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Spatial and temporal overlap between foraging shorebirds and spawning horseshoe crabs (*Limulus polyphemus*) in the Cape Romain-Santee Delta Region of the U.S. Atlantic coast

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ABSTRACT—Shorebird use of horseshoe crab (*Limulus polyphemus*) eggs as food items has been well documented along the Atlantic coast of the United States at northeastern stopover sites such as the Delaware Bay. However, the relationship between migratory shorebirds and horseshoe crab eggs has not been well studied in the South Atlantic Bight. The objective of our study was to assess the spatial and temporal overlap between the density of horseshoe crab eggs and the relative abundance of foraging shorebirds during spring migration in the Cape Romain-Santee Delta Region (CRSD), South Carolina, USA. The CRSD is a site of international importance for shorebirds that supports ~100,000 shorebirds annually. We also sought to determine if horseshoe crab eggs were present in the diets of shorebirds at these sites. We monitored study plots between March and June 2015–2016 at predicted horseshoe crab spawning sites on beaches throughout Cape Romain National Wildlife Refuge. We conducted weekly shorebird surveys and collected core samples of beach substrate twice per month to measure densities of horseshoe crab eggs. We found a positive correlation between number of foraging shorebirds and horseshoe crab eggs for both years. In a molecular analysis of shorebird fecal samples, 95% of the samples tested contained DNA from horseshoe crab eggs. The spatial and temporal overlap between shorebirds and horseshoe crab eggs, and the dietary analysis of fecal samples, suggest that there are areas of localized horseshoe crab spawning that shorebirds can utilize as a food source during spring in Cape Romain National Wildlife Refuge. *Received 14 January 2021. Accepted 9 June 2021.*

Key words: Atlantic flyway, diet, migration, Red Knot, Sanderling, shorebird conservation, South Atlantic Bight.

Traslape espacial y temporal entre playeros forrajeando y cangrejos cacerola (*Limulus polyphemus*) en la región de Cape Romain-Santee Delta de la costa atlántica de los Estados Unidos

RESUMEN (Spanish)—El uso de los huevos del cangrejo cacerola (*Limulus polyphemus*) como recurso alimenticio por playeros, ha sido bien documentado a lo largo de la costa atlántica de los Estados Unidos en sitios de abastecimiento como Delaware Bay. Sin embargo, la relación entre playeros migratorios y huevos de cangrejo cacerola no ha sido bien estudiada en la zona South Atlantic Bight. El objetivo de nuestro estudio fue determinar el traslape espacial y temporal entre la densidad de huevos de cangrejo cacerola y la abundancia relativa de playeros que forrajeaban durante la migración de primavera en la región Cape Romain-Santee Delta (CRSD) en South Carolina, EUA. El CRSD es un sitio de importancia internacional para playeros que da soporte a ~100,000 playeros anualmente. Buscamos también determinar si los huevos del cangrejo cacerola se encontraban presentes en la dieta de playeros en esos sitios. Monitoreamos sitios de estudio entre marzo y junio 2015–2016 en sitios previstos de puesta en playas a lo largo del Cape Romain National Wildlife Refuge. Llevamos a cabo reconocimientos semanales y obtuvimos muestras de sustrato de playa dos veces al mes para medir las densidades de huevos de cangrejo cacerola. Encontramos una correlación positiva entre el número de playeros forrajeando y los huevos de cangrejo cacerola en ambos años. Por medio del análisis molecular de muestras fecales de playeros, el 96% de éstas contenía ADN de los huevos de cangrejo cacerola. El traslape espacial y temporal entre playeros y huevos de cangrejo cacerola, y el análisis de la dieta a través de las muestras fecales, sugieren que existen áreas localizadas de puesta del cangrejo cacerola que las playeras usan como recurso alimenticio durante la primavera en el Cape Romain National Wildlife Refuge.

Palabras clave: *Calidris alba*, *Calidris canutus*, conservación de playeros, dieta, migración, ruta migratoria del Atlántico, South Atlantic Bight.

Shorebirds in North America are declining rapidly, particularly long-distance migrants that nest in the Arctic (Munroe 2017, Rosenberg et al. 2019). Many shorebirds use only a few refueling stops during northward migration to Arctic nesting grounds and therefore a determination of the

spatial and temporal patterns of use that occur there are important to management (Piersma and Baker 2000, Brown et al. 2001). Along the U.S. Atlantic Coast, the Delaware Bay is generally recognized as a critical stopover site for shorebirds migrating from wintering sites in the southern hemisphere to breeding sites in the Arctic (Clark et al. 1993, Niles et al. 2009). Approximately 0.5–1.5 million shorebirds arrive and subsequently depart during the boreal spring during a narrow window of 3–4 weeks, and this stopover is synchronized with peaks in spawning activity of horseshoe crabs (*Limulus polyphemus*; Tsipoura and Burger 1999,

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Botton et al. 2003, Mizrahi and Peters 2009). The eggs of horseshoe crabs (HSC) are abundant and lipid rich and therefore provide food that is high in quantity and quality for shorebirds in need of energy replenishment (Botton et al. 1994, Tsipoura and Burger 1999, Gillings et al. 2007, Haramis et al. 2007). The abundance and distribution of HSC eggs also is positively correlated with shorebird distribution and abundance within the Delaware Bay, with shorebirds found more frequently on beaches with higher concentrations compared to lower concentrations of HSC eggs (Botton et al. 1994, Karpanty et al. 2006). Therefore, site use by shorebirds at both the landscape and patch scale appears to be linked to access to HSC eggs in this critical stopover site.

While the importance of HSC eggs to migrating shorebirds has been well established in the Delaware Bay, examinations of this relationship outside of that region are less common, with most other efforts occurring in the mid- and north-Atlantic regions of the U.S. (e.g., Placyk and Harrington 2004 [Connecticut], James-Pirri et al. 2005 [Massachusetts], Cohen et al. 2010 [Virginia], Beekey et al. 2013 [Connecticut]; but see Schwarzer 2011 [Florida]). To date, data gaps still exist regarding the interrelationships of migratory shorebirds with HSC in the South Atlantic Bight (i.e., South Carolina, Georgia, and northeast Florida). The region supports 3 sites of varying classifications of importance to migratory shorebirds within the Western Hemisphere Shorebird Reserve Network (WHSRN): Altamaha River Delta (ARD; regional importance), Georgia Barrier Islands (GBI; landscape of hemispheric importance) in Georgia, and Cape Romain-Santee Delta in South Carolina (CRSD; international importance). The ARD and GBI combined support ~450,000 shorebirds annually and ~30% of the migratory population of Red Knots (*Calidris canutus*) (WHSRN 2021a, 2021c). The CRSD supports ~10% of the migratory population of Red Knots but ~100,000 shorebirds annually (WHSRN 2021b). CRSD is designated as internationally important and is one of the most abundant sites for migratory shorebirds on the Atlantic Coast of North America. The CRSD region also supports an active harvest of HSC for limulus amebocyte lysate (Smith et al. 2017).

Given the lack of available data on interactions of shorebirds and HSC in the South Atlantic Bight,

we sought to obtain baseline data on the occurrence and overlap of the 2 taxa during the boreal spring within the CRSD region. Our objectives were to (1) temporally track the relative abundance of migratory shorebirds at low energy beaches that were known or suspected HSC spawning sites during spring migration, (2) temporally track the density of HSC eggs within plots on these same beaches, and (3) verify the occurrence of HSC eggs in the diets of migratory shorebirds at these same sites. Our data provide a unique assessment of the relationship between migratory shorebirds and HSC within a core migratory region for shorebirds along the Atlantic coast of the United States.

Methods

Study area

Our research was focused on the Cape Romain National Wildlife Refuge (CRNWR) within the CRSD (33.03°N, 23.38°W; Fig. 1) during the boreal spring migration of 2015 and 2016. CRNWR supports ~11,000 shorebirds during spring migration (Wallover et al. 2015). CRNWR is composed of barrier islands, shallow bays, tidal creeks, salt marsh (dominated by *Spartina alterniflora*), shell mounds, fresh and brackish water impoundments, mudflats, oyster (*Crassostrea virginica*) reefs, and maritime forest. The region has a strong tidal cycle, with mean high tide 1.7 m and spring tides over 2 m (NOAA 2021).

Shorebird and horseshoe crab surveys

To measure the abundance of shorebirds and the density of HSC eggs, we established survey plots on bayside beaches of 3 barrier islands (Bulls, Cape, and Lighthouse islands) and 2 bay islands (Little Bulls and Marsh islands; Fig. 1 [abbreviations for plot names included therein]). The 5 islands were not chosen randomly but instead were chosen based on prior observations of spawning activity of HSC, logistics of access, and presence of low energy beaches where HSC typically spawn. The sampling design accounted for all of the barrier and bay islands in CRNWR except one island that did not have a bayside or end beach that was accessible. We established 10 survey plots in 2015 and 12 in 2016 among the 5 islands prior to peak shorebird migration each year ($n = 1\text{--}3$ plots

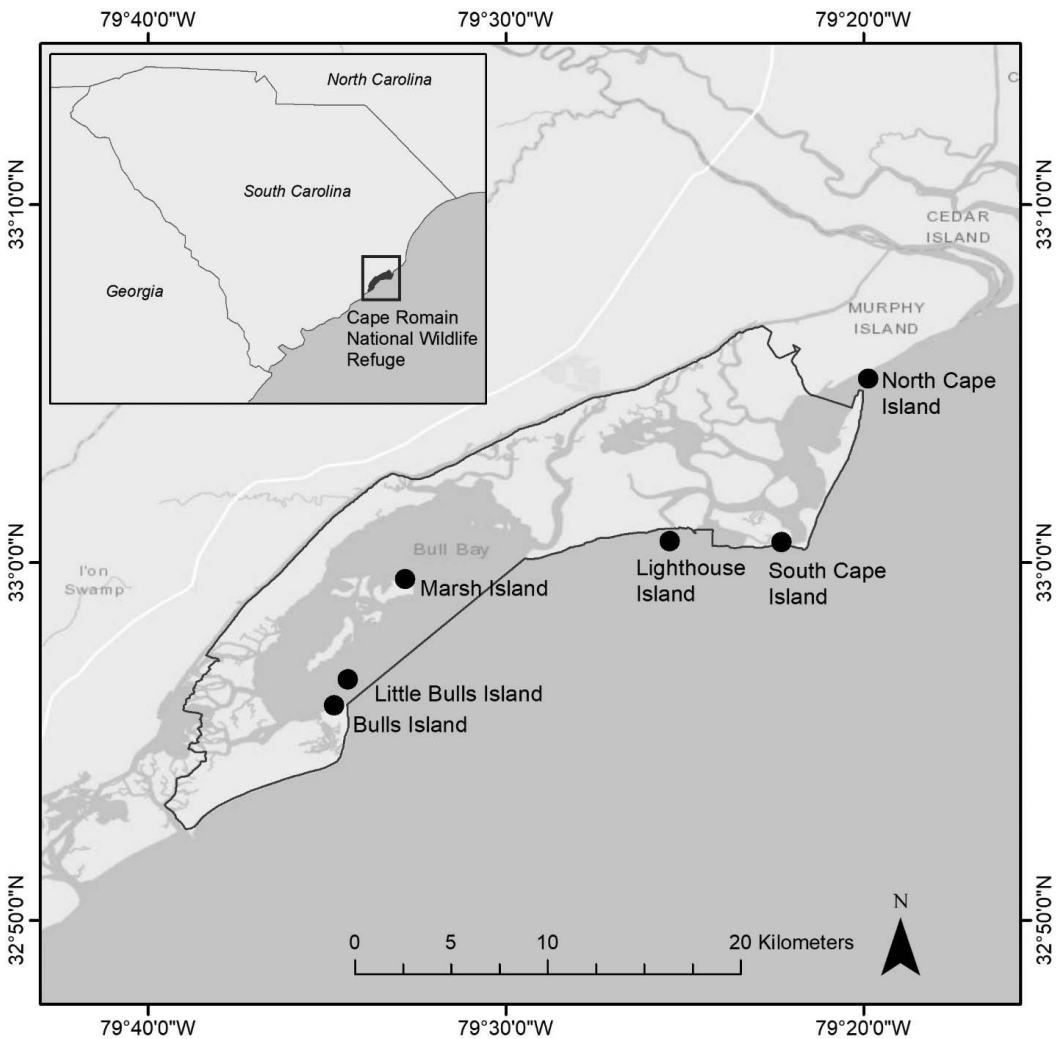


Figure 1. Shorebird and horseshoe crab survey plots, Cape Romain National Wildlife Refuge, South Carolina, April–June 2015, March–June 2016. Number of plots within each site for 2015 and 2016, respectively, were Bulls Island $n = 3$ (Bulls-A-15, B-15, C-15), $n = 2$ (Bulls-A-16, B-16); Little Bulls Island $n = 1$ (LBulls-15), $n = 2$ (LBulls-A-16, B-16); Marsh Island $n = 1$ (Marsh-A-15), $n = 2$ (Marsh-A-16, B-16); Lighthouse Island $n = 2$ (LH-A-15, B-15), $n = 3$ (LH-A-16, B-16, C-16); South Cape Island $n = 1$ (SCape-15), $n = 1$ (SCape-16); North Cape Island $n = 2$ (NCape-A-15, B-15), $n = 2$ (NCape-A-16, B-16). Plot location differed between years.

per island). Because HSC spawn during high tides, especially during the maximum high tides each month near the full and new moons (Barlow et al. 1986), we located plots adjacent to and immediately below the high tide line. Although we used the same islands each year, exact plot locations varied between years to accommodate changes in beach profiles. The length of each plot was 200 m (with the exception of one plot at Lighthouse Island and one plot at Marsh Island in 2016, which

were each 100 m in length due to logistical constraints), measured as relatively parallel to the water line. Width (maximum width = 32 m) differed among plots due in part to tide and weather conditions and we therefore measured plot width using a laser rangefinder at the start of every survey. We spaced plots 50 m apart at islands containing multiple plots.

We conducted 105 shorebird surveys at plots within ± 2 h of predicted high tide between 13

April and 13 June 2015 (mean $\pm$ SD = 1.17 ± 0.18 surveys per plot per week), and 202 surveys between 28 March and 11 June 2016 (mean $\pm$ SD = 1.54 ± 0.17 surveys per plot per week). Shorebirds feed on a diversity of prey items throughout the entire tidal cycle in the Cape Romain NWR (Jamieson 2019). To focus on shorebird use of HSC eggs, we conducted surveys during high tides on beaches because the substantial tidal inundation in the CRSD region leaves typical foraging habitat (e.g., tidal marshes, mudflats, oyster reefs) unavailable while eggs of HSC appear to remain available on beaches during this tide phase (Barlow et al. 1986).

At the start of each survey, we measured temperature and wind speed using a hand-held anemometer (Kestrel 2000; Kestrel Meters, Boothwyn, Pennsylvania, USA). We conducted counts adjacent to or within plots (74% of surveys) or by boat within 15 m of shore (26% of surveys). We found no significant difference in shorebird counts by survey type each year (2015: $t_{13} = 0.3$, $P = 0.8$; 2016: $t_{118} = 0.4$, $P = 0.6$) and therefore pooled survey types for all subsequent analyses. Shorebirds that were actively foraging within the survey plot were enumerated to species by 2 observers each using a spotting scope. Each observer counted a different species within the plot. We included gull species (<2% of total birds counted each year) in our counts because they also forage on HSC eggs (Botton et al. 1994, Karpanty et al. 2006). Duration of shorebird surveys ranged from 1 to 42 min depending on the extent of counting needed, and we excluded any surveys during which birds were flushed either by our presence or any other activity. We did not conduct surveys during heavy rainfall or when winds exceeded 10 m/s. We report count data as the sum of all birds/200 m² (to align with plot length) and normalized these data using a $\log(x + 0.5)$ transformation. We analyzed data separately by year to account for differences in plot locations between years.

We measured abundance of HSC eggs available to shorebirds within the same plot locations as described above and within ± 2 h of predicted high tide. Although HSC deposit most eggs at 10–20 cm deep, wave action and erosion unbury eggs and at 0–5 cm depth HSC eggs are potentially available for shorebird consumption (Botton et al. 1994). We collected samples of HSC eggs coinciding with full or new moon stages when

HSC are most likely to spawn (Smith et al. 2002b, Ehlinger et al. 2003). During 2015 we sampled eggs from 10 study plots on 3 occasions (3–11 May, 16–19 May, and 1–5 Jun) with each occurring within 2.3 ± 3.4 d of a full or new moon. During 2016, we sampled eggs from 11 study plots on 5 occasions (11–18 and 23–25 Apr, 7–9 and 22–24 May, and 6–9 Jun) with each occurring within 3.5 ± 2.8 d of a full or new moon. The order of sampling among sites was randomly set within each moon cycle. Therefore, our samples likely reflect an approximate maximum availability of eggs of HSC on survey plots given surveys occurred near new and full moon phases. Furthermore, our estimates are representative of the plots sampled within each beach and based on sampling design, and therefore extending the scope of inference to the entire beach would be inappropriate. We collected HSC eggs within 1 m of the most recent high tide line using a PVC core (10 cm diameter $\times$ 5 cm deep; Karpanty et al. 2006). We collected a composite sample of 3 cores every 10 m over a 100 m transect. The 100 m transect was randomly selected from within the 200 m plot. Samples of eggs were placed in plastic bags and stored in coolers in the field, then in a refrigerator for up to 24 h, after which they were sifted through a 1 mm mesh sieve to remove larger sand particles. In 2015 we preserved samples in 95% ethanol. In the lab, we counted the number of eggs for each sample. In 2016, processed samples were examined for the presence of eggs immediately and then frozen. We report the count data from the composite cores as eggs/composite core or eggs/m² for ease of comparison to other studies. Data were analyzed separately by year due to differences in sampling procedures (i.e., timing of surveys) between years.

We measured grain size of sediments at survey plots to assess its potential relationship with the density of shorebirds and HSC eggs. To collect a representative sample of the sediment grain size in the upper 0–5 cm of sand across our study plots, we used a PVC corer (10 cm diameter $\times$ 5 cm deep) to collect 5 sand cores from each plot at 40 m intervals in the upper section of the beach (within 1 m of high-water line). We collected samples from 10 to 26 April 2016 for 11 plots (all plots excluding Marsh B; $n = 55$). We stored samples in plastic bags in a freezer until analyses were conducted. Upon thawing we dried sand

samples in an oven at 110 °C for 24–72 h. Before sieving, organic material was removed from samples and sand clumps were disaggregated. The oven-dried samples were then poured into a stacked series of 15.2 cm diameter sieves with descending mesh sizes (4, 2, 0.5, 0.25, 0.125, and 0.063 mm) and processed on a Humboldt motorized sieve shaker for 15 min. The weight of the sample retained on each sieve was recorded to within 0.01 g to calculate percent of the total weight in each size class. We calculated mean particle size of sediments following Folk (1974), using the package GRADISTAT 8.0 (Blott and Pye 2001). Mean particle size is reported in micrometers and in phi units. Phi units (Φ) are a standardized unit commonly used to compare sediment grain sizes. Phi is calculated by a logarithmic conversion of mm to the Wentworth (1922) grade scale of particle size as $\Phi = -\log_2$ (diameter of the particle in mm).

Diet determination

To assess the presence of HSC eggs in diets of shorebirds, we collected fecal samples following shorebird surveys in 2016. Fecal samples were only collected when survey periods lasted >45 min. Doing so increased the probability that collected samples represented local foraging activity based on the gut passage rate for common shorebird species (~30–60 min; Castro et al. 1989, Quaintenne et al. 2010, Onrust et al. 2013). This sampling restriction resulted in 7 collection events from 2 sites: 2 plots at Marsh Island and 1 plot at Bulls Bay. We collected samples from mixed species flocks due to the absence of large, single-species flocks during surveys. We recorded the species composition of each flock for each fecal sample. We only collected fresh samples (i.e., appeared moist) because DNA degrades with exposure to sunlight and substrate (King et al. 2008). We were careful to collect the fecal sample itself and not the sand from under or around the sample to minimize the opportunity for any DNA in sand to contaminate the fecal sample. Tweezers were disinfected with a bleach solution between collections. We stored samples in 95% ethanol and then froze them until PCR analysis could be performed (Oehm et al. 2011).

We isolated the DNA using a Qiagen QIAamp Mini Stool Kit (Qiagen, Germantown, Maryland,

USA). We used the primers developed by Novcic et al. 2015 (Limf92 and Limr300) to amplify a 236 base pair segment of the cytochrome oxidase (COI) region of the mitochondrial DNA (mtDNA). The traditional PCR method used in Novcic et al. (2015) was adapted for real time qPCR by T. Darden and M. Walker (South Carolina Department of Natural Resources), who developed an HSC-specific probe with a fluorescent reporter. We ran 10 samples per qPCR reaction. Each sample had 8 replicates (to increase the chance of detecting positive results in samples with low initial copy number). In addition to these samples, we also ran a negative control, with 8 negative replicates, to ensure that there were no contaminants in the qPCR materials. We also ran a positive control, using tissue taken from an HSC leg, with 2 replicates.

Data analyses

We developed linear mixed models to assess the relationships between temporal factors and the relative abundance of shorebirds on our survey plots. We did not include an interaction term between time and plot in any of our models because not all plots were visited during each time period due to logistical or weather constraints. We did not include island as a variable but instead treated each plot as a separate sampling unit and included plot as a random factor. We used a model-selection approach and included 4 models: week as a continuous variable, week as a continuous and quadratic variable, week as a categorical variable, and a null model. We ranked models using Akaike information criteria corrected for small sample sizes (AIC_c) and used the highest ranked model for coefficient estimates. We assessed the relative abundance among plots by comparing densities among plots from the highest ranked model each year using intercept and SE terms for random effects (*ranef* and *se.ranef* functions in the *lme4* package in R 3.5.2; R Development Core Team 2019).

We sought to normalize the distribution of the HSC egg count data using a $\log(x + 1)$ transformation but were unsuccessful. Therefore, to account for the non-normal distribution and unequal variances observed in the data set (both years), differences in egg abundance across plots and sampling periods were compared with Krus-

Table 1. Shorebird count data for surveys conducted in Cape Romain National Wildlife Refuge, South Carolina, April–June 2015 and 2016. Only species within the top 90% of total birds counted each year are included (e.g., Dunlin not within 90% in 2016, so no data included).

Species	2015				2016			
	% of all birds counted	Mean count per survey	SD	Maximum single count	% of all birds counted	Mean count per survey	SD	Maximum single count
Sanderling	38.3	26.0	54.8	291	47.8	27.2	65.8	502
Ruddy Turnstone	15.7	10.7	29.0	236	15.5	8.8	27.7	228
Semipalmated Sandpiper	15.4	10.5	40.3	378	15.5	8.8	25.7	154
Dunlin	13.0	8.8	46.1	450				
Red Knot	5.5	3.8	15.4	120	5.8	3.3	16.0	144
Short-billed Dowitcher	5.1	3.5	21.4	209	5.5	3.2	12.1	79

kal-Wallis nonparametric test and subsequently with Wilcoxon multiple comparison tests (analyses conducted separately for plot and sampling period). We also compared the same data using ANOVA followed by LSD tests. Because the significance results were the same using Kruskal-Wallis or ANOVA, we report the ANOVA and LSD results for ease of interpretation. Data for 2015 and 2016 were analyzed separately to account for differences in sampling between years.

The relationship between shorebird abundance and densities of HSC eggs was modeled as a simple linear relationship. Individual study plots served as sampling units (2015, $n = 10$; 2016, $n = 12$), with mean shorebird density from all surveys within plots as the dependent variable and mean density of eggs of HSC from all surveys within plots as the independent variable. Plot locations were included as a random variable in the model. Shorebird abundance and densities of HSC eggs were log-transformed prior to analysis to normalize those data. We were unable to successfully normalize the HSC egg data (see above) but opted to use the log-transformed data to maintain consistency in scale with the shorebird data. Data for 2015 and 2016 were analyzed separately to account for differences in sampling between years.

Difference in size of sand grains among plots was compared using an ANOVA followed by LSD tests. We assessed the relationship between sand grain size and abundance of HSC eggs, and between sand grain size and shorebird density, using simple linear regression models. Individual study plots served as sampling units ($n = 11$), with mean shorebird density and egg abundance as the dependent variables and mean sand grain size as

the independent variable. Plot locations were included as a random variable in the model. Shorebird and HSC egg abundance were log transformed in all linear regressions to normalize the data (but see above).

All mean values are reported as mean $\pm$ standard deviation in untransformed data for ease of comparison. All statistical analyses were performed R 3.5.2 (R Development Core Team 2019).

Results

Shorebird occurrence

We conducted 105 shorebird surveys between 13 April and 12 June 2015, and 202 shorebird surveys between 28 March and 11 June 2016. We recorded 14 species of shorebirds and 2 species of gulls during surveys in 2015, and 18 species of shorebirds and 3 species of gulls in 2016. Six species accounted for 90% of the total count (7,126 birds) across all surveys in 2015: Sanderling (*Calidris alba*), Ruddy Turnstone (*Arenaria interpres*), Semipalmated Sandpiper (*Calidris pusilla*), Dunlin (*Calidris alpina*), Short-billed Dowitcher (*Limnodromus griseus*), and Red Knot (Table 1). Five species accounted for 90% of the total count (11,485 birds) in 2016: Sanderling, Semipalmated Sandpiper, Ruddy Turnstone, Red Knot, and Short-billed Dowitcher (Table 1). The same 3 species (Sanderling, Ruddy Turnstone, and Semipalmated Sandpiper) were ranked as the 3 most common species each year (Table 1).

Densities of shorebirds in our study were best predicted by models that included survey week as a categorical variable in 2015 (AIC_c weight = 0.91)

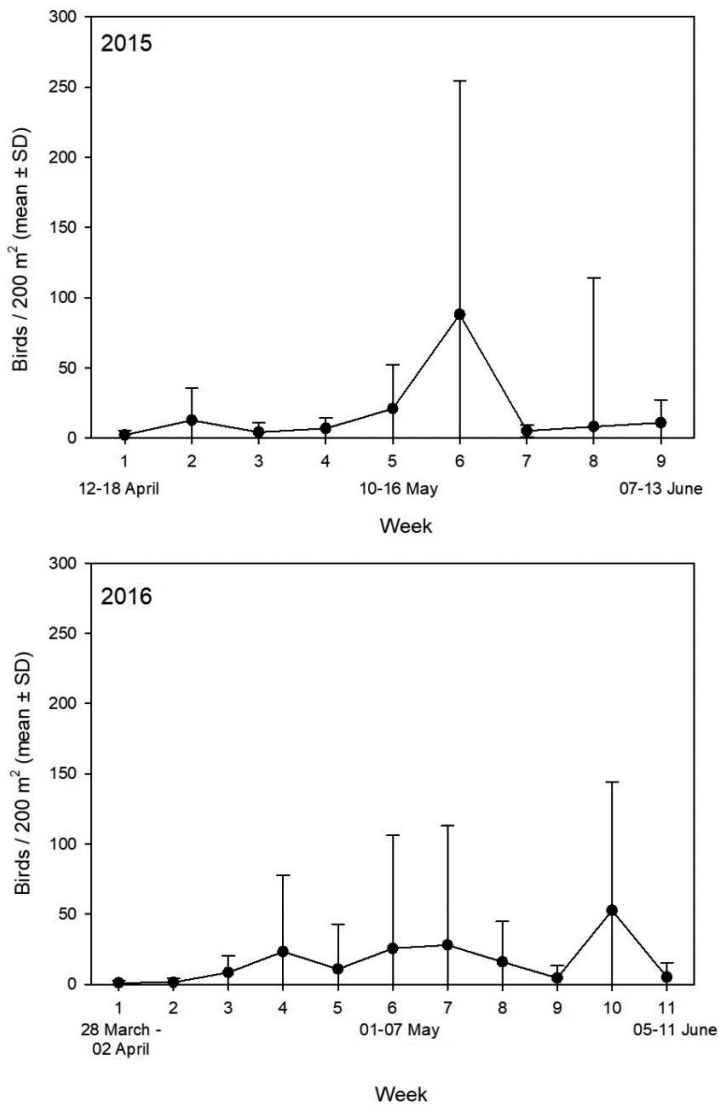


Figure 2. Shorebird densities (birds/200 m² ± SD) across 9 sampling weeks for combined 10 study plots in 13 April–12 June 2015, and across 11 sampling weeks for combined 12 study plots, 28 March–11 June 2016, in Cape Romain National Wildlife Refuge, South Carolina (2015 week 6 significantly different from all other weeks except week 5 [LSD < 0.05]; 2016 week 10 significantly different from all other weeks except week 7, week 7 significantly different from all other weeks except weeks 3, 4, and 8 [LSD < 0.05]).

and in 2016 (AIC_c weight = 0.99). Density of shorebirds differed throughout spring migration by week in both 2015 ($F_{8,87} = 5.4$, $P < 0.0001$) and 2016 ($F_{10,180} = 5.9$, $P < 0.0001$). In 2015 (Fig. 2), densities of shorebirds did not differ between week 1 (week 1 = reference level) and week 3, but the density in all other weeks (i.e., 2, 4–9) > week 1 ($t_{1,87} > 2.1$, $P < 0.04$ for each). Density appeared

to peak during the weeks of 17–23 May (88.3 ± 166.4 birds/200 m²; Fig. 2). In 2016, shorebird density appeared to be lowest during weeks 1 and 2 as well as weeks 9 and 11, and density appeared to be higher in all other weeks ($t_{1,180} > 1.7$, $P < 0.10$ for each; Fig. 2). Density appeared to peak during the week of 29 May–4 June (week 6, 52.6 ± 91.6 birds/200 m²; Fig. 2).

Table 2. Shorebird surveys (intercept $\pm$ SE of random variable term, and range of) for 10 study plots, 13 April–12 June 2015, and for 12 study plots, 28 March–11 June 2016, in Cape Romain National Wildlife Refuge, South Carolina. Density is reported as birds/200m². The intercept $\pm$ SE is of the random variable term from the best-performing model and shows the difference between the value for that plot and an “average plot.”

2015			2016		
Plot	Intercept $\pm$ SE	Density range	Plot	Intercept $\pm$ SD	Density range
Bulls-A-15	-0.17 ± 0.14	0 – 7.6	Bulls-A-16	-0.04 ± 0.11	0 – 72.5
Bulls-B-15	0.13 ± 0.14	0.3 – 47.5	Bulls-B-16	-0.08 ± 0.12	0 – 72.0
Bulls-C-15	-0.43 ± 0.14	0 – 9.0	LBulls-A-16	-0.36 ± 0.12	0 – 9.7
LH-A-15	0.13 ± 0.13	0 – 134.0	LBulls-B-16	-0.16 ± 0.11	0 – 39.4
LH-B-15	-0.02 ± 0.13	0 – 28.3	LH-A-16	-0.40 ± 0.12	0 – 11.0
LBulls-15	0.54 ± 0.14	4.5 – 549.5	LH-B-16	-0.24 ± 0.12	0 – 34.0
Marsh-A-15	0.19 ± 0.16	1.4 – 102.0	LH-C-16	-0.37 ± 0.12	0 – 9.3
NCape-A-15	-0.09 ± 0.15	0 – 50.2	Marsh-A-16	0.56 ± 0.13	0 – 98.0
NCape-B-15	-0.16 ± 0.15	0 – 11.0	Marsh-B-16	1.20 ± 0.14	3 – 420.0
SCape-15	-0.12 ± 0.13	0 – 11.3	NCape-A-16	0.09 ± 0.12	0 – 39.0
			NCape-B-16	-0.06 ± 0.12	0 – 63.3
			SCape-16	-0.14 ± 0.12	0.2 – 18.0

Based on an assessment of intercept values for each plot (where plot appeared as a random variable in the highest-ranked model from each year), the densities of shorebirds appeared to differ among plots in 2015 and 2016. In 2015, densities of shorebirds appeared highest at Little Bulls Island (LBulls-15) and lowest at Bulls-C-15 compared to average densities (Table 2). In 2016, densities of shorebirds appeared highest at Marsh-B-16 and lowest at Lighthouse A (LH-A-16), Little Bulls A (LBulls-A-16), and Lighthouse C (LH-C-16) compared to average densities (Table 2).

Abundance of horseshoe crab eggs

In 2015, we collected 300 composite samples of HSC eggs over 3 sampling periods from 3 May to 5 June, and in 2016 we collected 590 composite samples across 5 sampling periods between 11 April and 9 June. Abundance of HSC eggs was higher ($F_{2,297} = 5.0$, $P = 0.007$) during 16–19 May 2015 (26.1 ± 90.4 eggs per composite core) compared to 3–11 May (6.5 ± 29.0 eggs per composite core) and 1–5 June (6.4 ± 31.8 eggs per composite core). Abundance of HSC eggs (10.9 ± 54.5 eggs per composite core) did not differ among sampling periods in 2016 ($F_{4,574} = 0.4$, $P = 0.4$). Abundance of HSC eggs differed among plots in 2015 ($F_{9,288} = 8.96$, $P < 0.0001$) and 2016 ($F_{11,574} = 53.83$, $P < 0.0001$; Table 3). In 2015, abundance was greater at LBulls-15

compared to all other plots except Bulls-B-15 (LSD tests, $P < 0.05$). In 2016, Marsh-A-16 and Marsh-B-16 had higher abundances of eggs (LSD tests, $P < 0.05$) compared to all other plots.

We found a positive relationship between the average abundance of HSC eggs within plots and the average density of foraging shorebirds within plots in 2015 ($t_8 = 2.5$, $r^2 = 0.44$, $P = 0.035$; Fig. 3a) and 2016 ($t_{10} = 6.2$, $r^2 = 0.79$, $P = 0.0001$; Fig. 3b). For any shorebird that comprised $>5\%$ of total birds for either year (6 species), we also analyzed the species-specific relationship with abundance of HSC eggs. The density of Sanderling, Semipalmated Sandpiper, Red Knot, and Ruddy Turnstone were each positively correlated with abundance of HSC eggs in 2015 ($0.42 \leq r^2 \leq 0.68$ for each), while the densities of Sanderling, Semipalmated Sandpiper, Red Knot, Ruddy Turnstone, and Short-billed Dowitcher were positively correlated with abundance of HSC eggs in 2016 ($0.60 \leq r^2 \leq 0.98$ for each).

Mean size of sand grains differed among plots at Cape Romain NWR ($F_{10,43} = 8.57$, $P \leq 0.0001$). Particle size was largest at SCape-16 (346.4 ± 56.04) compared to all other plots (range of means for all other plots = 150.44 ± 0.42 to 224.04 ± 6.82 ; LSD tests, $P \leq 0.05$). The relationships between sand grain size and abundance of HSC eggs ($t_9 = -0.4$, $P = 0.7$) and between sand grain sizes and densities of foraging shorebirds ($t_9 = -1.4$, $P = 0.2$) were not significant.

Table 3. Surveys for horseshoe crab eggs (N = number of composite cores) from 10 study plots, 3 May–5 June 2015, and for 12 study plots, 11 April–9 June 2016, in Cape Romain National Wildlife Refuge, South Carolina.

2015				2016			
Plot	N	Mean $\pm$ SD	Max.	Plot	N	Mean $\pm$ SD	Max.
Bulls-A-15	30	1.9 ^{dc} $\pm$ 6.5	31	Bulls-A-16	50	0.7 ^b $\pm$ 3.6	25
Bulls-B-15	30	56.3 ^{ab} $\pm$ 137.1	545	Bulls-B-16	50	0.5 ^b $\pm$ 2.6	18
Bulls-C-15	30	2.0 ^{dc} $\pm$ 10.4	57	LBulls-A-16	50	0.2 ^b $\pm$ 1.3	8
LBulls-15	40	40.5 ^a $\pm$ 95.3	492	LBulls-B-16	50	0.7 ^b $\pm$ 3.1	16
LH-A-15	30	10.5 ^{cd} $\pm$ 46.5	256	LH-A-16	50	0.0 ^b $\pm$ 0.2	1
LH-B-15	30	0.1 ^c $\pm$ 0.3	1	LH-B-16	50	0.0 ^b $\pm$ 0.0	0
Marsh-A-15	20	3.3 ^{dc} $\pm$ 10.9	49	LH-C-16	50	0.0 ^b $\pm$ 0.0	0
NCape-A-15	30	6.9 ^{bc} $\pm$ 14.0	72	Marsh-A-16	40	79.2 ^a $\pm$ 140.9	496
NCape-B-15	30	0.1 ^c $\pm$ 0.3	1	Marsh-B-16	50	71.5 ^a $\pm$ 121.7	646
SCape-15	30	2.5 ^c $\pm$ 13.9	76	NCape-A-16	50	0.1 ^b $\pm$ 0.9	6
				NCape-B-16	50	0.2 ^b $\pm$ 1.7	12
				SCape-16	50	0.6 ^b $\pm$ 4.0	28

^{a,b,c,d,e} Means with same superscript letter are not statistically different (ANOVA; means compared by LSD tests; $P < 0.05$).

Horseshoe crab eggs in diet

We analyzed 100 fecal samples for the presence of HSC DNA collected across 7 sampling events in 2016. Sanderling was the dominant species in the first 6 sampling events while Semipalmated Sandpiper was the dominant species in the final sampling event (Table 4). Of the 100 samples tested, 95% were positive (at least 1 positive replicate per sample) for HSC DNA (Table 5). HSC DNA was present in 80–100% of the shorebird fecal samples at all 3 of the plots we tested, and for each date between April and June (Table 5).

Discussion

Occurrence of shorebirds and eggs of horseshoe crabs

Our research is the first examination of the relationship between migratory shorebirds and HSC eggs within the South Atlantic Bight. The CRSD region is regarded as an important stopover site for migratory and breeding shorebirds (Dodd and Spinks 2001, Wallover et al. 2015); thus understanding the dynamics of food resources in this area may have positive impacts on shorebirds through the flyway.

Foraging shorebirds were regularly observed on our survey plots during both years of our study. Our counts varied widely throughout each survey season, ranging from a few individuals to ~1,000 per survey. We detected a distinct peak in

abundance in 2015 during the week of 17–23 May while in 2016 the peak was not as distinct and occurred ~5–10 d later. Although we could not test this difference directly, we suggest that the occurrence of a tropical storm in late May 2016 may have contributed to it.

HSC eggs in our study area provide food for shorebirds preparing for migration to breeding grounds but also for shorebirds that are forgoing breeding perhaps because they are immature. For example, shorebird numbers peaked in early June in 2016, when Arctic nesting shorebirds such as Red Knots have left temperate areas (Niles et al 2012). Large numbers of knots, dowitchers, and other Arctic nesting species are present throughout the summer in CRSD (Dodd and Spinks 2001, Wallover et al. 2015) and June spawning of HSC may be a valuable food source for this group of nonbreeding shorebirds.

Of the 20 species recorded during surveys, only 4 species were regular breeders in the study area (Willet [*Tringa semipalmata*], American Oystercatcher [*Haematopus palliatus*], Wilson's Plover [*Charadrius wilsonia*], and Laughing Gull [*Leucophaeus atricilla*]) while the rest were migratory. These species nest in April and May in South Carolina; thus HSC eggs also provided food for local nesting shorebirds. Approximately 70% of the birds counted included Sanderlings (38% and 48% by year), Ruddy Turnstones, and Semipalmated Sandpipers (each of the latter comprising ~15% of the individuals counted each year). All 3

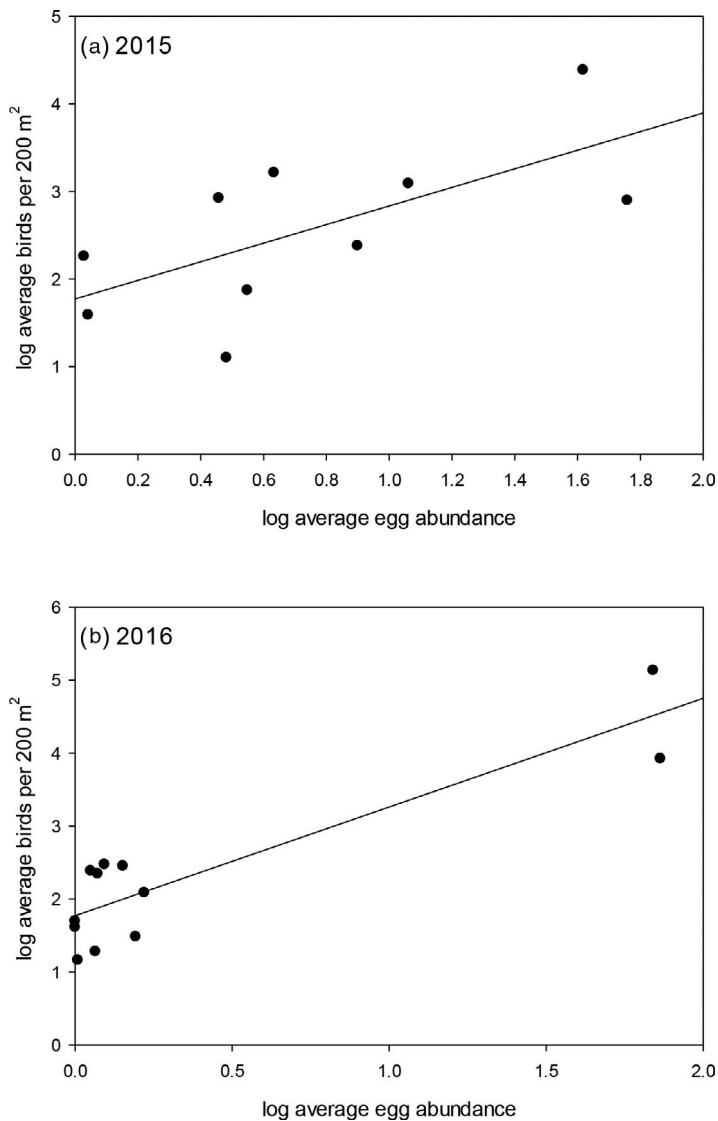


Figure 3. Density ($\log + 1$ birds/200 m²) of shorebirds in relation to density ($\log + 1$) of horseshoe crab eggs (per composite core) for (a) 10 study plots, 13 April–12 June 2015 ($\log \text{ shorebird density} = 1.7 + 1.06 * \log \text{ density of horseshoe crab eggs}$), and (b) 12 study plots, 28 March–11 June 2016 ($\log \text{ shorebird density} = 1.7 + 1.53 * \log \text{ density of horseshoe crab eggs}$), Cape Romain National Wildlife Refuge, South Carolina.

species are Arctic breeders that migrate and/or stop over at various locations along the U.S. Atlantic coast. For example, these same 3 species, along with Red Knots, were also abundant during spring migration in Delaware Bay where diet samples demonstrated that eggs of HSC comprised the majority of their diet (Tsipoura and Burger 1999).

Eggs of HSC provide a high-quality food resource for shorebirds that also can be locally

abundant (Smith et al. 2002b). Eggs of HSC were present in at least 1 plot per island, suggesting a consistent prey resource throughout the study area, although one that clearly fluctuated over time and space. The density of eggs peaked in 2015 during surveys from 16 to 19 May while in 2016 there was no significantly higher density during any sampling period. Given the high fidelity to migratory routes and stopover sites by some

Table 4. Species compositions of foraging flocks (percentage of total summing to 90%) for shorebird fecal samples collected by date and plot, 19 April–9 June 2016, Cape Romain National Wildlife Refuge, South Carolina.

Date (2016)	Plot	Total birds counted	Ruddy Turnstone	Sanderling	Willet	Marbled Godwit ^a	Semipalmated Sandpiper	Red Knot	Short-billed Dowitcher
19 April	Bulls B	363		82.6					7.2
21 April	Marsh B	245		81.6		11.4			
11 May	Marsh B	840	27.1	38.3			17.5		9.2
15 May	Marsh B	877	23.8	43.0			17.6	6.8	
23 May	Marsh A	89	30.3	33.7			15.7		13.5
8 June	Marsh B	208	16.4	52.9				20.2	
9 June	Marsh A	225	6.7		6.7		36.0	9.3	31.6

^a Marbled Godwit (*Limosa fedoa*)

shorebirds (Burton and Evans 1997, Leyrer et al. 2006), our results suggest that among years shorebirds consuming HSC eggs in CRNWR may experience different foraging environments characterized by substantial temporal variability.

Egg availability varies spatially as well, and at multiple scales. For example, Thompson (1998) suggested that within CRNWR Bulls Island and Marsh Island would be suitable habitat for HSC based on a predictive habitat model that included factors such as the presence of >1 ha of sand flats and beach that is accretional. In 2015, we recorded the highest densities of eggs on Bulls Island and in 2016 on Marsh Island, suggesting that these 2 islands were still providing usable habitat for spawning HSC.

Counts of eggs in CRNWR were significantly lower compared to those in Delaware Bay, where Botton et al. (1994) measured egg densities of 100,000 eggs/m² in the top 5 cm of substrate. Karpanty et al. (2006) calculated that when densities of eggs in Delaware Bay were greater than ~21,000 eggs/m², there was a 50% chance of

Red Knots occurring at the site. Across both years of our study, only 4 plots (LBulls-15, Bulls-B-15, Marsh-A-16, and Marsh-B-16) had maximum counts of eggs that exceeded this threshold.

During our study, we observed that HSC were being harvested for production of limulus ameobocyte lysate (LAL) on our survey plots. HSC are removed as they arrive at spawning beaches and harvest information is confidential. Although the Southeast HSC stock assessment is considered good (Atlantic States Marine Fisheries Commission 2019), it is unclear how the removal of HSC from our survey plots during our examination of HSC egg density effected local egg density, distribution, and annual fluctuations.

Our results also demonstrated that the abundance of shorebirds at CRNWR temporally overlapped with the abundance of HSC eggs for both years of our study. In 2015, we identified a single peak in overlapping abundance between shorebirds (10–23 May) and HSC eggs (16–19 May). The temporal overlap of birds and HSC egg abundance that we observed has also been

Table 5. Presence of DNA from horseshoe crabs in fecal samples (*n* = 100) collected from shorebirds, as determined from qPCR analysis during 19 April–9 June 2016 at Cape Romain National Wildlife Refuge, South Carolina.

Date	Plot	Total samples tested	No. samples with ≥1 replicate with HSC DNA	% samples with HSC DNA
19 April	Bulls-B-16	20	18	90
21 April	Marsh-B-16	10	8	80
11 May	Marsh-B-16	20	20	100
15 May	Marsh-B-16	10	10	100
23 May	Marsh-A-16	11	11	100
23 May	Marsh-B-16	14	13	93
9 June	Marsh-A-16	15	15	100
	Total	100	95	

documented in the Delaware Bay, where there is a well-established relationship between the occurrence of shorebirds and the availability of HSC eggs (Botton et al. 2003, Mizrahi and Peters 2009). Unlike in 2015, shorebird abundance in 2016 did not display a singular peak. In 2016, HSC spawning began early in the season, with spawning events observed as early as 12 March, and the abundance of HSC eggs remained similar throughout the sampling periods. Early spawning events may occur with warmer temperatures, and in March 2016, the average air temperature was 3.3 °C above average for South Carolina, and ocean temperatures as measured in Charleston, South Carolina, were also ~3–5 °C higher in March 2016 compared to March 2015 (NOAA 2016). Thompson (1998) described a similar early spawn for HSC in South Carolina in March 1998 during a relatively warm spring. We suggest that this early onset of spawning and the lack of a clear temporal peak in the abundance of HSC may have contributed to the difference in the temporal pattern of shorebirds between years.

We also found that bird distribution was positively correlated to HSC egg abundance among our study plots. In 2015, the correlation was driven by a consistent pattern of shorebird density vs. abundance of HSC eggs (i.e., a relatively consistent scatter among low, moderate, and high values of each variable) while in 2016 the correlation was highly leveraged by 2 plots (Marsh-A-16 and Marsh-B-16). The difference in patterns between the 2 years suggests that shorebirds are able to track availability of HSC eggs among beaches whether that difference is slight (e.g., 2015) or more severe (e.g., 2016). Karpanty et al. (2006) also found that Red Knots used sites with higher abundances of eggs, while Gillings et al. (2007) employed a controlled field experiment to demonstrate that shorebirds aggregated at sites in response to both surface and subsurface egg densities. Alternatively, a study in the Delaware Bay found that abundance of HSC eggs at a site could not predict shorebird densities (Botton et al. 1994). Botton et al. (1994) theorized that there was not a relationship with shorebird density because eggs were superabundant over the entire study area, and therefore egg densities exceeded a threshold level of abundance that was acceptable to shorebirds across all sites in the study.

Our measures suggest that egg densities in CRNWR are moderate compared to Delaware Bay's (Karpanty et al. 2006) and therefore foraging shorebirds may be responding positively to sites with higher densities of eggs in an area of comparatively lower densities. Species that were significantly correlated to HSC egg abundance for both years of our study were Sanderling, Semipalmated Sandpiper, Red Knot, and Ruddy Turnstone. Similarly, Tsipoura and Burger (1999) found that Red Knots, Semipalmated Sandpipers, and Sanderlings foraged consistently on HSC eggs in Delaware Bay.

Eggs of horseshoe crabs in shorebird diet

Along with documenting a spatial and temporal overlap between shorebirds and HSC eggs, we also verified that birds were consuming HSC eggs at our study plots, and likely during or immediately prior to our surveys. The high percentage of fecal samples testing positive for HSC DNA suggest that HSC eggs are a regular food source in the shorebird diet during spring migration at Cape Romain NWR. The analyses of shorebird feces for the presence of DNA of HSC demonstrated that shorebirds consumed eggs at CRNWR as early as April, when spawning begins. We also found that the occurrence of HSC DNA in samples appeared to be more consistent in May and June ($\geq 93\%$) compared to April (80–90%). This pattern appears similar to findings by Novcic et al. (2015) who found higher percentages of HSC eggs in the diet of shorebirds in Delaware Bay at the end of May compared to the beginning of May. Although we designed our sampling methods to specifically measure HSC egg abundance, we observed other potential food sources in our core samples including amphipods, polychaetes, and insects (Takahashi 2016).

Although the size of sand grains on beaches may affect shorebird distribution during foraging (Colwell 2010), we did not find that sand grain size was related to the density of foraging shorebirds during our study. Botton et al. (1994) hypothesized that less energy is required by shorebirds to forage on HSC eggs (non-mobile, high detectability due to color contrast) compared with polychaetes and mussels, and therefore differences in sand grain sizes are not a driving factor in foraging decisions. We also examined

sand grain size as a factor affecting the distribution of HSC eggs across study plots. Sand grain size affects both the drainage and oxygen content of sand and subsequently has been found to affect where HSC lay their eggs (Anderson and Shuster 2003, Smith et al. 2011). Previous studies have found that the optimal sand grain size for development of HSC eggs is 350–500 μm (Smith et al. 2002a, Avissar 2006), which are larger particle sizes than we observed in our study. We did not find that the difference in sand grain size among our plots affected the distribution of eggs among our plots, which may be partly attributed to sampling surface sediment instead of including sediment of deeper depths where eggs are originally deposited.

However, other variables may be more important than differences in sand grain size for determining distribution of eggs at Cape Romain NWR. For example, we found a patchy distribution of HSC eggs within and among plots, which appears to be consistent with other studies (Smith et al. 2002c, Pooler et al. 2003). We posit that beach characteristics at the micro- and meso-scale may be driving the spatial distribution of HSC eggs. For example, the presence of marsh, peaty substrates, or high wave energy reduce suitability for spawning of HSC (Botton et al. 1994, Penn and Brockmann 1994, Anderson and Shuster 2003) and these characteristics can differ within a single beach and among nearby beaches in CRNWR, potentially contributing to within- and among-site variability. These suppositions suggest that a more detailed habitat model may be warranted for spawning sites for HSC in CRNWR that considers tide height, temperature, lunar phase, wind direction, time of day, salinity, and beach composition (Rudloe 1980, Barlow et al. 1986, Penn and Brockmann 1994; Smith et al. 2002b, 2002c; Brockmann and Johnson 2011).

Although the overall densities of HSC in CRNWR appear lower compared to the Delaware Bay, our study site clearly provides foraging opportunities for migratory and local shorebirds. We suggest that the morphology of the islands here creates small areas of dense spawning activity by HSC, which may provide the type of disturbance needed to make eggs available to shorebirds. For example, HSC eggs may be laid at a depth that is inaccessible to most shorebirds (10–20 cm; Anderson and Shuster 2003). The spawning

activity of other crabs (Smith 2007, Mattei et al. 2010) provides sufficient disturbance to allow the eggs to become available to shorebirds without eliminating availability entirely. While the Delaware Bay appears to offer a relatively high density of spawning activity throughout the region to accomplish this (Botton et al. 2003, Smith 2007), CRNWR may provide some sites of sufficient density. For example, during diurnal spawning surveys conducted on Marsh Island and Little Bull Island in 2016, Takahashi (2016) recorded ~ 1.1 crabs/ m^2 , a density measurement similar to that found in Delaware Bay (Smith et al. 2002c). Therefore, CRNWR appears to provide sites where localized, high densities of HSC spawn and may therefore increase the availability of eggs to shorebirds. To better understand the availability of HSC eggs to migratory shorebirds in this region, surveys of egg densities could be expanded to a wider geographical area and into the autumn migration stage.

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