FINAL REPORT

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Project Title: Seasonal patterns of Horseshoe Crab spawning and the relative importance of Horseshoe Crab eggs within the diets of South Carolina shorebirds

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Progress: Completed

Objective 1A: Assessing seasonal patterns of the availability of Horseshoe Crab eggs to foraging shorebirds

In South Carolina (SC), Horseshoe Crabs spawn from April to June, with the largest spawning events typically occurring in May. During spawning events, females come ashore and lay eggs in the sediment, usually at a depth of 10 – 20cm. At this depth, eggs are not available to most shorebirds (Botton et al. 1994); however, when large spawning events occur, females may excavate previously laid eggs and bring them to the surface where they are available for shorebird consumption (Smith 2007). Additionally, over time, storms and tides will shift sediments and raise eggs closer to the surface where they can be accessed by shorebirds (Jackson et al. 2014). As in other locations, the utilization of these eggs by shorebirds depends on the overlap in timing between Horseshoe Crab spawning activity and shorebird presence.

Many migratory shorebirds, including Rufa Red Knots, stopover in SC (Pelton et al. 2022), and yet how their migratory timing corresponds with Horseshoe Crab spawning and egg availability near the sediment surface is unclear. When a mismatch in that timing occurs, shorebirds must rely on other prey items. Quantifying seasonal patterns in Horseshoe Crab egg availability is necessary to understand the availability of these eggs to shorebirds and other species that rely on them. To do so, data is needed on both the timing of Horseshoe Crab spawning and when these eggs become accessible to shorebirds (i.e. occur in the top 5cm of the sediment).

Accomplishments: From late March through early July 2023, we conducted bi-weekly Horseshoe Crab egg surveys on four beaches – Turtle Island, Parris Island, Otter Island, and Pine Island. In 2024, we conducted these surveys at three beaches – Turtle Island, Otter Island, and Crab Bank. At each beach, we set up two 100-meter transects. In each transect, we took 40 sediment cores, spaced 2.5m apart. We first sieved the top 5cm of each core (the depth at which shorebirds can access Horseshoe Crab eggs, Botton et al. 1994) and then the remaining 20cm to look for Horseshoe Crab eggs in both portions. When found, Horseshoe Crab eggs were bagged, with eggs

found in the top 5cm section of the sediment kept in a separate bag from those found in the bottom 20cm of sediment. We brought all eggs back to SCDNR's MRRI facility where they were counted. A subset of eggs was then placed in 95% ethanol for potential future population genetic analyses.

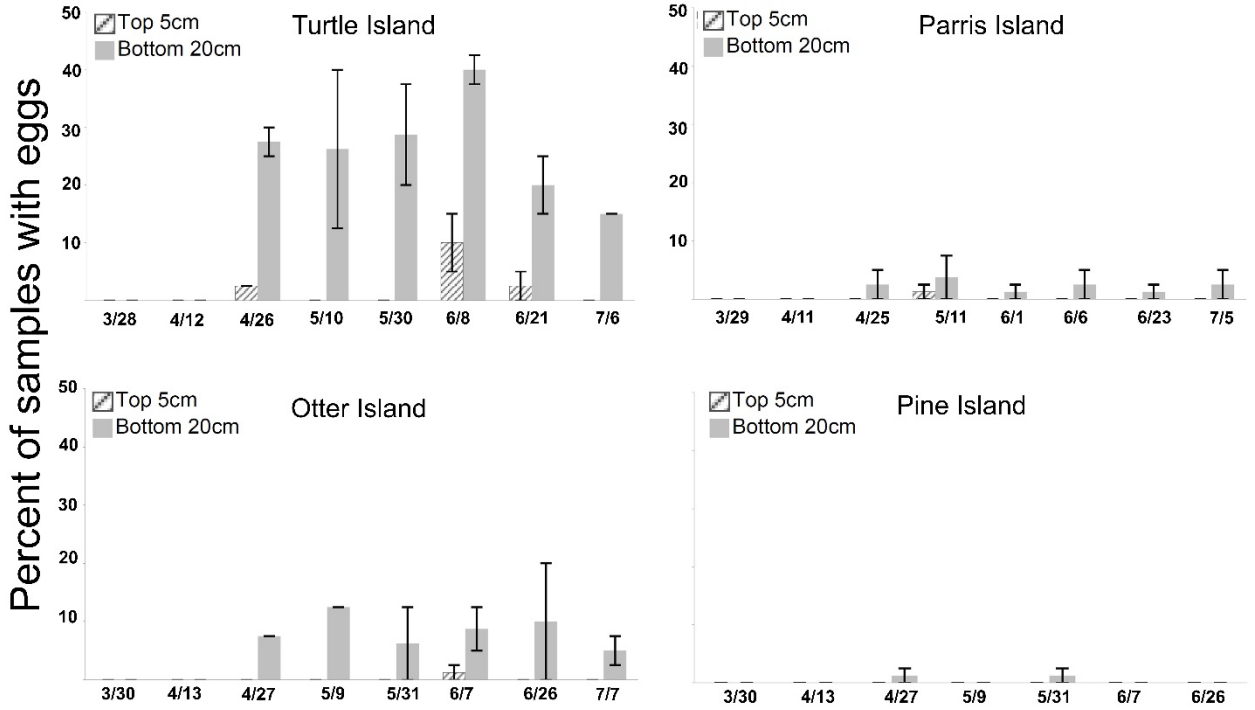

Figure 1. Horseshoe Crab egg abundance from March – July, 2023 at Turtle, Otter, Parris, and Pine Islands. We took 40 core samples from two transects at each island to look for Horseshoe Crab eggs. Striped bars indicate the percent of core samples that contained eggs in the top 5cm layer of the sediment; solid bars indicate the percent of core samples with eggs in the bottom 20cm.

In 2023, we found Horseshoe Crab eggs on all four beaches, although we found fewer samples with eggs throughout the season at Parris Island and Pine Island than on Turtle Island and Otter Island (Fig. 1). Egg abundance in the bottom 20cm of sediment peaked in early June on Turtle Island; on Otter Island, egg abundance at that depth reached its highest point in early May and then remained steady through the rest of the sampling season (Table 1, Figure 1). Egg abundance in the top 5cm of sediment peaked on both Turtle and Otter islands in early-June (Figure 1).

In 2024, we found Horseshoe Crab eggs at different prevalences on all three beaches (Fig. 2). The percentage of samples that had eggs was highest at Turtle Island, followed by Otter Island and then Crab Bank. The percentage of samples with eggs in the bottom 20cm of sediment peaked in late May on Turtle Island and Crab Bank and in late June for Otter Island. At all sites, the percentage of samples with eggs was highest from late May throughout the month of June (Table 2, Figure 2). Eggs were not found in the top 5cm of sampled cores on Otter Island in 2024, but eggs were present in this top layer of sediment in early June on Crab Bank and at low percentages from late May to early July on Turtle Island (Figure 2).

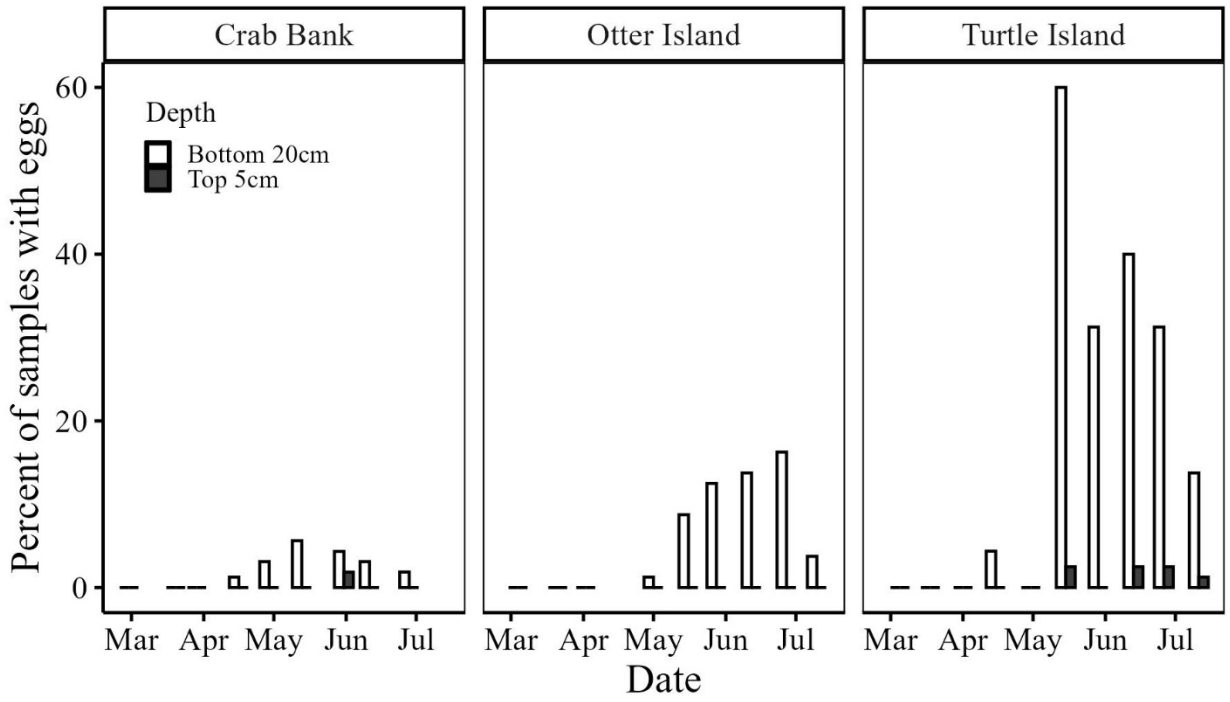

Figure 2. Horseshoe Crab egg abundance from March – July 2024 at Crab Bank, Otter Island, and Turtle Island. White bars indicate the percentage of core samples with eggs in the bottom 20cm layer of the sediment; grey bars indicate the percentage of core samples that contained eggs in the top 5cm layer of the sediment.

In both 2023 and 2024, when eggs were found in cores, more eggs were present in the bottom 20cm layer of sediment than in the top 5cm layer at every site (Table 1). These data show that the vast majority of Horseshoe Crab eggs are not at a depth reachable by shorebirds.

Objective 1B: Assessing seasonal patterns of Horseshoe Crab spawning activity

While Horseshoe Crab spawning activity occurs solely in the spring and summer in most locations along the Atlantic coast, Horseshoe Crabs are known to spawn in the fall in Florida and Georgia (e.g. Sasson et al. 2020); however, evidence for fall spawning in SC is lacking. Fall spawning in SC would represent another influx of nutrients from marine systems to terrestrial consumers.

Accomplishments: We surveyed Turtle Island on October 18, 2023 and Otter Island on October 31, 2023 for Horseshoe Crab eggs, using the same design as in Objective 1A. We did not find eggs in any of our core samples at either island during these sampling events. While this result does not necessarily mean that Horseshoe Crabs do not spawn in the fall in South Carolina, it does suggest that fall beach spawning, if it occurs, may not be nearly as prevalent as in the spring/summer.

Since we did not find eggs on any beaches in the fall of 2023, we did not conduct fall spawning surveys in 2024.

Table 1. Egg counts from the top 5cm and bottom 20cm of sediment cores at each location.

		2023			2024		
Location	Depth (2023 N, 2024 N)	Mean (± SD)	Median	Range	Mean (± SD)	Median	Range
Turtle Island							
	5 cm (3, 7)	174.7 ± 320.1	41	3 - 1152	61.9 ± 93.6	25	1 - 270
	20 cm (126, 144)	1052.3 ± 1876.4	294.5	1 - 11944	1711 ± 2275.6	689.5	1 - 12446
Otter Island							
	5 cm (1, 2)	72 ± 0	72	72	1.5 ± 0.7	1.5	1 - 2
	20 cm (39, 38)	849.2 ± 1570.2	111	1 - 7523	401.8 ± 760.2	61.5	1 - 3232
Pine Island							
	5 cm (0, N/A)	0	0	0	N/A	N/A	N/A
	20 cm (2, N/A)	16.5 ± 17.7	16.5	4 - 29	N/A	N/A	N/A
Parris Island							
	5 cm (1, N/A)	10	10	10	N/A	N/A	N/A
	20 cm (10, N/A)	639.7 ± 990.9	280	4 - 3085	N/A	N/A	N/A
Crab Bank							
	5 cm (N/A, 3)	N/A	N/A	N/A	293 ± 498.8	8	2 - 869
	20 cm (N/A, 30)	N/A	N/A	N/A	960.7 ± 1089.5	575.5	14 - 3769

Objective 2: Assessing seasonal patterns in the diet composition of shorebirds

Shorebird diets likely change throughout the season as different prey items become available. It remains unclear, however, how diets are impacted by the influx of Horseshoe Crab eggs during Horseshoe Crab spawning season. Horseshoe Crab eggs are highly nutritious and so have the potential to become the dominant prey item of shorebirds when they are readily available. As of yet, however, no studies in SC have quantified how and whether shorebird diets shift once Horseshoe Crab spawning begins. Quantifying the diet of shorebirds is necessary to understand their potential reliance on Horseshoe Crab eggs in SC. We take an approach to assessing shorebird diet through metabarcode sequencing of fecal samples. This approach allows small quantities of DNA to be sequenced and is sensitive enough to allow identification of prey items through the presence of their DNA in the fecal sample. We present the fecal metabarcoding data as both binary count data (detected/not detected) and relative read abundances. Relative read abundances can provide a more accurate view of population-level diet and have been found to benefit datasets where individual fecal samples are likely to contain many species and similar species across samples (Deagle et al. 2018).

Collection and analysis of fecal samples from Bulls, Kiawah, Seabrook, and Williamson Islands were supported by USFWS grant F23AC02257, *Characterizing Red Knot Foraging Habitat to Inform Management*, and are included here to aid in providing a more holistic dataset for interpreting the results of the combined projects.

Accomplishments: During the egg surveys at Turtle, Parris, Otter, and Pine Islands (see Objective 1A) in 2023, we collected bird fecal samples found within the transect areas. In total, we collected 887 fecal samples, mostly from Turtle and Otter Islands. We also collected fecal samples from Botany Bay and Kiawah and Seabrook Islands, as they are areas heavily utilized by roosting Red Knots (Pelton et al. 2022). A full list of locations and dates of sample collections is provided in Table 2.

During the egg surveys at Turtle, Otter, and Crab Bank (see Objective 1A) in 2024, we collected bird fecal samples found within the transect areas. In total, we collected 654 fecal samples (Table 2). We also collected 25 fecal samples from Williamson Island and Bulls Island, as they are areas heavily utilized by Red Knots (Takahashi et al. 2021).

Table 2. Number of shorebird fecal samples collected at sampling locations from March to July 2023 and February to July 2024.

Location	2023						2024							Grand Total
	Mar	Apr	May	Jun	Jul	Total	Feb	Mar	Apr	May	Jun	Jul	Total	
Botany Bay	17					17								17
Bulls Island										15			15	15
Crab Bank							20	28	121	107	19		295	295
Kiawah Island	26					26								26
Otter Island	46	83	63	117	14	323		6	24	56	39	2	127	450
Parris Island		2	44	3	1	50								50
Pine Island	14	125	82	10		231								231
Seabrook Island		35				35								35
Turtle Island	4	22	126	51	2	205		53	119	51	8	1	232	437
Williamson Island										10			10	10
	107	267	315	181	17	887	20	87	264	239	66	3	679	1566

Table 3. Number of bird fecal samples processed for DNA isolation and metabarcoding from March 2023 to July 2024.

Location	2023						2024							Grand Total
	Mar	Apr	May	Jun	Jul	Total	Feb	Mar	Apr	May	Jun	Jul	Total	
Bulls Island										15			15	15
Crab Bank							10	14	14	20	13		71	71
Kiawah Island	5					5								5
Otter Island	24	22	20	20	10	96								96
Pine Island		8				8								8
Seabrook Island		5				5								5
Turtle Island	4	19	20	20	2	65		20	39	24	8	1	92	157
Williamson Island										9			9	9
	33	54	40	40	12	179	10	34	53	68	21	1	187	366

Fecal samples were placed in 95% ethanol and brought back to the Hollings Marine Laboratory for processing. We used a subset of samples collected over the course of the season at Turtle and Otter Islands, as well as at Kiawah and Seabrook Island, for genetic sequencing and analyses. We extracted DNA from fecal samples using the QIAamp Fast DNA Stool Mini kit, following a modified protocol (Zeale et al. 2011). DNA isolates from each sample were subjected to PCR with the following cycling conditions: 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 51°C and 1 min at 72°C, with a final incubation of 5 min at 72°C. The primers targeted the cytochrome oxidase 1 (*COI*) gene using validated primers in previous studies for metabarcoding (Folmer et al., 1994; Leray et al., 2013; Zhang et al., 2018). Amplified products or fragments (~313 bp) were verified on a 1-2% agarose gel, cleaned with ExoSAP-IT Express to remove any residual components of the PCR reaction and normalized to 20ng/µL. Products were submitted to Genewiz (Azenta) for library preparation and amplicon sequencing (250bp paired-end) on an Illumina platform.

Raw sequence reads were assessed for quality using *FASTQC* (Andrews 2023) and had their forward and reverse primer sequence removed using *Cutadapt* (Martin 2011). Amplicon variant discovery was accomplished with DADA2 in the R environment (Callahan et al. 2016), in which raw reads were first filtered using default parameters (maxN = 0, trunQ = 2, maxEE=2). For each sample, filtered forward and reverse reads were denoised with an error model generated for the data and then merged. The assembled sequences were further filtered for chimeras to generate a final list of unique amplicon sequence variants (ASVs) for taxonomic assignment. The generated ASVs were then aligned against a custom database, containing 3,259,654 COI genetic sequences (Meglécz 2023), and the entirety of the nucleotide sequence database on NCBI using blastN with the following parameters: - perc_identity 98, -max_target_seqs 5, -evalue 1e-50, producing the 5 closest matches with at least 98% identity between query ASV and candidate organisms.

In total, 179 samples from the 2023 collection and 187 samples from the 2024 collection were successfully processed and metabarcode-sequenced (Table 3). Across all sampling sites and both years, we detected 18 species of bird. Horseshoe Crab (*Limulus polyphemus*) DNA was detected during each sampling month except for February on Crab Bank (Figure 4). Relative read abundances of prey items (at taxonomic order level) reveal a shift in shorebird diet throughout sampling months with unique diet compositions across sampling sites (Figure 5). Relative read abundance of Horseshoe Crab (Order Xiphosura) in bird fecal samples increases in May (Crab Bank and Otter Island) and June (Turtle Island) which corresponds with Horseshoe Crab peak spawning season. When comparing relative read abundance across all samples, there is a clear increase in Horseshoe Crab relative read abundance during peak Horseshoe Crab spawning months (Figure 6). This increase in relative read abundance during spawning season is statistically significant (Figure 7; difference in mean relative read abundance = 0.16478, p = 0.00028).

In an effort to understand the relationship between relative read abundance of Horseshoe Crab and bird species, we assigned fecal samples to a host bird species based on which bird species had the highest read abundance in that fecal sample. Most fecal samples had a clear host (i.e. read abundances were orders of magnitude higher than any other bird in that same fecal sample). Diet composition varies across bird species, even within genus. Horseshoe Crab mean relative read abundance is largest in Ruddy Turnstone (*Arenaria interpres)*, Sanderling *(Calidris alba)*, Semipalmated Sandpiper (*Calidris pusilla)*, Gull-billed Tern *(Gelochelidon nilotica)*, Laughing

Gull *(Leucophaeus atricilla),* and Unknown which is presumably a composite of several species (Figure 8). When we look at the relative read abundance of Horseshoe Crab alone, we see that all but three host birds have fecal samples that include Horseshoe Crab (Figure 9). The Ruddy Turnstone (*Arenaria interpres*) had the highest mean relative read abundance (56%) and Piping Plover (*Charadrius melodus*) had the lowest greater-than-zero mean relative read abundance (6%). Only three species contained no Horseshoe Crab sequences at all: Royal Tern (*Thalasseus maximus*), Short-billed Dowitcher (*Limnodromus griseus*), and Wilson's Plover (*Charadrius wilsonia*).

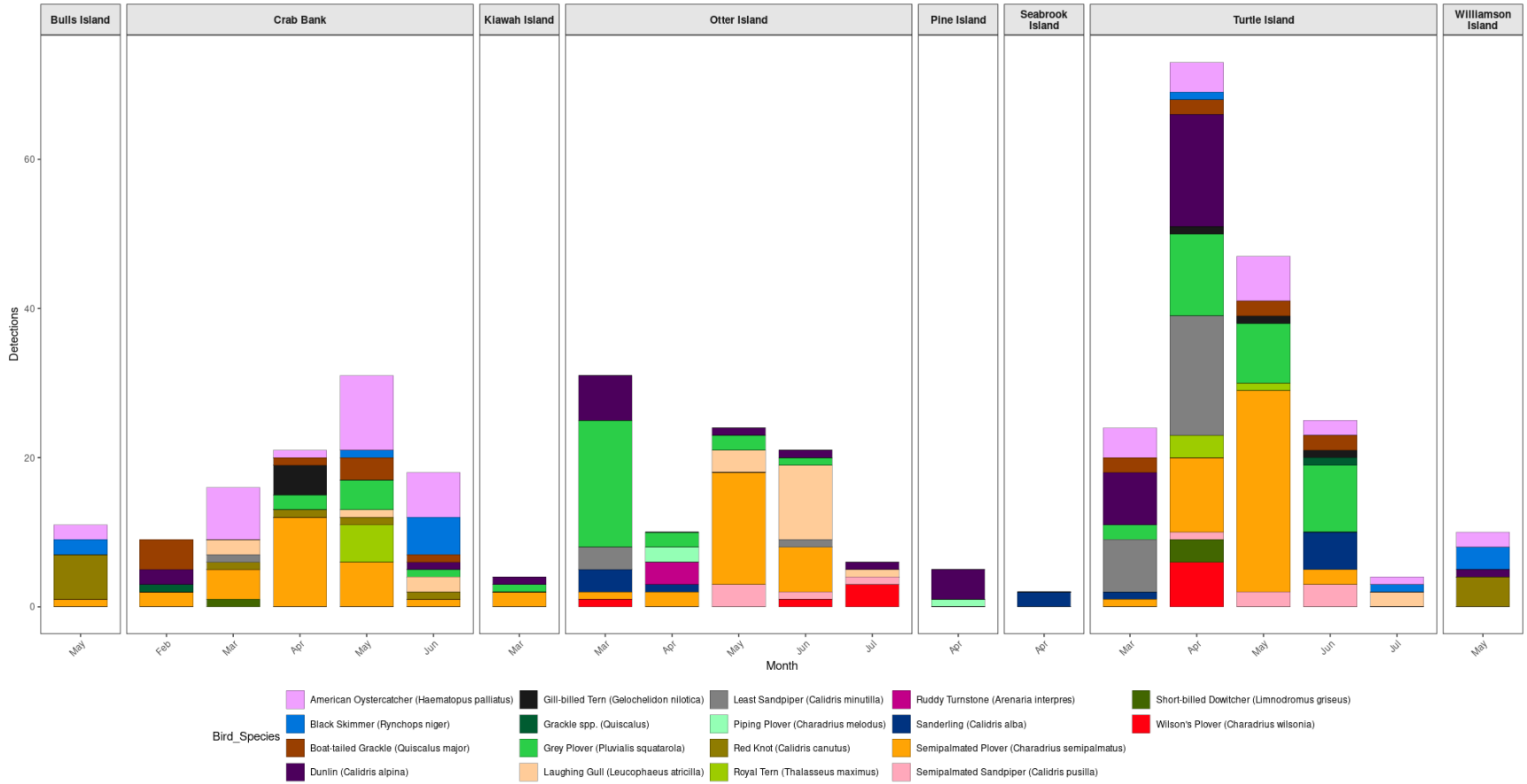

Figure 3. Number of bird detections in all processed fecal samples color-coded by species for each location and collection month over all sampling years.

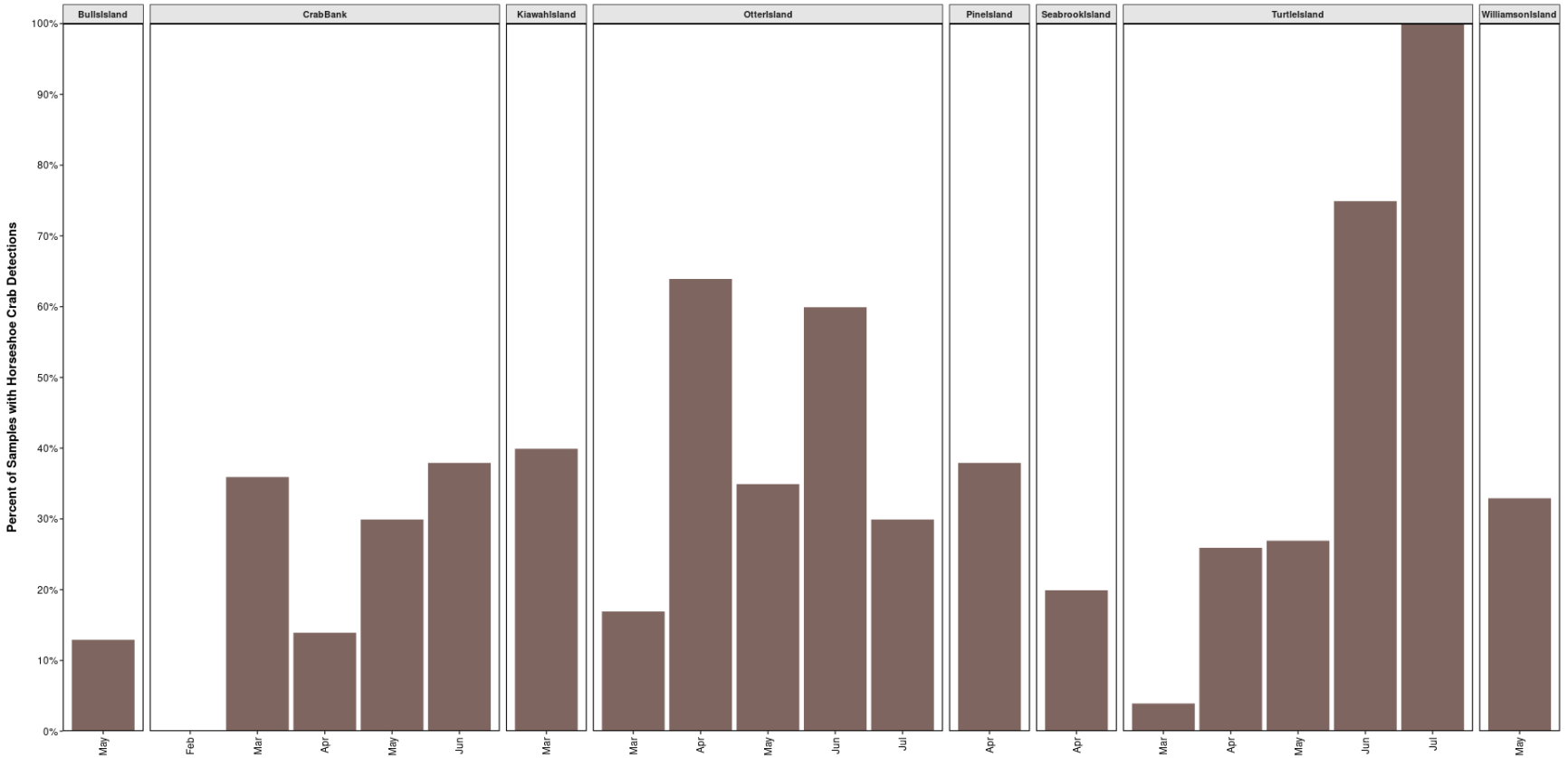

Figure 4. Percentage of fecal samples at each location, by month, in which Horseshoe Crab DNA was detected.

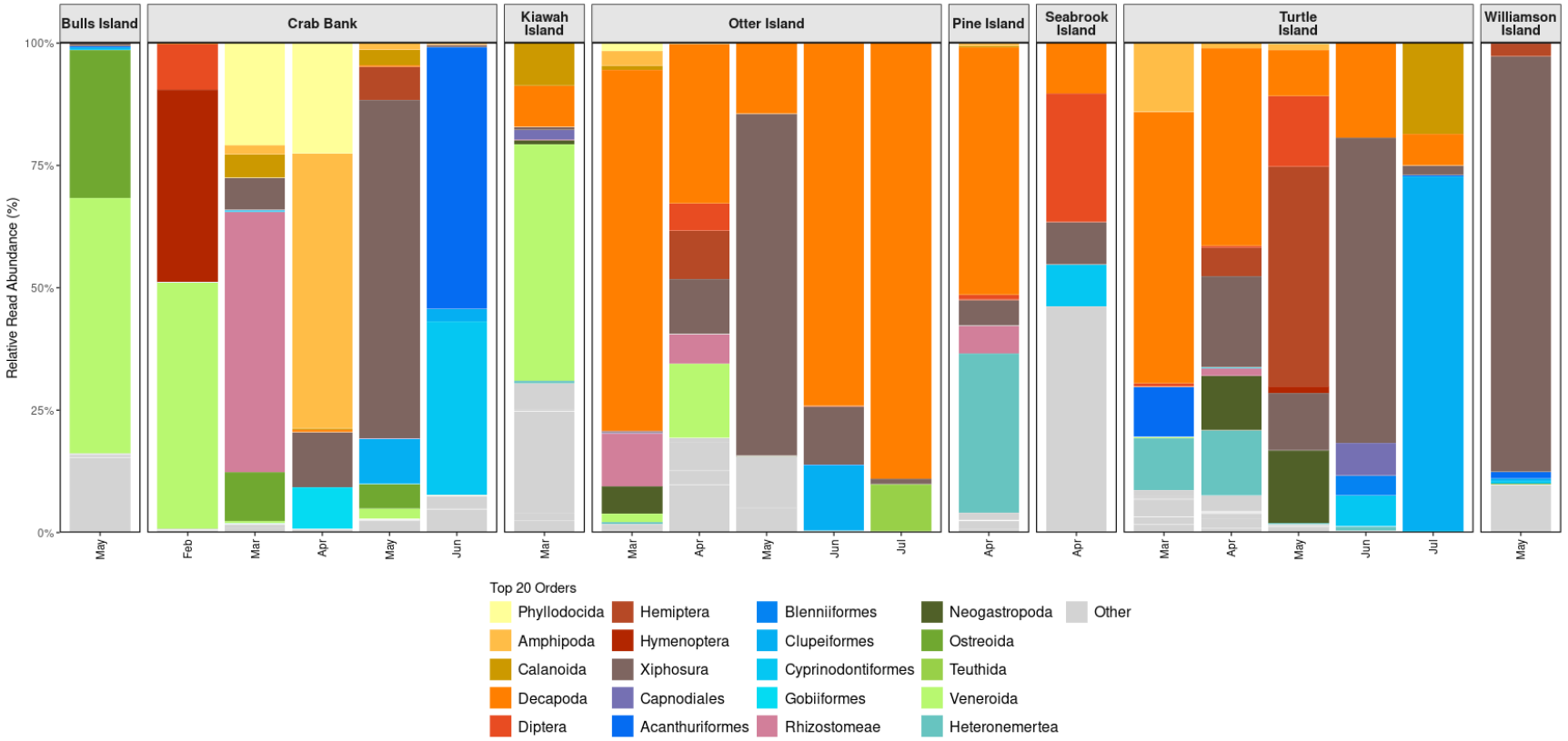

Figure 5. Relative read abundance in all processed fecal samples color-coded by OTU, at taxonomic order level, for each location and collection month over all sampling years. Only the 20 most abundant orders are visualized. All other orders were combined in the category 'Other'. The legend and color-coding are organized so that orders in the same phylum are grouped and visualized in the same color family (e.g. all arthropods are colored in the orange-brown color family).

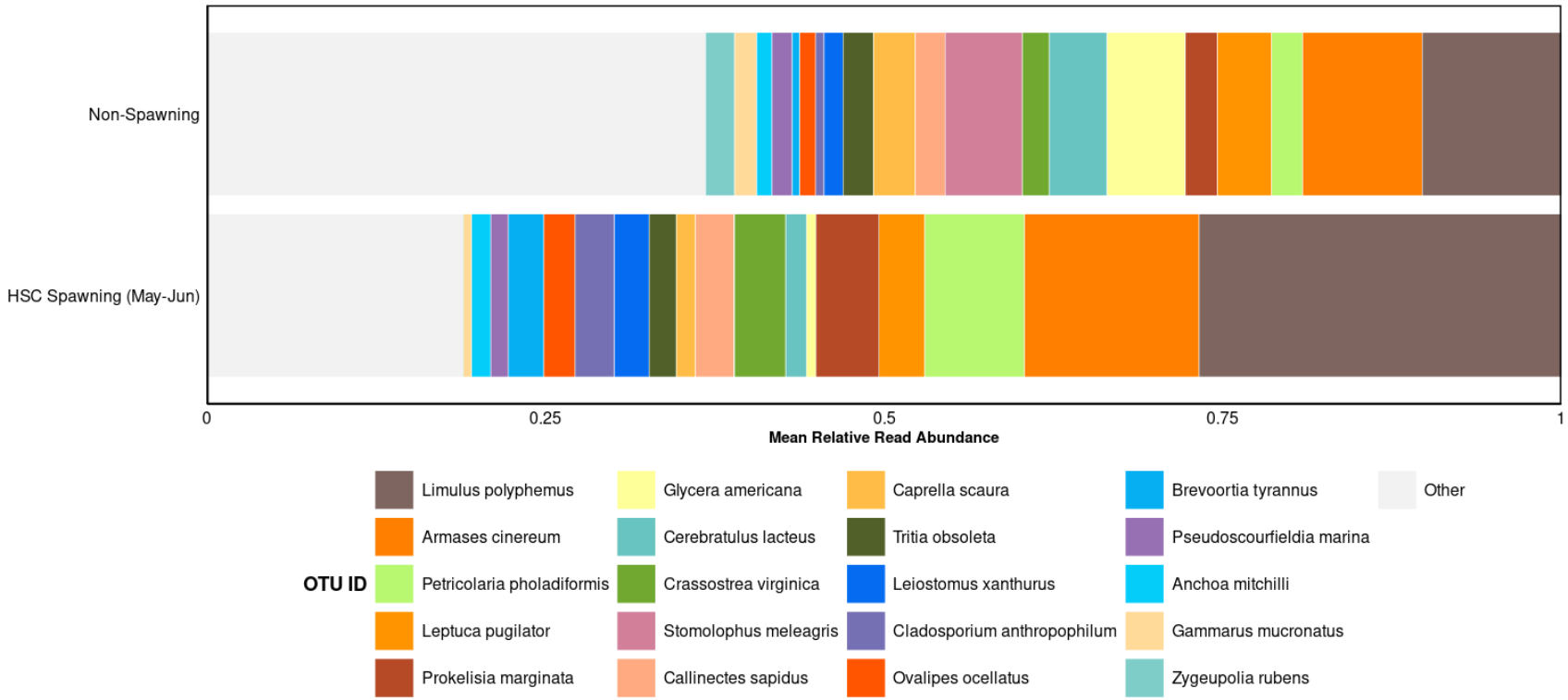

Figure 6. Mean relative read abundance across all samples in Horseshoe Crab peak spawnging season (May-June) compared to non-spawning season. The 20 most abundant OTU, at taxonomic species level, are visualized (color refelects taxonomic orders as in Figure 5) while all other OTU are grouped as ‘Other’

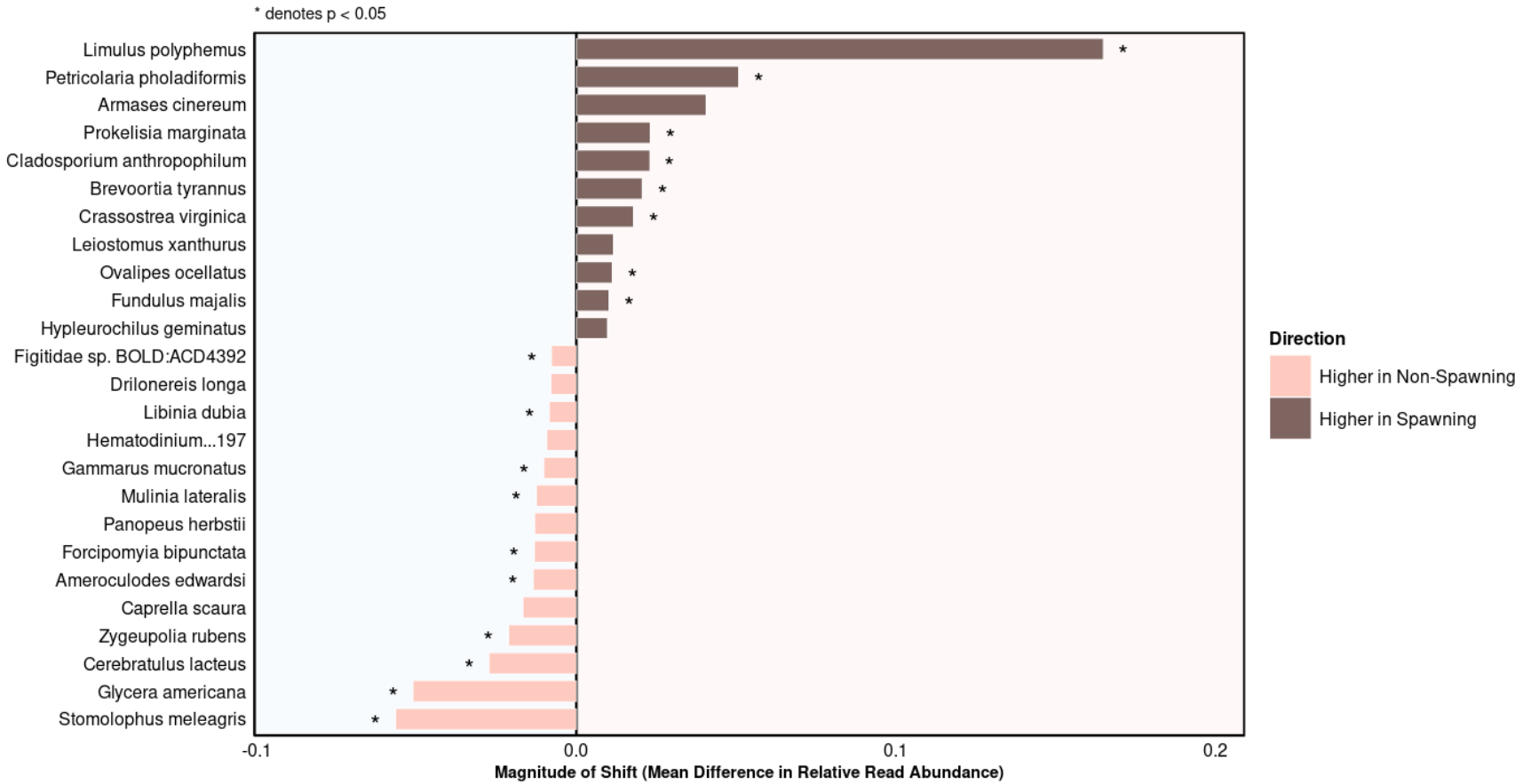

Figure 7. Magnitude of change in relative read abundance for the top 20 most abundant OTU in the dataset across all samples in peak Horseshoe Crab spawning season (brown) versus non-spawning season (peach). Asterisks represent significant differences in relative read abundance based on a Wilcoxon Signed Rank Test.

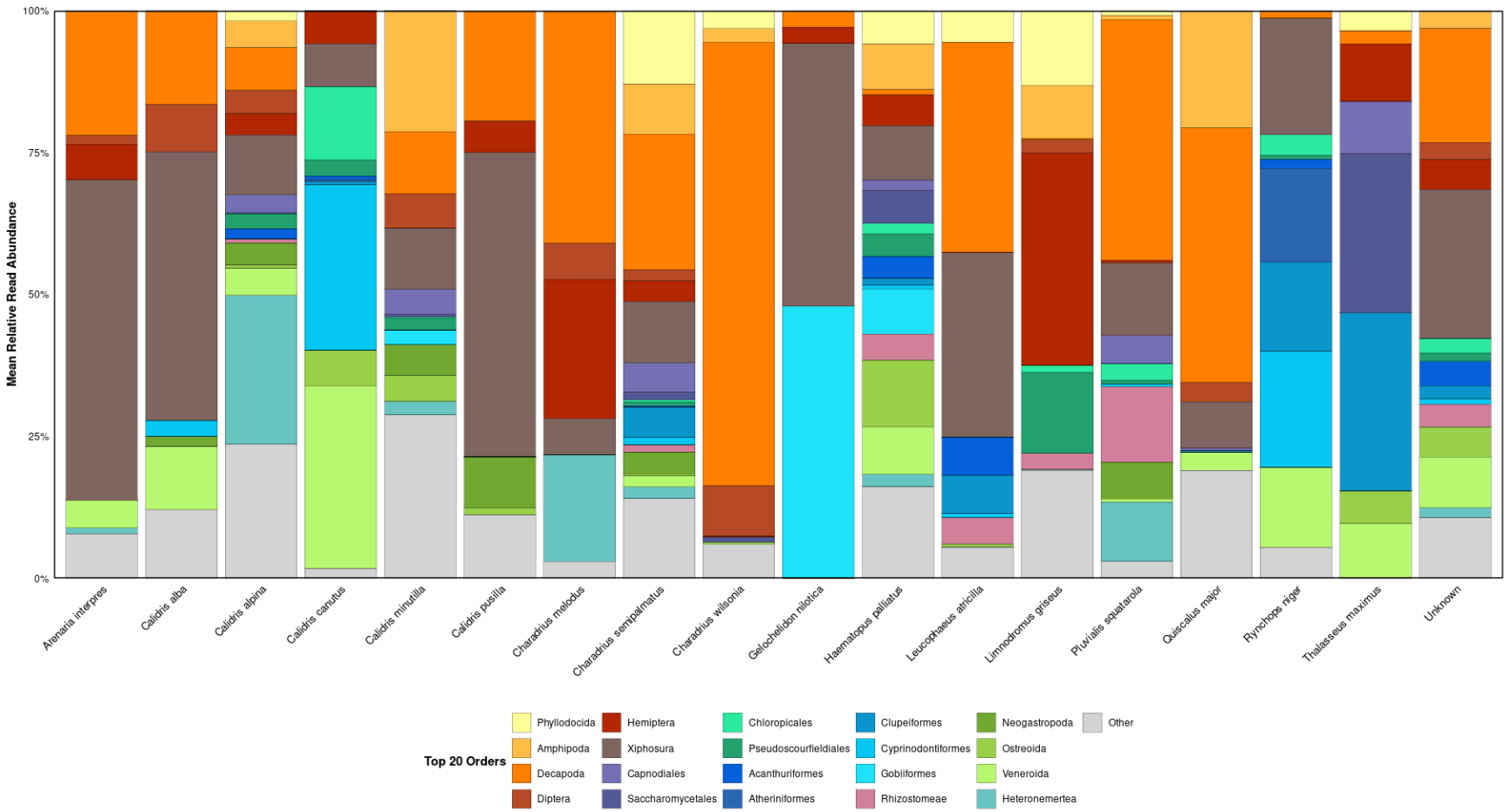

Figure 8. Relative read abundance in all processed fecal samples color-coded by OTU, at taxonomic order level, for each most likely host bird species over all sampling years. Samples with no clear host bird species are grouped as “Unknown”. Only the 20 most abundant orders are visualized. All other orders were combined in the category ‘Other’. The legend and color-coding are organized so that orders in the same phylum are grouped and visualized in the same color family (e.g. all arthropods are colored in the orange-brown color family).

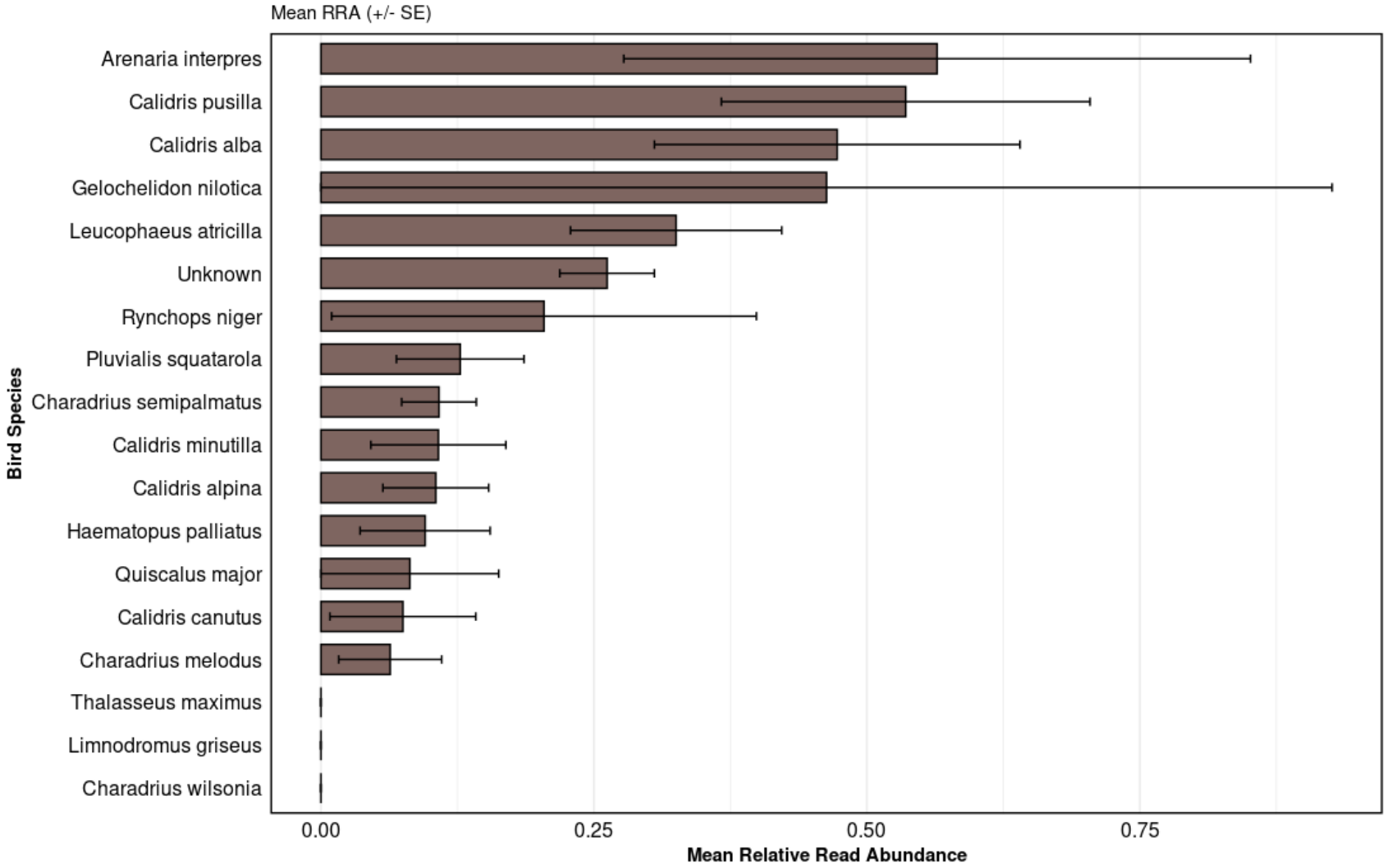

Figure 9. Mean relative read abundance (with standard error) of Horseshoe Crab in fecal samples grouped by most likely host bird species. Samples with no clear host bird are grouped as “Unknown”

Objective 3: Assessing the nutritional value of intertidal prey organisms as they relate to important shorebird species

Shorebirds in SC consume a suite of prey items, including Horseshoe Crab eggs and other intertidal marine organisms. Current assessments of resource availability are largely based on abundance data, but expanding knowledge to include information on resource quantity (i.e. biomass) and quality (i.e. nutritional value) on a per unit area and per organism type basis will provide an important layer of information for determining habitat value. Comparing the availability and nutritional value of prey items will allow us to better understand the importance of Horseshoe Crab eggs to the diets of shorebirds by estimating the relative value of Horseshoe Crab eggs, in both quantity and quality, compared to other available prey items.

Accomplishments: In the spring of 2023, 159 benthic core samples were collected at three habitat types: inlet-facing beach, ocean-facing beach, and marsh relict habitats. Inlet-facing beach habitats were the same areas sampled for the Horseshoe Crab-specific sampling. Ocean-facing beaches consisted of sandy, energetic beaches of Kiawah and Seabrook Islands. At each site, precise location, elevation data, biomass (ash-free dry weight), and sediment characteristics were quantified. At least 12 cores were collected at each site. Ocean-facing beaches and marsh relicts were sampled in areas where Red Knots were actively foraging. These samples were analyzed to compare differences between sites and understand prey-related factors that may influence foraging area selection. In 2024, additional marsh relict samples were collected on Bulls Island and Little Caper's Island to better understand spatial and interannual variability.

To gather information on potential nutritional value of these prey items, seven samples from 2023 were sent to an analytical lab (University of California Davis Analytical Lab, Davis, CA) to characterize nutrient types available in common prey items: Donax Clam (shell on), Donax Clam (shell off), amphipods, polychaete worms (two replicates), early-stage Horseshoe Crab eggs, and late-stage Horseshoe Crab eggs. These were analyzed for protein and lipid, as well as nitrogen and phosphorus when sufficient quantities could be obtained. From 2024, additional samples were sent for nutritional analysis, and these included False Angelwings (shell on), False Angelwings (shell off), Scorched Mussel (shell on), and Scorched Mussel (shell off). Data were also compiled on biomass to allow for calculation of nutritional value per unit area of habitat.

Generally, Horseshoe Crab eggs provide a greater percentage (8%) of fats than the other invertebrate prey items sampled (Table 4). The beachfront bivalves, comprised of False Angelwings, Scorched Mussel, and Bean Clam, averaged 5% fat, while amphipods and worms averaged below 3% by weight. Protein content was the dominant nutritional parameter analyzed, averaging nearly 50% of the mass of animals analyzed. Scorched Mussels and Bean Clams contained the greatest protein concentrations, in excess of 60%, while Horseshoe Crab eggs averaged 51% protein. There was also a slight decrease in nutritional parameters in early versus late-stage eggs, indicating possible consumption or conversion of these materials during egg development. Future research should explore these data in the context of area-adjusted metrics of nutritional content to enable comparison of available nutritional density across habitat types.

Table 4. Nutritional content, as a percentage of dried organism mass, per individual for various shorebird prey taxa. Data are also shown adjusted for differing biomass values, presented in parentheses as milligrams of analyte per individual.

Species	Protein	Crude Fats	Nitrogen	Phosphorus	Carbon	C:N	C:P	Biomass Per Indiv.	Biomass Source
	% (mg ind^{-1})	% (mg ind^{-1})	% (mg ind^{-1})	% (mg ind^{-1})	% (mg ind^{-1})			mg	
False Angel Wing Clams, shucked (*Petricolaria pholadiformis*)	47.5 (6.09)	4.7 (0.60)	7.6 (0.98)	0.7 (0.09)	34.9 (4.48)	4.59	51.40	12.83 ± 1.84	Gibson thesis, n=80, 1390 ind.
Scorched Mussels, shucked (*Brachidontes exustus*)	66.3 (0.75)	6.1 (0.07)	10.6 (0.12)	1.3 (0.01)	40.4 (0.45)	3.81	31.10	1.16 ± 0.10	Gibson thesis, n=36, 274 ind.
Bean Clams, shucked (*Donax variabilis*)	62.4 (1.47)	4.1 (0.10)	9.9 (0.24)	n.d.	36.7 (0.86)	3.68	n.d.	2.36 ± 0.34	Folly Beach study, n=238, 1305 ind.
Horseshoe Crab Eggs, early stage (*Limulus polyphemus*)	53.1 (0.95)	8.3 (0.15)	8.5 (0.15)	0.4 (0.01)	45.5 (0.81)	5.35	119.74	1.79 ± 0.05 (not staged)	Gibson thesis, n=6, 948 ind.
Horseshoe Crab Eggs, late stage (*Limulus polyphemus*)	50.0 (0.90)	7.5 (0.13)	8.0 (0.14)	0.4 (0.01)	38.3 (0.69)	4.79	106.39		
Amphipods	44.6 (0.08)	2.8 (0.01)	7.1 (0.01)	n.d.	30.6 (0.06)	4.29	n.d.	0.18 ± 0.02	This study, n=61, 572 ind.
Polychaetes and Oligochaetes	16.3 (0.21)	2.2 (0.03)	2.6 (0.03)	n.d.	11.3 (0.14)	4.35	n.d.	1.26 ± 0.29	This study, n=71, 339 ind.

Objective 4: Development of outreach and educational materials on Horseshoe Crab, shorebird, and benthic prey interactions in SC habitats (Years 1 & 2)

Accomplishments:

Education staff hosted several educational field trip programs that incorporated current research about Horseshoe Crabs and shorebirds. In 2023, this included three programs that reached 70 students and teachers. In 2024, this included two programs that reached 58 students and teachers. Staff also created an infographic detailing the Horseshoe Crab/shorebird connection in South Carolina. This infographic is used during educational events and can be found online (https://www.dnr.sc.gov/marine/mrri/shellfish/hscrabs.html).

Significant Deviations:

None

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