitat (Timmerman & Chapman 2004, Bradley & Morris 1990). Furthermore, a recent experiment documented lower rates of embryonic development in Horseshoe Crabs reared in mud compared to sand (Castro 2019). Despite potentially slower developmental rates, salt marshes still have the potential to contribute substantially to Horseshoe Crab recruitment.

Many shorebirds rely on Horseshoe Crab eggs laid in sandy beach habitats as a food resource. This includes the 31 species listed within the migratory shorebird guild of the SC State Wildlife Action Plan (SWAP) and Supplemental Volume (SCDNR 2015; https://www.dnr.sc.gov/swap/index.html), such as the federally listed *Rufa* Red Knot, *Calidris canutus rufa* (Botton *et al.* 2009). The shorebird guild listed in the SC SWAP is represented by 3 families: Scolopacidae (sandpipers), Charadriidae (plovers), and Recurvirostridae (avocets). These birds generally forage by probing in the sand or mud for food items. Some of these birds can migrate large distances. For example, the *Rufa* Red Knot makes a trans-polar migration from

the southern tip of South America to breeding grounds in the Canadian Arctic. During this migration, most Red Knots stop along the U.S. Atlantic Coast where they spend two or more weeks bulking up in order to complete their migration and successfully breed. Shorebirds can consume large amounts of Horseshoe Crab eggs with bird stomach contents often dominated by this prey item (Tsipoura & Burger 1999). Given the reliance of migratory Red Knot on Horseshoe Crab eggs, declines in Red Knot populations in the Delaware Bay region have been linked to a reduced availability of Horseshoe Crab eggs (Baker *et al.* 2004).

While shorebirds, such as the *Rufa* Red Knot, are known to use a variety of coastal habitats, including open beaches and marshes, these birds move on to beaches to forage during periods when Horseshoe Crab eggs are most abundant (Burger *et al.* 1997). The ability of Red Knots to forage on these eggs depends on the abundance of spawning Horseshoe Crabs and the depth of the eggs. Female Horseshoe Crabs bury eggs to a depth up to 20 cm (Botton *et al.* 1994), but Red Knots are generally thought to be restricted to feeding in the top 5 cm of the sediment (Botton *et al.* 1994; Yang *et al.* 2013; Smith *et al.* 2020; Takahashi *et al.* 2021). Wave action and bioturbation can both contribute to the shifting of eggs from deep (*e.g.* 20 cm) into shallow (≤ 5 cm) areas of the sediment (Nordstrom *et al.* 2006).

Horseshoe Crabs and certain migratory shorebird species, including the *Rufa* Red Knot, are listed as priority species within the SC SWAP, warranting a detailed description of the nexus between Horseshoe Crab spawning habitat and shorebird foraging areas. By identifying habitats used by both spawning Horseshoe Crabs and shorebirds, researchers will have more targeted information that could allow for the designation of habitat that is critical to maintaining populations of these species and important trophic linkages in the coastal environment.

Methods

Objectives 1 and 2

Public reports: Information on locations where Horseshoe Crab spawning occurred were collected from state-wide public reports using an online reporting form. Most spawning reports included the following information: date/time, GPS coordinates, location description, photograph, approximate number of Horseshoe Crabs, and observer contact information. These reports were reviewed and habitat type (*i.e.* beach, marsh or neither) was determined. Social media (Twitter, Facebook, Instagram) was used to promote the use of this online survey tool.

Spawning Surveys: Surveys of Horseshoe Crab spawning activity were conducted at three locations during the spawning season in 2021: Harbor Island, Coffin Point, and Grice Beach, SC. At Harbor Island and Coffin Point, we conducted these surveys in both beach and marsh habitat. Grice Beach was a new area where systematic spawning surveys had not recently been conducted but where we had received reports of spawning activity. All spawning surveys were conducted at high tide on days on, or around, the full and new moon in early April and in June. In late April and May, additional surveys were conducted as part of a USFWS grant award (SC-T-F16AF01121). Together, these two grants allowed for a full bi-weekly sampling of spawning activity from early April to June. Only results associated with grant award SC-T-F19AF00749 are reported here. At each location, two people walked along a 2 m wide transect and recorded

the presence and description (sex, mating status) of any Horseshoe Crabs within the transect. Once the survey was complete, Horseshoe Crabs that were not actively spawning (*i.e.* not dug in) were tagged with Horseshoe Crab tags provided to SCDNR from USFWS and their condition recorded.

Egg Sampling surveys: To document the range of habitats used by spawning Horseshoe Crabs, we surveyed beach and marsh habitats in St. Helena Sound, SC for evidence of spawning activity, (*i.e.* presence of Horseshoe Crab eggs and embryos, in both 2020 and 2021). In 2020, we divided St. Helena Sound, SC into five regions that contained both marsh and beach habitat with sampling taking place from June 29 to July 22 (Fig. 1). Within each region, we sampled 10 randomly selected points in beach habitat and 10 randomly selected points in salt marsh habitat. Random points were selected using the U.S. Geological Survey (USGS) National Hydrology Dataset (NHD). Hydrologic GIS data for the level 4 USGS hydrologic unit 0305 were downloaded in ESRI Geodatabase format from the NHD website. Polygon data from the "NHDArea" feature class were extracted for the area of interest in the St. Helena Sound, SC. These data were converted to polylines to create a set of lines representing both banks of hydrologic features, as appropriate. Data were observed over USDA National Agriculture Imagery Program 2017 imagery and edited using GIS methods to improve the match of NHD lines to the imagery, and to remove features that were obviously erroneous or were impounded and therefore inaccessible from public boat ramps. All lines were assigned to "marsh" and "beach" habitat categories. Features were simplified using a GIS

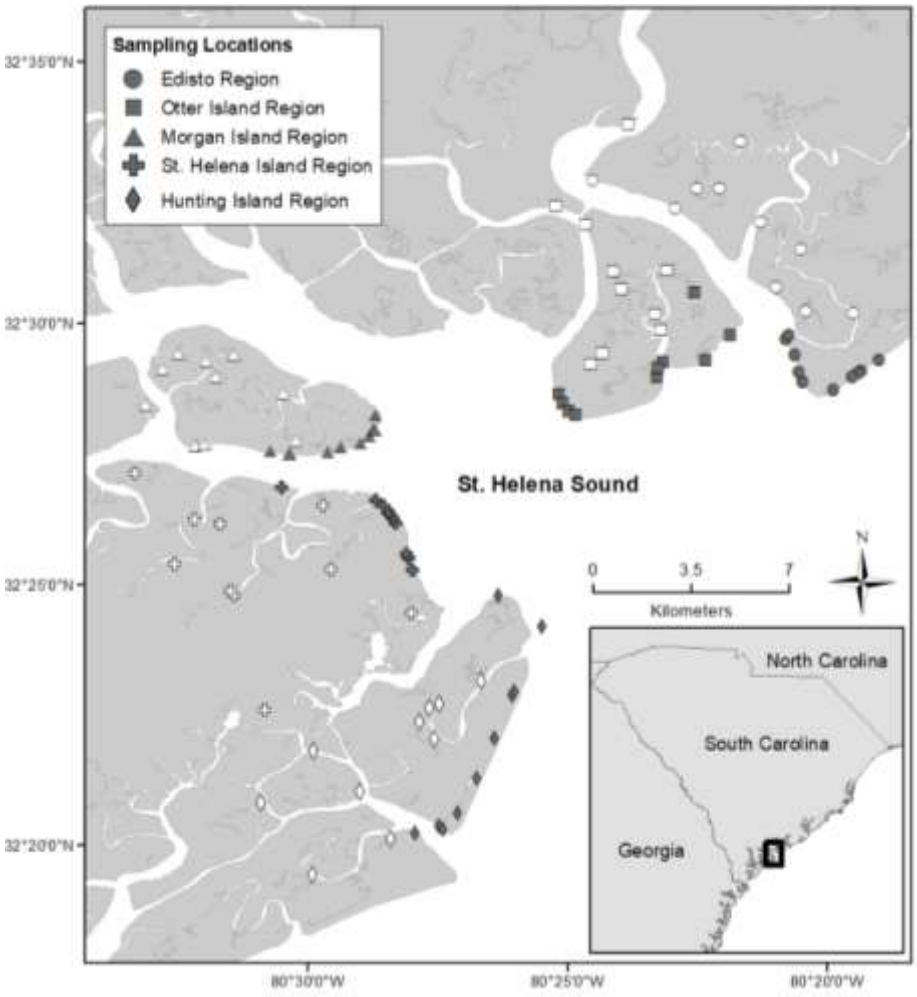

Figure 1. Sites from each of the 5 regions in St. Helena Sound, SC sampled for Horseshoe Crab eggs/embryos in 2020. White (open) symbols represent marsh sites, and grey (closed) symbols represent beach sites.

dissolve to single lines for each habitat category. Within each region, GIS methods were used to place 30 evenly spaced points along each habitat line type (*i.e.* 30 beach and 30 marsh points in each region). The distance between these 30 points differed by region based on available habitat. From each region and habitat type, 10 points were randomly selected as sampling locations.

In 2021, we conducted a more intensive survey within the Edisto/Otter/Pine Island region, as that region yielded the majority of Horseshoe Crab eggs in the previous year between May 13 to June 14 in 2021 (see Results). Within that region, we sampled 50 random points on beach habitat and 50 random points in salt marsh habitat. Random points were selected using a GIS polyline data layer of coastal shorelines. Shoreline data were extracted for the area of interest in the St. Helena Sound. Within the area of interest, shorelines were assigned to "marsh" and "beach" habitat categories. GIS methods were used to randomly place 50 points along the shorelines of each habitat line type. The distance between each point was no less than 100 m.

For both years, if a site was deemed inaccessible in the field, the nearest comparable and accessible site was surveyed by the field crews. GPS coordinates were recorded for all sampled sites. Upon arrival at each site, hand trowels were used to dig in areas near the high tide wrack line (beach) or within the marsh levee (marsh) to search for Horseshoe Crab eggs and embryos. Crews searched for eggs/embryos by digging holes 10-20 cm deep, which corresponds to the depth eggs are laid (Botton *et al.* 1994, Smith *et al.* 2002). We sampled at each site until we found eggs/embryos or until we had sampled for 30 person-minutes (*e.g.* one person sampling for 30 minutes, two people for 15 minutes, etc.), whichever occurred first. We documented the presence or absence of eggs/embryos and the linear distance or area sampled. In 2020, a subset of 4 marsh samples and 2 beach samples were retained and brought back to the lab for staging based on Kendrick *et al.* (2021). For the 2021 field season, we measured the hardness of the sediment using a pocket penetrometer (Forestry-Suppliers, model: LR – 280) which measured the pressure (in kg cm^{-2}) required for the device to penetrate the sediment surface. We took three sediment hardness measurements, one each at the beginning, middle, and end of the area sampled at each site and then took the mean of those measurements.

Fall surveys: In order to assess whether Horseshoe Crabs spawn in the fall, we searched for the presence of Horseshoe Crab eggs/embryos on Turtle Island on September 30, 2020, and at Pine Island, Big Bay Creek, and Dawes Island in October and November 2021. At each site, we dug 10 – 20 cm deep holes along the high tide line to look for the presence of Horseshoe Crab eggs/embryos.

Embryonic development: From each sample collected in 2020, we removed a random subset of embryos that were previously preserved in ethanol to stage developmental progress. We assigned each embryo into one of seven stages of development, following protocols published in Kendrick *et al.* (2021). Additionally, we counted the number of discolored embryos found in the subset of embryos. We stopped quantifying developmental progress and stopped counting discolored embryos once we had categorized the stages of 50 unstained embryos.

Egg viability: Experimental development trials were conducted in 2020 to compare the viability of eggs extracted from beach and marsh habitats. For two marsh locations and one beach location where eggs were found, eggs were retained, put on ice, brought to the Marine Resources

Research Institute (MRRI) and placed in a refrigerator. We removed 60 early stage (*i.e.* stages A & B) embryos from each sample and divided them up evenly between two glass dishes filled with 250 ml of artificial seawater at 25.6 ppt (± 0.19 SD). We counted the number of embryos that developed to the trilobite stages every seven days for 21 days. We changed the water twice a week (Tuesdays and Thursdays).

To test the viability of discolored eggs, we set up two rearing dishes as described above, each holding 30 discolored eggs. We changed the water in these dishes twice a week and counted the number of embryos that developed to the trilobite stage every seven days for 21 days.

Objective 3

To compare Horseshoe Crab spawning phenology with the presence of shorebirds, we conducted egg surveys and shorebird surveys in both beach and marsh habitat in 2022. Surveys were conducted along transects at Deveaux Bank and Otter Island at sites selected because they were known Horseshoe Crab spawning areas (SCDNR unpubl. Data, Cushman *et al.* 2019) with a large shorebird presence (*e.g.* Sanders *et al.* 2021).

Horseshoe Crab spawning phenology: To quantify how Horseshoe Crab spawning effort changes over the season, we conducted egg surveys every other week between April 4 and July 7, 2022, at one beach and one marsh site on both Deveaux Bank and Otter Island, totaling four sample sites. Sampling occurred along transects at mid to low tide approximately one week after each new and full moon cycle, when Horseshoe Crabs spawn in their greatest abundance. At beach sites, we selected transect locations based on previous sightings of Horseshoe Crab spawning activity in those areas. At marsh sites, we used ground truthing efforts to find transect locations along the marsh platform edge that matched marsh characteristics of areas where Horseshoe Crab eggs had been found in previous years (Kendrick *et al.* 2021). Transects at the Otter Island beach and marsh sites and at the Deveaux Bank beach site were 100 m in length and placed along the high tide wrack line (beach) and within the marsh levee (marsh). We sampled every 2.5 m along these transects, resulting in 40 total samples taken from each transect. At the Deveaux Bank marsh site the transect was only 40 m in total length due to site restrictions, thus samples were taken every 1.0 m instead of 2.5 m. We used a mud auger (AMS One-Piece Auger) to take core samples to a depth of 20 cm. The top 5 cm of each core sample (where eggs are thought to be accessible to shorebirds) was first carefully sorted by hand to determine if Horseshoe Crab eggs were present, then the remaining core sample (from 5 cm to 20 cm) was sorted. At beach sites, core samples were sieved in the field using 1 mm stainless steel mesh sieves (the top 5 cm of the core sample was sieved first, followed by the rest of the core) and any eggs found were placed in labeled Ziploc bags. At marsh sites, if eggs were found in the sample, the entire core sample was placed in a labeled Ziploc bag (making sure to separate sediment from the top 5 cm and the remaining core sample in different labeled bags). We placed all samples in a cooler with ice for transport back to the MRRI for further processing. All samples were placed in a walk-in refrigerator at the MRRI after field sampling until processing could be completed to prevent the development of the eggs/embryos. After sieving and sorting, eggs were counted in a clear, glass dish, using a clicker counter. A subsample of 50 eggs from each sample was then placed in 95% ethanol and archived at the MRRI.

Shorebird surveys: Shorebird surveys were conducted within the same transect areas as the egg surveys. Surveys were conducted between April 2022 and June 2022 at Deveaux Bank and Otter Island beach sites. Surveys were conducted within a week of each egg survey (average time between egg and shorebird survey = 2.4 ± 1.7 days). Surveys typically lasted between 10 and 15 minutes, and all species and numbers of birds observed in the transect area were recorded along with individual bird behaviors such as roosting or foraging. Additionally, we collected avian fecal samples found within the transect during our egg surveys and brought these samples back to the MRRI for archiving. In the future, these samples may be used to document the presence of Horseshoe Crab egg/embryo material in the diet of shorebirds using genetic techniques. SCDNR Shorebird Biologist Felicia Sanders as well as SCDNR Wildlife Biologist Matt King conducted most of the shorebird surveys, with assistance from Maina Handmaker. Sanders also assisted with the project design.

Feasibility of using Unoccupied Aerial Vehicles for surveys: Uncrewed aerial vehicle (UAV) flights were conducted to test the applicability of this technology to document both Horseshoe Crab and species-specific shorebird abundance on beaches. On February 1, 2022, staff conducted UAV flights over flocks of foraging birds at Botany Bay, SC. The goals of these flights were to determine: 1) the height at which UAVs can be flown without causing foraging birds to scatter; and 2) whether bird species could be distinguished and counted using videos recorded by the UAV. Multiple passes were made by the UAV (DJI Phantom 4 Pro v2.0 with a 20-megapixel camera) over the bird aggregations, first starting at 200 ft in altitude and reducing altitude for each subsequent pass by 20 ft until the UAV was flown at a minimum altitude of 80 ft. We recorded videos of the birds at each pass, and an observer on the ground used a spotting scope to record whether the birds flushed or stopped foraging during the flight. SCDNR Shorebird Biologists Felicia Sanders and Janet Thibault assisted with the design and implementation of the UAV surveys.

Data analysis

Objectives 1 & 2

To compare habitat use by spawning Horseshoe Crabs, we conducted multiple analyses. In 2020, we compared the likelihood of finding eggs/embryos in the beach and marsh habitats with a binomial generalized linear mixed model using the 'glmer' function in the lme4 package in R version 3.5.1. Within the model, the presence or absence of eggs was the dependent variable, habitat type (*i.e.* beach or marsh) was a fixed effect, and region was a random effect. In 2021, statistical analyses were conducted using R version 3.6.1. We compared the likelihood of finding egg/embryos in the beach and marsh habitat with a chi-square test with eggs found (Y/N) as the dependent variable and habitat type as the independent variable. We compared the hardness of sediments in the beach and salt marsh using a one-way ANOVA. We also compared the hardness of marsh sediments where eggs were found to the hardness of marsh sediments at sites without eggs using a *t*-test. Note: We could not do a similar comparison at beach sites due to the paucity of beach sites with eggs that had a hardness measurement.

To compare the likelihood of finding eggs/embryos in core samples taken from the beach and the marsh during egg surveys conducted in 2022, we conducted a generalized logistic regression

model with a binomial distribution using the 'glm' function from the lme4 package in R version 4.2.1 This model was run separately for Deveaux Bank and Otter Island. The presence of eggs in each core sample (Y/N) was the dependent variable and habitat type was the independent variable. These analyses were conducted for both the overall likelihood of finding eggs and the likelihood of finding eggs in the top 5cm of the sediment. To determine whether the number of Horseshoe Crab eggs found in core samples differed between beach and marsh habitats, we conducted generalized linear models with a negative binomial distribution using the 'glm.nb' function from the lme4 package in R for each location. In the model, the number of eggs in each core sample was the dependent variable and habitat type was the independent variables. Only core samples with eggs/embryos were included (*i.e.* we did not use zero counts in the analyses). We only conducted this analysis for eggs found at Otter Island as only one core sample in the Deveaux Bank marsh contained eggs.

Objective 3

To understand the phenology of Horseshoe Crab spawning and egg availability to shorebirds in both beach and marsh habitats, we tracked changes in Horseshoe Crab egg occurrence (as presence/absence) and density over the Horseshoe Crab spawning season. For these models, we conducted generalized logistic regression models using the 'lme4' package in R version 4.2.1 with date (*i.e.* Julian day) as the independent variable. For occurrence we used the 'glm' function with a binomial distribution and 'logit link' function, and for egg density we used the 'glm.nb' function with a negative binomial distribution. In these analyses, only core samples with eggs were included (*i.e.* we did not use zero counts in the analyses). In the above analyses, marsh and beach samples were combined, but separate models were conducted for Deveaux Bank and Otter Island.

Egg/shorebird presence overlap: To compare the overlap of foraging shorebirds and Horseshoe Crab eggs, we conducted a generalized linear model with a negative binomial distribution using the 'glm.nb' function from the 'lme4' package in R to test for a relationship between the likelihood of finding eggs/embryos in core samples (measured as the percent of core samples containing eggs) and the number of foraging shorebirds in beach transects. We conducted this analysis only for the beach transects on Otter Island and Deveaux combined. We did not conduct a similar analysis for the marsh as shorebirds were never observed foraging in the marsh (Pers. Obs.).

Results

Objectives 1 & 2

Public reports: From April 10 – July 1, 2021, we received 55 reports of spawning Horseshoe Crabs spanning the SC coast. Although difficult to discern with certainty based on GPS coordinates and location descriptions, 6 of the 55 reports appear to be of Horseshoe Crabs spawning in marsh habitats. Two observers reported seeing previously tagged Horseshoe Crabs.

Spawning surveys: Our spawning surveys in early April 2021 at Coffin Point documented, for the first time during standardized SCDNR spawning surveys, adult Horseshoe Crabs spawning in

the salt marsh in SC. In fact, we recorded more Horseshoe Crabs spawning in the salt marsh during that survey (6 females, 17 males) than we saw during the concurrent spawning survey at the nearby Coffin Point beach site (3 females, 6 males). Our spawning surveys at Grice Beach generally saw low numbers of Horseshoe Crabs.

Egg surveys: In 2020, we found eggs at 2 out of 50 beach sites sampled and at 4 out of 50 marsh sites sampled. The two beach sites with eggs were both found in the Edisto region, while the four sites in the marsh with eggs were spread across three regions (Edisto, Otter Island, and Hunting Island). We found no significant difference between the two habitats in the likelihood of finding eggs (z-value = 0.83, p = 0.41). In 2021, we found eggs/embryos at 5 out of 50 randomly selected beach sites and at 7 out of 50 randomly selected marsh sites (Fig. 2). There was no significant difference in the likelihood of finding eggs and embryos in either habitat (χ^2 = 0.42, df = 1, p =0.51). As expected, sediments at beach sites were significantly harder than those at marsh sites (F-value = 20.3, df = 1, $p < 0.0001$). Within marsh habitats, we found no significant difference in the hardness of the sediment at sites where eggs were found compared to sites where eggs were not found (F-value = 2.0, p = 0.16). We documented no evidence of fall Horseshoe Crab spawning at any of the four locations surveyed in 2020 and 2021.

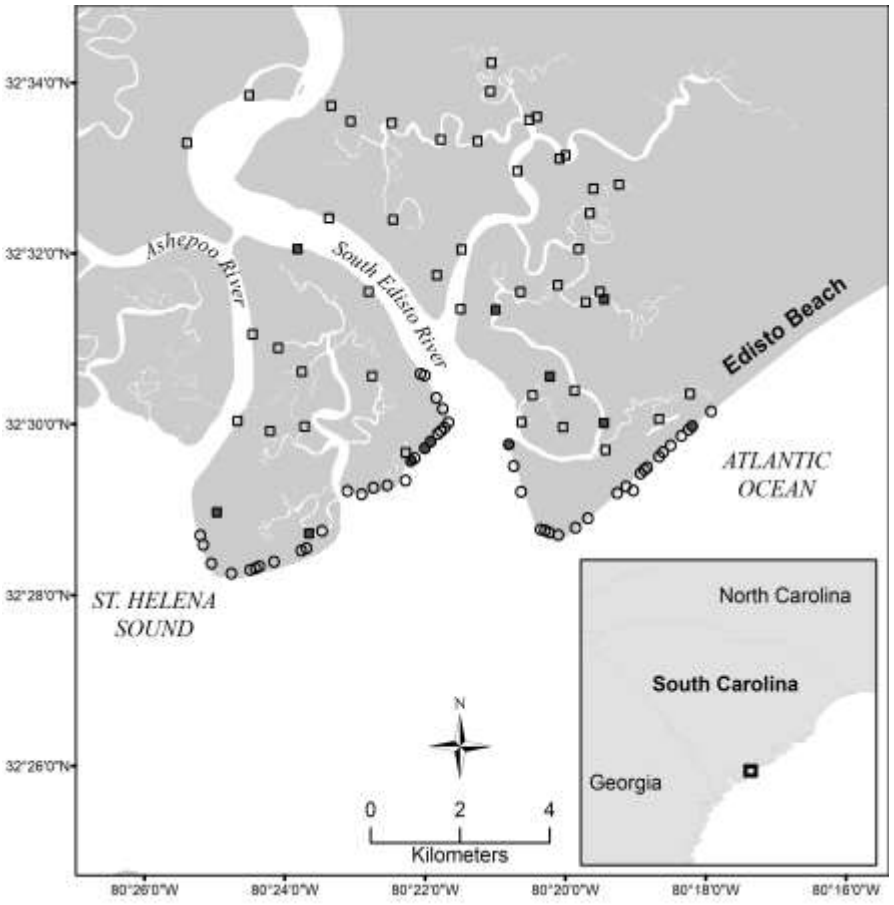

Figure 2. Locations of the 2021 Horseshoe Crab egg and embryo sampling sites in the Edisto/Pine/Otter Island region within St. Helena Sound, SC. Circles represent sandy beach sites and squares salt marsh sites. Filled in shapes indicates eggs/embryos were found at that site.

We found no difference in the likelihood of finding eggs between beach and marsh sites in our 2022 egg surveys at Otter Island (overall: z-value = -0.96, p = 0.337, top 5cm: z-value = 0.96, p = 0.34). We were, however, overall more likely to find eggs on the beach than in the marsh at Deveaux Bank (z-value = -2.1, p = 0.04), although this pattern was not found for eggs in the top 5cm of

the sediment (z-value = -0.05, p = 1.0). We found no difference in the number of eggs found per core sample between beach and marsh habitats on Otter Island (Fig. 3, z-value = 1.2, p = 0.22).

Embryonic development: 100% of the embryos collected from beach sites in 2020 were in the trilobite stage of development (*i.e.* stages F and G) compared to only 8.5% of marsh embryos. In contrast with embryos collected from beach sites, samples collected from the marsh sites had embryos at all stages of development. While we did not find discolored embryos in any of the beach samples collected, three of the four samples collected in the marsh contained discolored embryos.

Egg viability: In general, embryos reared at the MRRI had high rates of development. Over 90% of the embryos reached the trilobite stage after 21 days in two of the three samples. The third sample, taken from a marsh site, had lower development; only 63% of embryos reached the trilobite stage. We found low proportions of development for discolored embryos. Only 12 out of 60 (20%) discolored embryos reached the trilobite stage after 21 days.

Objective 3

Horseshoe Crab spawning phenology: A total of 41 core samples with eggs were found at Otter Island and 10 cores with eggs at Deveaux Bank. Three of the samples at Otter Island and one of the samples at Deveaux Bank contained eggs in the top 5 cm. The first cores with eggs were found at Otter Island on April 19, but not until May 3 at Deveaux Bank (Fig. 3). All the samples in the top 5 cm were found after June.

The likelihood of finding eggs in the entire core sample did not change with time at Otter Island (Fig. 4, z-value = -0.167, p = 0.867). The likelihood of finding eggs in the entire core sample increased with time at Deveaux Bank (z-value = 1.986, p = 0.047). There was not a significant effect of day on the number of eggs found in core samples at Otter Island (z-value = -1.657, p = 0.097) nor at Deveaux Bank (z-value = -0.576, p = 0.564).

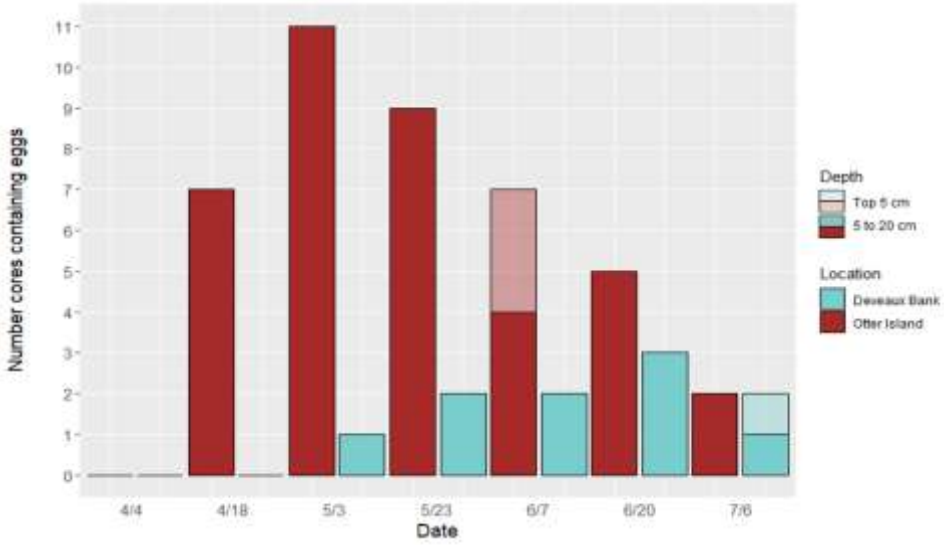

Figure 3. Mean number of eggs per core sample at Otter Island and Deveaux Bank in beach and marsh habitats found in the top 5 cm of the sediment and the rest of the core sample (5 – 20 cm).

Bird surveys: A total of 9 bird species were recorded in our Otter Island beach transect and 16 species in our beach transect at Deveaux Bank between April 2022 and June 2022 (Table 1). Only two species were seen in the marsh transect on Otter Island - Willet and Whimbrel – and both were seen only in one survey. No birds were seen in the marsh transect at Deveaux Bank. Shorebirds were seen foraging on the beach at both sites during all surveys; however, it could not be determined if these birds were foraging specifically on Horseshoe Crab eggs. No Red Knots were observed inside the transect areas, although one individual was observed foraging at the Otter Island beach outside the survey area.

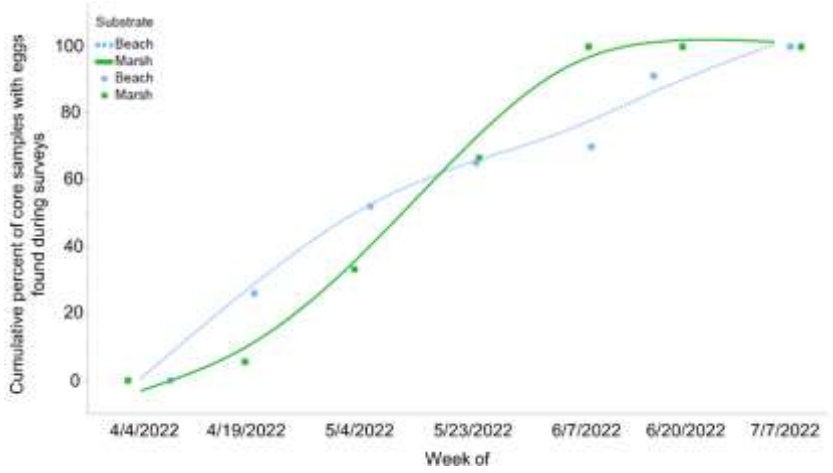

Figure 4. Cumulative total percent of core samples with eggs found at Otter Island in the beach (blue dashed line, blue circles) and marsh (green solid line, green squares) in 2022. The likelihood of finding eggs did not change with time at Otter Island in either habitat.

We collected 794 bird fecal samples from the beach and marsh transects at Otter Island and Deveaux Bank. These samples have been archived for future analyses.

Egg/shorebird presence overlap: We found that the likelihood of finding eggs in core samples on Otter and Deveaux Islands was not significantly correlated with the number of foraging shorebirds observed in the transect areas (z-value = 0.20, P = 0.84).

Feasibility of using UAVS for surveys: Aggregations of foraging shorebirds were disturbed and showed flushing behavior once a height of 80 ft was reached by the overhead UAV. Bird numbers from UAV footage at 200 ft and below could be counted with reasonable accuracy, but species identification of shorebirds was difficult at even the lowest altitudes.

Discussion

Multiple lines of evidence from this study indicate that Horseshoe Crabs in SC use salt marsh habitats to spawn, potentially at similar densities as they spawn on the beach. Staging data collected shows that eggs laid in the marsh develop similarly to those laid in the beach, although earlier research suggests that eggs developing in the marsh may be less viable (Kendrick et al. 2021). Given that in SC salt marsh habitat makes up over 95% of the coastline, if even a proportion of that coastline is used for spawning at levels similar to those found in this study, a considerable portion of Horseshoe Crabs that recruit into the SC population likely originate from eggs laid in salt marsh habitats.

Our surveys in early April 2021 were the first standardized spawning surveys to be conducted by the SCDNR in salt marsh habitat. During our surveys at Coffin Point and Harbor Island, we conducted simultaneous surveys in beach and marsh habitats. We did not observe any Horseshoe Crabs in the marsh at Harbor Island, and only a few on the beach at Harbor Island. At Coffin Point, we recorded more Horseshoe Crabs in the marsh than we did on the beach. Spawning surveys are often used as a metric of population trends over time. Since we now have evidence from multiple sources that Horseshoe Crabs spawn regularly in the marsh, it may behoove us to account for this activity in our population assessments of Horseshoe Crabs. Surveys in the marsh do, however, pose some challenges. During our egg surveys from the prior two years, we found that most eggs tend to be laid on the marsh platform edge. Spawning surveys are generally conducted at the peak high tide which corresponds to when spawning densities are highest (Rudloe 1980), but the marsh platform where eggs are laid is often under a few feet of water during that time. This makes spawning Horseshoe Crabs much more difficult to detect at high tide in the marsh than on the beach. Thus, it is likely that our marsh spawning surveys underestimated the numbers of Horseshoe Crabs present. Furthermore, accessing marsh areas can be logistically difficult. Many marsh areas that may be conducive to spawning can only be reached by boat, requiring greater resources to incorporate these areas into regular spawning surveys compared to sites accessible on foot. For these reasons, it may be that other methods, such as egg surveys (Smith et al. 2002), which do not rely on the immediate presence of Horseshoe Crabs and that can be conducted during a broader timeframe, may be preferable for monitoring spawning trends in the marsh.

As expected, the sediments at marsh sites were significantly softer than those found at beach sites. Based on anecdotal observations during spawning and egg surveys, Horseshoe Crabs seem to spawn preferably in marsh areas where sediments are relatively hard, but our results found no significant difference between the hardness of the marsh sediments in areas we found and did not find eggs/embryos. This result could be due to the low sample size (only seven marsh sites had eggs the year we measured hardness) or the fact that we averaged three hardness measurements across the entire sampling site. Even across short distances, the firmness of the marsh platform can change markedly. In future efforts, it may be better to record the hardness of the sediment in the immediate area where the eggs were found for these comparisons.

We were able to leverage the public reporting database to identify locations and habitats where Horseshoe Crabs spawn. Of the 55 reports submitted by the public in the spring and summer of 2021, six reports documented Horseshoe Crabs spawning in marsh habitat, as best as we can determine from the GPS coordinates and location descriptions. Five of the six observations were in locations where we previously did not know that Horseshoe Crabs spawn. Since marsh spawning has only recently been recognized as important in SC, most locations where marsh spawning occurs likely remain unknown. Thus, the public reporting survey may be an important tool that allows us to identify new spawning locations for future studies.

The results of the egg surveys conducted in 2022 may indicate that spawning was relatively steady throughout the season from April through the end of June, at least on Otter Island. We found no effect of time in the spawning season on our likelihood of finding eggs or the number of eggs that we found. However, Horseshoe Crab embryos can take up to four weeks to develop, so it is possible that eggs collected later in the season were laid early but not found until they

were further along in their development. Staging the eggs would allow us to distinguish between these possibilities. Unlike on Otter Island, however, we found that the likelihood of finding eggs at Deveaux Bank increased as the season progressed. This result likely stems from finding no eggs in our first two sampling trips.

Anecdotal reports suggested that Horseshoe Crabs may be spawning in SC in the fall. While fall spawning has been documented in Florida (Rudloe 1980, Sasson et al. 2020) and Georgia (Fletcher Smith, personal communication), it has not been verified in other states. In October and November of 2021, we conducted egg surveys in three areas—Pine Island, Big Bay Creek, and Dawes Island—where Horseshoe Crabs are known to spawn in the spring. We did not find any eggs in these surveys. While this does not indicate that there is no fall spawning in SC, it does suggest that the prevalence of spawning in the fall may be low. There are, however, a few caveats here: in general, our ability to find eggs when searching at random sites is low, as shown by our egg surveys over the last two years. Additionally, as we have documented, Horseshoe Crabs may actually spawn in the marsh more often than on the beach. Our fall egg surveys were conducted at beach sites and so we may be missing areas where fall spawning occurs. Egg surveys at marsh sites with abundant spring spawning may be more fruitful when searching for fall spawned eggs.

UAVs have been considered a possible tool for shorebird surveys. Our test flights indicated that shorebirds were prone to flushing once the UAV flew at an altitude of 80 ft or below. Unfortunately, identifying shorebirds to specific species from the recorded videos was difficult at higher altitudes, especially when mixed flocks of birds were closely packed together along the shore. While a higher resolution camera could improve the ability to differentiate bird species during flights, conducting species-level surveys of shorebirds does not seem feasible with the equipment used in this study.

We did not find a significant relationship between the likelihood to find eggs and the presence of foraging shorebirds within in our transects. This finding stands in contrast to previous work at other sites in South Carolina that did find a positive relationship between Horseshoe Crab eggs and shorebird presence (Takahashi et al. 2021). In the previous study, and contrary to this current project, Horseshoe Crab eggs were abundant in the top 5cm layer of sediment where they are accessible to most foraging shorebirds (Botton et al. 1994; Yang et al. 2013; Smith et al. 2020). Given that we found very few core samples with eggs in the top 5cm layer of sediment in this study, it is perhaps not surprising that we did not observe a similar pattern. Furthermore, shorebird surveys did not occur during the latter half of the season, which was when Horseshoe Crab eggs began to appear in the upper layers of the sediment. The results of the bird surveys conducted in 2022 indicate that multiple shorebird species use and forage at beach habitats on both Otter Island and Deveaux Bank during the Horseshoe Crab spawning season. Furthermore, birds were seen foraging in the transect area in which eggs were found during egg surveys, indicating that these areas are important habitats for both shorebirds, including the *Rufa* Red Knot, and Horseshoe Crabs. The availability of Horseshoe Crab eggs to shorebirds depends on their location in the sediment. In general, some shorebirds (particularly Red Knot) can only access eggs in the top 5cm of the sediment. Our egg surveys in 2022 only found eggs in the top 5cm later in the spawning season, and in only one case were these eggs found in beach habitats. Eggs migrate to the surface on beaches due to wave action and the spawning activity of other

Horseshoe Crabs that excavate previously spawned eggs (Nordstrom et al. 2006). The lack of eggs close to the surface on beaches in this study may indicate that either spawning densities are not high enough or wave action is not strong enough, or both, to regularly unearth buried eggs. Whether enough eggs reach the surface in time for migratory shorebirds, such as Red Knots, to rely on them as part of their diet in SC requires further study.

The results from this study add to the evidence that Horseshoe Crabs spawn in salt marsh habitat as frequently, if not more so, than on beach habitat in SC. These findings are important steps in understanding the role of salt marshes in Horseshoe Crab reproduction in SC. Follow-up studies should continue to identify marsh sites where Horseshoe Crabs spawn in high densities and rigorously quantify the impact of salt marshes on Horseshoe Crab embryonic viability through rearing and reciprocal transplant experiments. These next steps will allow us to better understand whether salt marshes represent essential Horseshoe Crab spawning habitat or potential population sinks.

Deviations from original plan

In 2020, sampling beach and marsh habitats was originally planned for the months of May and June. Due to Covid-19 restrictions, however, our sampling efforts were pushed back to June 29th to July 22nd, 2020. Since Horseshoe Crab eggs take between 2 – 4 weeks to develop, we would not have found any eggs that were laid between May through mid-June, the most active time for Horseshoe Crab spawning in South Carolina. Thus, our rates of finding embryos in the field and our sample sizes for the rearing and staging experiments in 2020 were lower than would be expected under normal circumstances.

Recommendations

Close the grant.

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Table 1. List and counts of shorebirds observed at Otter Island and Deveaux Bank during bird surveys and the prevalence of Horseshoe Crab eggs (defined as the percent of cores with positive occurrence).

Location	Habitat	Date	Species counts	Count of foraging birds	Horseshoe Crab egg prevalence
Otter Island	Beach	4/20/22	Dunlin (150); Willet (9); Short-billed Dowitcher (2); Sanderling (1)	9	17.5
Otter Island	Beach	5/5/22	Dunlin (12); Sanderling (1)	12	15
Otter Island	Beach	5/20/22	Sanderling (20)	20	7.5
Deveaux Bank	Beach	4/21/22	Marbled Godwit (4); Ruddy Turnstone (1)	5	0
Deveaux Bank	Beach	5/5/22	Marbled Godwit (7); Ruddy Turnstone (4); Sanderling (4); Short-billed Dowitcher (2)	17	2.5
Deveaux Bank	Beach	5/13/22	Sanderling (8); Ruddy Turnstone (2)	10	N/A
Deveaux Bank	Beach	5/30/22	Dunlin (11); Sanderling (6); Semipalmated Plover (4); Ruddy Turnstone (3); Short-billed Dowitcher (1)	25	5
Deveaux Bank	Beach	6/3/22	American Oystercatcher (5); Sanderling (3); Willet (3); Ruddy Turnstone (1)	0	N/A
Deveaux Bank	Beach	6/9/22	Marbled Godwit (5); American Oystercatcher (3); Ruddy Turnstone (1); Semipalmated Sandpiper (1)	0	2.5