IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH CAROLINA COASTAL CONSERVATION LEAGUE, Plaintiffs,	)	
v.	)	
ROBERT H. BOYLES, JR., in his official capacity as Director of the South Carolina Department of Natural Resources, and CHARLES RIVER LABORATORIES, INTERNATIONAL, INC., Defendants.	)	Civ. No. 2:22-CV-112-RMG

PLAINTIFFS' COMBINED MOTION FOR PRELIMINARY INJUNCTION AND MEMORANDUM IN SUPPORT

Plaintiffs Defenders of Wildlife ("Defenders") and South Carolina Coastal Conservation League (the "League") respectfully move the Court for a preliminary injunction against Defendant South Carolina Department of Natural Resources' ("SCDNR") authorization of horseshoe crab containment ponds and Defendant Charles River Laboratories International, Inc.'s ("Charles River") use of horseshoe crab containment ponds. For the reasons set forth herein, and based on the evidence and affidavits filed in support of this motion, Plaintiffs have made a clear showing on each of the four elements warranting preliminary injunctive relief.[1]

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[1] Pursuant to Local Civil Rule 7.02, prior to filing this motion, Plaintiffs' counsel conferred with counsel for both Defendants regarding this motion. The parties were unable to reach an agreement. SCDNR takes no position on this motion and Charles River opposes it.

Regarding the confidential documents discussed herein, Plaintiffs have redacted all information designated as "Confidential" by SCDNR or Charles River in this brief and in all exhibits. Plaintiffs are providing an unredacted brief with exhibits to the Court for *in camera* review. As Plaintiffs explained to opposing counsel, however, courts routinely hold that the First Amendment applies to preliminary injunction motions and supporting materials and requires a

INTRODUCTION

As the Court is well aware, this is a case about a migratory shorebird called the *rufa* red knot—a species in rapid decline and listed as "threatened" under the Endangered Species Act ("ESA"). Each spring, the robin-sized birds traverse the globe to reach their Arctic breeding grounds. Red knots stop on South Carolina beaches during this migration for a critical reason—because our State's beaches are the site of large aggregations of spawning horseshoe crabs each April through June. A super-abundance of horseshoe crab eggs on these beaches is critical to migrating red knots' survival and reproduction, as each red knot must consume roughly <u>several hundred thousand</u> of the tiny eggs during its South Carolina stopover to survive its journey to, and breed in, the Arctic.

In South Carolina, there is only one business responsible for horseshoe crab harvesting, which is poised to begin in the next month—Defendant Charles River. At the critical time when horseshoe crabs emerge from the sea to spawn and red knots arrive to feast on their eggs, Charles River takes roughly [redacted] spawning crabs from South Carolina beaches annually. The company, acting under permits issued by SCDNR, stores between [redacted] [redacted]

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higher burden to keep preliminary injunction proceedings redacted or sealed (versus the discovery motions that Plaintiffs have filed in this manner prior to now). *See, e.g., Ctr. for Auto Safety v. Chrysler Grp., LLC*, 809 F.3d 1092, 1096–1103 (9th Cir. 2016) (applying the "compelling reasons" test to sealing preliminary injunction proceeding, and rejecting "less exacting 'good cause' standard" applicable to sealing discovery motions); *Publicker Indus. Inc. v. Cohen*, 733 F.2d 1059 (3rd Cir. 1984) (finding a First Amendment public right of access to preliminary injunction proceedings); *Bayer Cropscience Inc. v. Syngenta Crop Prot., LLC*, 979 F. Supp. 2d 653, 656 (M.D.N.C. 2013) ("The Court concludes that the briefing and exhibits filed in connection with motions seeking injunctive relief are subject to the public's First Amendment right of access."); *RLI Ins. Co. v. Nexus Servs., Inc.*, No. 5:18-CV-00066, 2018 WL 10602398, at *1 (W.D. Va. Oct. 30, 2018) (holding that "more stringent First Amendment right of public access applies" to sealing a motion for preliminary injunction and supporting evidence). The Court, if it so chooses, would thus be justified in requiring SCDNR and Charles River to meet the more stringent standard discussed in these cases for keeping this motion and supporting evidence from public view.

[redacted]—about [redacted] crabs in recent years—in artificial containment ponds located away from the State's beaches during the spawning season.[2]

For its convenience, Charles River [redacted], where it drains up to half of the crabs' blood for pharmaceutical use. Horseshoe crabs are harmed in the harvesting process, by transport to and from containment ponds, storage in the ponds, and release from the ponds, causing significant harms above and beyond those inherent in the bleeding process itself. Documents obtained in discovery demonstrate widespread non-compliance with South Carolina law requiring that crabs be "handled so as to *minimize injury*" and "*returned unharmed* to state waters of comparable salinity and water quality . . . ." S.C. Code § 50-5-1330(C) (emphasis added). In 2022, Charles River also [redacted] a new permit term requiring that [redacted]. *See* IC Ex. A at 2, 4 (2022 bleeding permit terms); *see also id.* at 6, 8, 10, 12 (2022 pond permit terms).[3] All of these harms flowing from the containment pond process harm individual horseshoe crabs and the population as a whole, thus decreasing their ability to spawn the super-abundance of eggs that threatened red knots rely on.

In addition, harvested horseshoe crabs are not spawning on South Carolina beaches but in artificial containment ponds, depriving red knots of their critical food source at stopover sites during migration and unlawfully "taking" these birds by causing death and impaired breeding, in violation of the ESA. As the United States Fish and Wildlife Service ("Service") commented just this week, Charles River's harvest from a South Carolina wildlife refuge "would lead to a

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[2] Notably, this is around [redacted] the annual pond totals alleged in the Complaint, ECF No. 1 ¶ 95.

[3] As with prior filings, Plaintiffs use the prefix "IC" to denote exhibits submitted *in camera* as a result of a Defendant's confidentiality designations.

dramatic reduction in horseshoe crab eggs laid and therefore reduce the availability of this preferred food source for red knots," which "would significantly impact the migratory shorebirds" and "could negatively affect [breeding] success and long-term population viability." U.S. Fish & Wildlife Serv., *Draft Compatibility Determination for the Harvest of Horseshoe Crabs, Cape Romain National Wildlife Refuge*, at 14 (Mar. 14, 2023) (hereinafter "Draft CD for Cape Romain").[4] The same is true of the company's harvest-for-ponds across South Carolina.

For its part, Defendant SCDNR has, for years, been beholden to Charles River and has allowed the company to run roughshod over state regulations.[5] SCDNR has consistently failed to ensure that crabs are handled and stored so as to minimize harm, as the law requires; last year, the agency turned a blind eye when [redacted]. Charles River's influence over the state agency has apparently been without limit. When faced with public scrutiny of its practices, a

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[4] *Available at* https://www.fws.gov/DraftCD-CapeRomainNWR, https://perma.cc/XBE5-U6GP (permanent link).

[5] In 2022, for example, the Post and Courier published an article on this topic as part of its "Uncovered" series. *See* Tony Bartelme & Shamira McCray, *A South Carolina Agency is Hooked on ... MONKEYS AND BLOOD—SC agency rakes in millions from pharma company it regulates*, The Post & Courier (March 4, 2022), *available at* https://www.postandcourier.com/uncovered/sc-agency-rakes-in-millions-from-pharma-company-it-regulates/article_60a80662-82e1-11ec-94d8-2791d3f53dcd.html, https://perma.cc/A3XZ-LUJE (permanent link). Numerous other media stories and editorials have noted this questionable relationship between SCDNR and Charles River. *See* The Post and Courier Editorial Board, *Editorial: Time to untangle an unintended conflict along SC's shore* (Mar. 12, 2022), *available at* https://www.postandcourier.com/opinion/editorials/editorial-time-to-untangle-an-unintended-conflict-along-scs-shore/article_c3058d62-9f28-11ec-9e27-dbedb95087bd.html, https://perma.cc/6JEM-VCVU (permanent link); Chiara Eisner, *Of McMaster and marshes: Inside the 500K Proposal to Bleed Protected South Carolina Horseshoe Crabs*, The State (May 5, 2022), *available at* https://www.thestate.com/news/state/south-carolina/article257214377.html.

high-ranking Charles River official remarked that [redacted]" IC Ex. B.

Plaintiffs' motion is timely. Over the last several months, Plaintiffs have engaged in discussions with SCDNR to attempt to resolve this matter prior to this year's horseshoe crab spawning season without the need for injunctive relief. But after weeks of negotiations, Charles River's apparent influence over the agency proved to be so great that settlement discussions finally fell apart on Friday, March 3, 2023, necessitating the prompt filing of this motion. *See Solar Eclipse Inv. Fund XXXV, LLC v. $5,000,000*, No. 9:19-1176-RMG, 2019 WL 1930752, at *2 n.2 (D.S.C. Apr. 30, 2019) (Gergel, J.) (granting temporary restraining order and holding that four-month delay did not defeat finding of irreparable harm where plaintiff had used the time to try to resolve the matter, with the last settlement outreach occurring within a month); *see also Kan. Health Care Ass'n, Inc. v. Kan. Dep't of Soc. & Rehab. Servs.*, 31 F.3d 1536, 1543–44 (10th Cir. 1994) (holding that a preliminary injunction was timely because the plaintiff had tried to reach a settlement with the defendant and acted within three months of failing to reach such a settlement).

SCDNR's authorization—and Charles River's use—of horseshoe crab containment ponds in South Carolina is causing an unlawful "take" of red knots under the ESA. Plaintiffs will suffer irreparable harm if Charles River is authorized to proceed with harvesting crabs for storage in containment ponds during this spring's spawning season, while any harm to Charles River is speculative and, at most, purely monetary. The public interest favors the protection of a threatened species over Charles River's profits. Plaintiffs' motion should be granted.

FACTUAL BACKGROUND

I. Red Knot Migrations and the Critical Role of Super-Abundant Horseshoe Crab Eggs.

Each spring, red knots embark on "one of the longest distance migrations known in the animal kingdom," from wintering grounds on the southern tip of South America to breeding grounds in the Canadian Arctic. Endangered and Threatened Wildlife and Plants; Proposed Threatened Status for the Rufa Red Knot (Calidris canutus rufa), 78 Fed. Reg. 60,024, 60,027 (Sept. 30, 2013). Many red knots rely on only one or two "stopovers" along a nearly transpolar flight. Declaration of Dr. Lawrence Niles ("Niles Decl.") ¶ 14.[6]

For many of the red knots that remain, a short stop on the beaches of South Carolina is a "critical" stop along the way. *Id.* ¶¶ 20–22. The Service has proposed a rule designating stopover beaches across South Carolina as red knot critical habitat. Proposed Rule, Endangered and Threatened Wildlife and Plants; Designation of Critical Habitat for Rufa Red Knot (Calidris canutus rufa), 86 Fed. Reg. 37,410, 37,425–27 (July 15, 2021). According to SCDNR, up to two-thirds of visiting red knots fly directly to the Arctic after stopping on our State's beaches. Niles Decl. ¶ 21 (citation omitted).

Red knots have evolved to time their stopover to coincide with horseshoe crab spawning season. *Id.* ¶ 26. Each spring, just as red knots arrive, the crabs emerge from the sea to lay their tiny eggs in beach sands. *Id.* ¶ 25; Declaration of Dr. H. Jane Brockmann ("Brockmann Decl.") ¶ 12.[7]

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[6] Redacted declaration attached as Ex. A; unredacted version submitted as IC Ex. N.

[7] Redacted declaration attached as Ex. B; unredacted version submitted as IC Ex. O.

The timing is no coincidence. According to the testimony of Dr. Lawrence Niles, one of the world's leading experts on red knots, each bird must consume several hundred thousand horseshoe crab eggs during its South Carolina stopover to survive its arduous migration and breed in the Arctic. Niles Decl. ¶ 29; *see also id.* ¶¶ 26–39. Red knots unable to consume their share of crab eggs are significantly less likely to survive their migration and reproduce. *Id.* ¶¶ 29, 37–39.

Horseshoe crab eggs are fatty, nutrient-rich, and easily digestible. *Id.* ¶ 28; Brockmann Decl. ¶ 12. They are the only source of food available to red knots on South Carolina beaches that allows the birds to reliably build up the fat and protein reserves needed to fly to the Arctic, mate, and reproduce. Niles Decl. ¶¶ 26–30. As SCDNR researchers confirm, "[h]ighly nutritious horseshoe crab eggs and embryos . . . provide a *critical* food resource to the federally threatened *rufa* red knot . . . as it stops to feed in areas, such as . . . South Carolina during its annual migration from southern South America to the Arctic." *See* Michael R. Kendrick et al., *Assessing the Viability of American Horseshoe Crab (*Limulus polyphemus) *Embryos in Salt Marsh and Sandy Beach Habitats*, 240 Biol. Bull. 145, 146 (2021) (emphasis added, citations omitted) (attached as Ex. C, at 2).

SCDNR is correct that the crab's eggs are a "critical" food source for red knots in South Carolina. Niles Decl. ¶¶ 26–39. After flying thousands of miles, the birds are often emaciated by the time they reach South Carolina. *Id.* ¶ 14. To refuel, power a flight to the Arctic, and build up energy to breed in frigid habitat, red knots must increase their body weight by roughly 50% during a stopover. *Id.* ¶¶ 15–17. The birds must gain weight quickly to reach the Arctic before cold weather makes the region inhospitable. *Id.* ¶¶ 18, 30. Red knots "require[] stopovers rich in easily digested food to achieve adequate weight gain that fuels the next migratory flight and, upon arrival in the Arctic, fuels a body transformation to breeding condition." 78 Fed. Reg. at 60,027 (citations omitted).

Female horseshoe crabs deposit their eggs 10–20 centimeters below the beach surface, Brockmann Decl. ¶ 13, where red knots cannot reach them due to the birds' size and small beak length. Niles Decl. ¶ 36. But when large congregations of crabs spawn on beaches, successive spawning events over several days deposit thousands of eggs in the same spot. Brockmann Decl. ¶¶ 13–14. This displaces earlier-laid eggs, causing them to rise to the beach surface, *id.*, where they become accessible to red knots. Niles Decl. ¶ 36. Thus, red knots require an "accessible egg super-abundance" created by "repeated . . . spawning events." Niles Decl. ¶ 36. It is no surprise, then, that the bird's numbers have dwindled as commercial crab harvesting has exploded on the Atlantic Coast. *See id.* ¶ 10; *see also* Kristin L. Hamilton et al., *Physiological impacts of time in holding ponds, biomedical bleeding, and recovery on the Atlantic horseshoe crab*, Limulus polyphemus, Part A, 239 Comparative Biochem & Physiology 1 (2020) (attached as Ex. D, at 1).

II. The Biomedical Harvest and Horseshoe Crab Containment Ponds.

In South Carolina, Charles River is the only entity engaged in horseshoe crab harvesting. Despite the red knot's federally-protected status, SCDNR permits Charles River to harvest large numbers of spawning horseshoe crabs and store them in artificial holding ponds during their spawning exactly when desperately hungry red knots are searching South Carolina beaches for horseshoe crab eggs.

Each spring, as the birds arrive and crabs come ashore to spawn, agents of Charles River, with permits from SCDNR, remove around [redacted] crabs from the State's beaches and store about [redacted] of them in containment ponds during the spawning season. *See* IC Ex. C at 3[8].

Instead of spawning on beaches, crabs spawn in ponds en masse. Brockmann Decl. ¶¶ 41–44.

Figure 1, above. Agents of Charles River remove crabs from Turtle Island, a red knot stopover beach proposed as critical habitat by the Service, in 2019. ECF No. 67 at 13. Large numbers of crabs are also harvested for ponds from red knot beaches across South Carolina. *See* IC Ex. D; Niles Decl. ¶¶ 48–55.

Figure 2, right. [redacted]

[redacted]

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[8] Plaintiffs earlier submitted *in camera* Exhibit C, SCDNR's First Supplemental Answers to Plaintiffs' First Set of Interrogatories, as *in camera* Exhibit H in support of Plaintiffs' motion to compel, ECF No. 67, and have re-submitted the exhibit *in camera* here, in case the Court no longer has hard copies of that exhibit. References to ECF No. 67—IC Ex. H in Plaintiffs' expert declarations are to IC Ex. C.

Although biomedical horseshoe crab harvesting occurs in several states, South Carolina is the only state that allows crabs to be stored in ponds before bleeding. Crabs are only held in ponds here for Charles River's convenience because [redacted].[9] Thus, crabs are stored in ponds during spawning season [redacted].

Charles River's bleeding operation is [redacted].[10] The company drains up to half[11] of each crab's blood to extract a substance, called Limulus Amebocyte Lysate ("LAL"), which it sells on the global market as a test for endotoxins in medical equipment and injectable drugs. There is a synthetic alternative to horseshoe crab blood-derived LAL, called recombinant Factor C ("rFC"), shown to be "'equivalent or even superior to LAL.'" ECF No. 1 at 19–20 & nn. 8–10 (collecting and quoting studies). The U.S. Food and Drug Administration approves the use of

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[9] *See* IC Ex. E (SCDNR analysis, [redacted] .

[10] *See* Ex. E (SCDNR's admission that biomedical bleeding "is the only non-research purpose for which crabs may lawfully be taken, transported, or held in ponds in South Carolina," and that "only one entity or person [is] permitted to biomedically bleed or process horseshoe crabs in South Carolina"); IC Ex. A at 1–4 ([redacted] 2022 bleeding permits); IC Ex. F (annual letters from [redacted] to [redacted], noting that [redacted]).

[11] Kristin Linesch Hamilton et al., Ex. D at 5 ("Our results indicate that as much as half of an individual [crab's] total hemolymph volume might be extracted during a standardized eight-minute biomedical bleeding, a substantial loss of hemolymph during an already stressful process.").

rFC if each tested drug or device meets purity standards.[12] Some of Charles River's competitors, such as Eli Lilly, have switched over to the synthetic, with Lilly conducting an estimated 90% of its endotoxin testing using rFC.[13] Charles River continues to fight the use of the synthetic in the United States, most recently petitioning the FDA to limit the use of this alternative.[14]

Rather than switching to the synthetic, Charles River has [redacted] horseshoe crab harvesting in South Carolina. From 2015 to 2018, Charles River harvested roughly [redacted] crabs per year in South Carolina. IC Ex. C at 3. During these years, Charles River stored roughly [redacted]% of its harvest—around [redacted] crabs—in ponds each year before bleeding. *Id.* [redacted]. *See* IC Ex. G; *see also* IC Ex. A at 3 ([redacted]). [redacted], [redacted]. *See* IC Ex. C at 3. From 2019 to 2022,

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[12] *See* US Dep't of Health and Human Services, Food & Drug Admin., *Guidance for Industry – Pyrogen and Endotoxins Testing: Questions and Answers*, at 5 (2012), https://www.fda.gov/files/drugs/published/Pyrogen-and-Endotoxins-Testing--Questions-and-Answers.pdf, https://perma.cc/AH6D-LT7T (permanent link). ("Yes, firms may use alternative methods and/or procedures" for endotoxin testing, and specifically mentioning "Recombinant Horseshoe Crab Factor C Assay," where "shown to achieve equivalent or better results" (citing various USP Chapters)).

[13] *See* Deborah Cramer, *Inside the Biomedical Revolution to Save Horseshoe Crabs and the Shorebirds that Need Them*, Nat'l Audubon Soc'y (2018), https://www.audubon.org/magazine/summer-2018/inside-biomedical-revolution-save-horseshoe-crabs, https://perma.cc/96SE-YRPA (permanent link); Ex. F at 3 (Eli Lilly Report, noting "[b]y 2020, Lilly intends to transition 90 percent of our endotoxin tests to the synthetic compound," saving "on the order of several thousand horseshoe crabs" per year).

[14] *See* Citizen Petition to FDA by Charles River Laboratories, August 2, 2022, *available at* https://www.regulations.gov/document/FDA-2022-P-1764-0001 (last visited Mar. 13, 2023).

Charles River harvested roughly [redacted] crabs per year from South Carolina, storing between [redacted] [redacted]% of its harvest—around [redacted] crabs—in ponds each year before bleeding. *Id.*

Charles River [redacted] [redacted]—roughly [redacted] [redacted]. *See* IC Ex. H at 1–2. This will likely [redacted] [redacted] (based on the percentage of crabs held in ponds between 2019 and 2022, *see* IC Ex. C at 3).

Most—if not all—of the crabs stored in ponds are taken from or adjacent to red knot stopover beaches and proposed critical habitat. *See* Niles Decl. ¶¶ 48–53 & n. 13. "Given the proximity of the harvest to stopover sites, harvesters are removing crabs for pond storage that would otherwise be laying eggs" on beaches that could be consumed by red knots. *Id.* ¶ 50. "The removal of these crabs for pond storage deprives red knots of their eggs." *Id.*

Figures 3 (above) and 4 (below) [PLTF045180 and PLTF045182]. Red knots, identifiable in the foreground of *Figure 4*, and other shorebirds forage in the surf around a few horseshoe crabs on Turtle Island, while harvesters push their boat by the flock and harvest crabs in the background. Given that the [redacted], IC Ex. C at 3, it is [redacted].

The evidence produced in discovery indicates that harvesters often take crabs for pond storage *while* red knots are present and foraging for their eggs. *See* Fig. 3–4; Niles Decl. ¶ 58. That harvesters take food directly from red knots causes harms beyond food deprivation, likely disturbing and possibly "flushing" the birds. Niles Decl. ¶ 58; *see also id.* n.15 (defining "flushing"). Red knots disturbed in this manner will perceive a threat and may stop foraging or leave the beach. *Id.* ¶ 58. This wastes critical time and energy during the short time when red knots must build body mass. *Id.* As an SCDNR shorebird biologist observed, "[r]esting and feeding undisturbed is crucial [for SC red knots] to make it to their nesting grounds and surviv[e].” Ex. G at 3. Yet harvesters for Charles River observed by the Service “flush any and all birds present” and “disturb red knots foraging.” Draft CD for Cape Roman at 3, 16.

Charles River provides [redacted] for storage in ponds, paying [redacted] [redacted]” IC Ex. I. Although SCDNR does not require pond operators to record the sex of crabs held in containment ponds, IC Ex. C at 3; ECF No. 42-1 at 4, evidence suggests that [redacted].[15] This would mean ponds have stored around [redacted] each spring since 2019, *see id.*,[16] when the [redacted] [redacted] and the totals of [redacted]. At those rates, ponds likely deprive red knots of [redacted] crab eggs each spring.[17] Because each red knot must consume several hundred thousand crab eggs during its stopover, Niles Decl. ¶ 29, removing [redacted] crab eggs, conservatively, removes the food needed by [redacted] red knots each spring at current harvest levels. What is more, red knots depend not simply on the

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[15] Assuming that the sex ratios of crabs held in ponds before bleeding are the same as the sex ratios of crabs delivered for bleeding—a plausible assumption given that [redacted] [redacted] [redacted], IC Ex. C at 3–4—then roughly [redacted] percent of crabs held in ponds are female. *See id.*

[16] The number of female crabs in ponds was estimated by multiplying annual containment pond totals since 2019, *see* IC Ex. C at 3, by the likely share of females, [redacted]%, *see supra* note 15.

[17] According to Dr. Brockmann, adult female crabs in South Carolina likely lay over 80,000 eggs in a spawning season, while ponds and [redacted] likely prevent crabs from spawning on beaches for the remainder of the season. Brockmann Decl. ¶¶ 14, 41–51. Even assuming that pond-held crabs nevertheless lay *half* their eggs on beaches, pond use would still remove nearly [redacted] eggs from beaches ([redacted] females in ponds x 40,000 eggs/female = [redacted] eggs).

number of horseshoe crab eggs available at a stopover site but on a super-abundance of such eggs, because horseshoe crab eggs buried beneath the sand are inaccessible to the birds. *Id.* ¶ 36.

As the graph below shows, the [redacted] . According to Dr. Niles, [redacted] red knots stopping over on South Carolina beaches must feed on a super-abundance of crab eggs to build weight. Niles Decl. ¶ 61. Depriving red knots of such vast quantities of a critical food source, in the super-abundance required to make this food source physically accessible, [redacted], "makes it highly likely that some red knots will not obtain the sustenance needed to survive their migration and breed in the Arctic." *Id.* ¶ 67. It is "highly likely that horseshoe crab containment ponds have caused the death of red knots and prevented others from reproducing by depriving the birds of a critical food source." *Id.* ¶ 69.[18]

[redacted]

Figure 5. [redacted]

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[18] These harms that will worsen if [redacted] . *Id.* ¶ 69.

III. The Harvest-for-Ponds Effectively Operates Outside the Law, with SCDNR's Tacit Approval.

The horseshoe crab fishery is an atypical one. Given the ecological importance of horseshoe crabs, the South Carolina legislature requires that crabs harvested for biomedical bleeding "must be handled so as to *minimize injury* to the crab" and "must be *returned unharmed* to state waters of comparable salinity and water quality as soon as possible after bleeding," S.C. Code § 50-5-1330(C) (emphasis added). SCDNR is tasked with enforcing the statute, *id.* § 50-5-1330(A)–(C), and issues separate permits each spring for harvesting crabs, ECF No. 1-4, and for storing them in ponds, ECF No. 1-1 at 22–23.

The use of containment ponds in South Carolina effectively operates outside the law. Charles River's agents treat crabs in a reckless manner from harvesting through pond storage and release of surviving crabs. Brockmann Decl. ¶¶ 23–61; ECF No. 67 at 12–16. Documents from SCDNR show that [redacted], including [redacted] [redacted], IC Ex. C at 5, with [redacted] [redacted]. *See* ECF No. 71 at 8–9 (see unredacted brief). SCDNR has long been aware of these problems. *See* IC Ex. C at 5 (SCDNR's own [redacted] data). Indeed, they have been publicly reported, including the unlawful handling of crabs and illegal harvesting on closed islands.[19] Yet there is no evidence that the agency has *ever* charged any horseshoe crab permittee or revoked any permit for any one of the systematic violations of statutory and permit conditions in this fishery. Instead, year after year, SCDNR has re-issued permits and sent letters to [redacted] finding that "[redacted]

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[19] *See, e.g.*, Chiara Eisner, *South Carolina restricted the horseshoe crab harvest. The lab moved north for more blood* (May 6, 2022), The State, https://www.thestate.com/news/state/south-carolina/article260986517.html; *see also* Ex. H at 8, 11–12, 14 (internal pagination cited here).

[redacted]" IC Ex. F at 1, 2, 3, 5, 7; *accord* ECF Nos. 85-1, 85-2 & 85-3 ([redacted] in public documents that "[n]o significant issues regarding handling crabs and holding facilities arose during this season.").

As noted, SCDNR issues permits that authorize the holding of crabs in ponds for Charles River. When Plaintiffs filed this case, SCDNR placed no restrictions on the sex of crabs held in ponds. ECF No. 1-1 at 23 (2021 pond permit). After Plaintiffs filed this case, SCDNR for the first time prohibited female crabs from being stored in ponds, requiring instead that females be taken directly from beaches to bleeding. ECF No. 42-1 at 4; *see also* Ex. E at 5.

SCDNR's own data indicates that [redacted]. IC Ex. C at 5. Although, as noted, SCDNR does not require pond permittees to record the sex of all crabs held in ponds, *id.* at 3, the available evidence suggests that [redacted]. *See* Brockmann Decl. ¶¶ 52–58. More broadly, after supposed permit improvements to protect crabs in ponds, *see* ECF No. 42-1 at 4, [redacted]. *See* IC Ex. C at 5.

In sum, pond storage ensures that [redacted] crabs are *not* "handled so as to minimize injury" or "returned unharmed" to state waters, S.C. Code § 50-5-1330(C), and horseshoe crab permit conditions are often ignored and ineffective. Instead of enforcing the law or revoking permits, *see* ECF No. 42-1 at 4 ("Failure to comply with any of the above conditions can result in revocation of this permit."), SCDNR has just indicated that it plans to re-issue new pond permits for the 2023 season later this week, Ex. I, apparently [redacted]. This creates an imminent likelihood of unauthorized incidental "take" of red knots, necessitating a preliminary injunction to prevent this take.

LEGAL FRAMEWORK

I. The Endangered Species Act.

"In response to growing concern over the extinction of many animal and plant species, Congress enacted the Endangered Species Act of 1973." *Gibbs v. Babbitt*, 214 F.3d 483, 487 (4th Cir. 2000) (citation omitted). Congress' response was "powerful and substantially unequivocal." *Loggerhead Turtle v. Cty. Council of Volusia Cty.*, 148 F.3d 1231, 1246 (11th Cir. 1998) (citation and quotations omitted). Indeed, the ESA is "the most comprehensive legislation for the preservation of endangered species ever enacted by any nation." *Tenn. Valley Auth. v. Hill*, 437 U.S. 153, 180 (1978) ("*TVA*").

"'The plain intent of Congress in enacting this statute . . . was to halt and reverse the trend toward species extinction, whatever the cost. This is reflected not only in the stated policies of the Act, but in literally every section of the statute.'" *Babbitt v. Sweet Home Chapter of Cmtys. for a Great Ore.*, 515 U.S. 687, 699 (1995) (quoting *TVA*, 437 U.S. at 184). "[E]xamination of the language, history, and structure of the legislation . . . indicates beyond doubt that Congress intended endangered species to be afforded the highest of priorities." *TVA*, 437 U.S. at 174.

Recognizing these species' "esthetic, ecological, educational, historical, recreational, and scientific value to the Nation and its people,"16 U.S.C. § 1531(a)(3), the ESA set out "to provide a means whereby the ecosystems upon which endangered species and threatened species depend may be conserved [and] to provide a program for the conservation of such endangered species and threatened species," *id.* § 1531(b). The purpose of the statute is to recover these species to the point at which they no longer require its protections. *See id.* § 1532(3) ("conservation" and "conserve" mean "to use and the use of all methods and procedures which are necessary to bring any endangered species or threatened species to the point at which the measures provided pursuant to [the ESA] are no longer necessary").

In pursuing these objectives, Section 9(a)(1)'s "take" prohibition is the "cornerstone" of the statute. *Gibbs*, 214 F.3d at 487. That section prohibits any person from committing the unauthorized "take" of any endangered species. 16 U.S.C. § 1538(a)(1)(B). It also prohibits any person to cause unauthorized take to be committed by another. *Id.* § 1538(g). This prohibition extends to state agencies issuing permits for otherwise-lawful activities that result in the unauthorized take of an endangered species. *Strahan v. Coxe*, 127 F.3d 155, 163–64 (1st Cir. 1997). The Secretary of the Interior may extend "take" prohibitions to threatened species, *id.* § 1533(d), as it has done for the threatened red knot, *see* Endangered and Threatened Wildlife and Plants; Threatened Species Status for the Rufa Red Knot, 79 Fed. Reg. 73,706, 73,728 (Dec. 11, 2014).

The legislative history "make[s] clear that Congress intended 'take' to apply broadly," *Babbitt*, 515 U.S. at 704—indeed, "in the broadest possible manner to include every conceivable way in which a person can 'take' or attempt to 'take' any fish or wildlife." S. Rep. No. 93-307, 1973 WL 12683, at *2,995 (1973).

Consonant with that intent, the ESA defines "take" as "to harass, harm, pursue, hunt, shoot, wound, kill, trap, capture, or collect, or to attempt to engage in any such conduct." 16 U.S.C. § 1532(19). Relevant here, take by "harass[ment]" means "an intentional or negligent act or omission which creates the likelihood of injury to wildlife by annoying it to such an extent as to significantly disrupt normal behavioral patterns which include, but are not limited to, breeding, feeding or sheltering." 50 C.F.R. § 17.3. And take by "harm" includes "significant habitat modification or degradation where it actually kills or injures wildlife by significantly impairing essential behavioral patterns, including breeding, feeding, or sheltering." *Id.*

"Congress understood § 9(a)(1)(B) to prohibit indirect as well as deliberate takings." *Babbitt*, 515 U.S. at 700. Thus, "activities not intended to harm an endangered species . . . may constitute unlawful takings under the ESA . . . ." *Id.* at 701.

The Service may authorize incidental take that results from an otherwise-lawful activity in one of two ways, either through an incidental take statement issued with a biological opinion following formal consultation on a federal agency action, 16 U.S.C. §1536(b)(4), or through an incidental take permit where no federal agency action is involved. *Id.* § 1539(a)(1)(B).

II. Standard of Review

a. Preliminary Injunction

A preliminary injunction "preserve[s] the relative positions of the parties until a trial on the merits can be held." *United States v. South Carolina*, 720 F.3d 518, 524 (4th Cir. 2013) (quoting *Univ. of Tex. v. Camenisch*, 451 U.S. 390, 395 (1981)). A plaintiff is entitled to that remedy upon showing "that he is likely to succeed on the merits, that he is likely to suffer irreparable harm in the absence of preliminary relief, that the balance of equities tips in his favor, and that an injunction is in the public interest." *Di Biase v. SPX Corp.*, 872 F.3d 224, 230 (4th Cir. 2017) (quoting *Winter v. NRDC*, 555 U.S. 7, 20 (2008)).

b. Endangered Species Act

As noted, the ESA "afforded the highest of priorities" to protecting listed species. *TVA*, 437 U.S. at 180. In ESA cases, the judgment of Congress guides the preliminary injunction analysis, where courts' highest priority is preventing harm to listed species while the case plays out. *See e.g.*, *Sierra Club v. Von Kolnitz*, No. 2:16-cv-03815, 2017 WL 3480777, at *8 (D.S.C.

Aug. 14, 2017) (Norton, J.) ("[G]iven the unique confines on preliminary injunctions in ESA actions," the "'equitable scales are always tipped in favor of the endangered or threatened species, and the balance of hardships and the public interest tips heavily in favor of protected species.'" (emphasis in original) (quoting *Red Wolf Coal. v. U.S. Fish & Wildlife Serv.*, 210 F. Supp. 3d 796, 806 (E.D.N.C. 2016)); *Am. Rivers v. U.S. Army Corps of Eng'rs*, 271 F. Supp. 2d 230, 249 (D.D.C. 2003) (on preliminary injunction, courts respect that "Congress spoke in the 'plainest of words' in enacting the ESA, 'making it abundantly clear that the balance has been struck in favor of affording endangered species the highest of priorities.'" (*quoting TVA*, 437 U.S. at 194)); *Cottonwood Env't L. Ctr. v. U.S. Forest Serv.*, 789 F.3d 1075, 1091 (9th Cir. 2015) ("[T]he equities and public interest factors always tip in favor of the protected species.").

ARGUMENT

Plaintiffs have made a clear showing on each of the four factors warranting preliminary injunctive relief.

I. Plaintiffs Are Likely to Succeed on the Merits.

a. Plaintiffs Have Standing.

As a threshold matter, Plaintiffs have standing to bring this case. An organization has standing to sue "either based on an injury to the organization in its own right or as the representative of its members who have been harmed." *Friends of the Earth, Inc. v. Gaston Copper Recycling Corp.*, 204 F.3d 149, 155 (4th Cir. 2000) (en banc). An organization has standing to sue on behalf of its members when "(1) at least one of its members would have standing to sue in his own right; (2) the organization seeks to protect interests germane to the organization's purpose; and (3) neither the claim asserted nor the relief sought requires the participation of individual members in the lawsuit." *Id.* (citation omitted). Under this prong, individual members have standing if "(1) [they have] suffered an injury in fact that is (a) concrete and particularized and (b) actual or imminent, not conjectural or hypothetical; (2) the injury is fairly traceable to the challenged action of the defendant; and (3) it is likely, as opposed to merely speculative, that the injury will be redressed by a favorable decision." *Sierra Club v. U.S. Dep't of the Interior*, 899 F.3d 260, 283 (4th Cir. 2018) (citation and quotations omitted).

"In the environmental litigation context, the standing requirements are not onerous." *Am. Canoe Ass'n v. Murphy Farms, Inc.*, 326 F.3d 505, 517 (4th Cir. 2003). "This is so because [t]he extinction of a species, the destruction of a wilderness habitat, or the fouling of air and water are harms that are frequently difficult or impossible to remedy by monetary compensation." *Beck v. McDonald*, 848 F.3d 262, 274 n.5 (4th Cir. 2017) (citation and quotations omitted). "Threats or increased risk … constitute[] cognizable harm." *Friends of the Earth*, 204 F.3d at 160; *accord Beck v. McDonald*, No. 3:13-cv-999-TLW, 2015 WL 13777969, at *9 (D.S.C. Mar. 31, 2015), *aff'd*, 848 F.3d 262 (4th Cir. 2017). "[T]he desire to use or observe an animal species, even for purely aesthetic purposes, is undeniably a cognizable interest for purposes of standing." *Lujan v. Defs. of Wildlife*, 504 U.S. 555, 562 (1992) "[E]nvironmental plaintiffs adequately allege injury in fact when they aver that they use the affected area and are persons for whom the aesthetic and recreational values of the area will be lessened by the challenged activity." *Friends of the Earth, Inc. v. Laidlaw Envtl. Servs. (TOC), Inc.*, 528 U.S. 167, 183 (2000) (citation and quotations omitted).

Plaintiffs' members visit beaches around South Carolina each spring to enjoy, observe, photograph, and, in the case of Mr. Crolley, conduct an ecotourism business involving migrating red knots and spawning horseshoe crabs. *See* Ex. J, Declaration of Chris Crolley ("Crolley

Decl.") ¶¶ 5–13, 18; Ex. K, Declaration of Daniel Prohaska ("Prohaska Decl.") ¶¶ 13–14; Ex. L, Declaration of Dana Beach ("Beach Decl.") ¶¶ 3–8, 11–12. Their interests are harmed by Charles River's removal and containment of around [redacted] crabs each spawning season, which depletes red knots' critical food source, horseshoe crab eggs. *See* Prohaska Decl. ¶¶ 17, 19; Crolley Decl. ¶¶ 14–21; Beach Decl. ¶¶ 9–16. These members' interests are also harmed by the [redacted] [redacted] versus on South Carolina beaches, where they would be able to observe red knots during planned future birding trips to look for birds feeding on horseshoe crab eggs. *See* Prohaska Decl.¶¶ 13–17; Beach Decl. ¶¶ 14–16; Crolley Decl. ¶¶ 16–18; Ex. M, Declaration of Christian Hunt ("Hunt Decl.") ¶ 16.

These members fear the harvest for containment ponds has contributed to the marked declines in red knot and horseshoe crab abundance that they have observed over the years, which lessens their enjoyment of their trips to South Carolina beaches and "lost clients" for an ecotourism business. Crolley Decl. ¶¶ 18–22; Prohaska Decl. ¶¶ 17–18; Beach Decl. ¶¶ 13–16. Because "reasonable concerns about the effects of [the challenged conduct] directly affected [members'] recreational, aesthetic, and economic interests," Plaintiffs' members have standing. *Laidlaw*, 528 U.S. at 183–84. These injuries are caused by Charles River's use and SCDNR's authorization of containment ponds and would be redressed if the Court enjoins such ponds. *See, e.g.*, Crolley Decl. ¶ 23; Beach Decl. ¶ 16.[20]

b. <u>Plaintiffs Are Likely to Succeed on Their Section 9 Claims.</u>

Plaintiffs are likely to succeed on their claims that SCDNR and Charles River are in violation of Section 9's prohibition against unauthorized incidental take of red knots. The harvest

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[20] Plaintiffs also have organizational standing to pursue their claims. *See generally* Hunt Decl. ¶¶ 8–11.

of spawning horseshoe crabs for storage in containment ponds while red knots are present on the beach causes unauthorized incidental take by harassing red knots and disrupting feeding and sheltering behaviors. The removal of spawning horseshoe crabs and their storage in containment ponds deprives red knots of a critical food source on stopover beaches, causing unauthorized incidental take via harm that actually kills or injures red knots by impairing their feeding and breeding ability. Because neither SCDNR nor Charles River has obtained authorization from the Service for this incidental take, both are in violation of Section 9.

Section 9(a)(1) prohibits causing or committing the unauthorized "take" of the red knot. 16 U.S.C. § 1538(a)(1)(B), (g). As noted above, the ESA defines "take" as "to harass, harm, pursue, hunt, shoot, wound, kill, trap, capture, or collect, or to attempt to engage in any such conduct." 16 U.S.C. § 1532(19). Relevant here, take by "harass[ment]" means "an intentional or negligent act or omission which creates the likelihood of injury to wildlife by annoying it to such an extent as to significantly disrupt normal behavioral patterns which include, but are not limited to, breeding, feeding or sheltering." 50 C.F.R. § 17.3. And take by "harm" includes "significant habitat modification or degradation where it actually kills or injures wildlife by significantly impairing essential behavioral patterns, including breeding, feeding, or sheltering." *Id.*

As explained above, containment ponds likely remove [redacted] crab eggs from red knot feeding beaches each spring—roughly the amount of food needed by [redacted] red knots. *See supra* at 14. Again, it is not just the sheer number of eggs that are removed, but also that removal of spawning crabs depletes the super-abundance of eggs necessary for red knots to successfully feed. Brockmann Decl. ¶ 51. According to Dr. Niles, depriving red knots of such vast quantities of a critical food source makes it "highly likely" that red knots stopping over in

South Carolina will be too frail to survive their migration to the Arctic and breed, causing death and lower rates of reproduction. Niles Decl. ¶ 67.

SCDNR's new permit conditions have not addressed the problems with containment ponds. Last year, when [redacted], they were still [redacted]. *See* IC Ex. C at 5; Brockmann Decl. ¶¶ 52–54. There is even documentation of spawning in containment ponds. *See* Brockmann Decl. ¶¶ 41–44. Documents from the pond operators [redacted] demonstrate that there has historically been [redacted]. *See* IC Ex. J. Moreover, horseshoe crab containment ponds, [redacted], likely prevent crabs from spawning for the remainder of the season even after release, Brockmann Decl. ¶¶ 48–51, further depriving red knots of critical crab eggs in the super-abundance needed to make them accessible. Niles Decl. ¶¶ 63–65.

Containment ponds thus cause incidental take of red knots by directly removing spawning horseshoe crabs from stopover beaches—where red knots need to feed on a super-abundance of accessible eggs to renourish and restore their bodies, survive their migration to the Arctic Circle and breed once they arrive—to containment ponds, where this critical food source is inaccessible to the birds. Neither SCDNR nor Charles River has sought or obtained federal authorization for this incidental take.

ESA regulations defining take by "harm" and "harass[ment]" specifically include impacts to an animal's feeding, breeding, and sheltering patterns. 50 C.F.R. § 17.3. By depriving red knots of the super-abundance of horseshoe crab eggs on stopover beaches they need, the use of containment ponds to store spawning horseshoe crabs is causing death and reduced reproductive ability. Niles Decl. ¶¶ 66–69. At the motion to dismiss stage, this Court held that Plaintiffs' allegations regarding the deprivation of critical horseshoe crab eggs as a result of containment ponds plausibly asserted a "take" of red knots. *Defs. of Wildlife v. Boyles*, 608 F. Supp. 3d 336, 346 (D.S.C. 2022) ("These facts allow the Court to draw the reasonable inference that Defendants' use and authorization of containment ponds significantly disrupt the normal feeding patterns of red knots."). Plaintiffs have now submitted evidence establishing that the harvest for containment ponds is [redacted] as Plaintiffs' Complaint alleged, and thus birds are being [redacted] as alleged in the Complaint.

On the stopover beaches, moreover, harvest of crabs for pond storage while red knots are present likely "annoy[s] [red knots] to such an extent as to significantly disrupt normal behavioral patterns," including feeding and sheltering. 50 C.F.R. § 17.3 (definition of "harass"); *see* Niles Decl. ¶ 58 (harvest for ponds while red knots are present may disturb red knots and cause them to flush); *accord* Draft CD for Cape Romain at 3, 16. The removal of spawning horseshoe crabs to containment ponds "actually kills or injures" red knots through "significant habitat modification or degradation [that] significantly impair[s] essential behavioral patterns, including breeding and feeding," 50 C.F.R. § 17.3 (definition of "harm"). Charles River is thus liable for the unauthorized incidental take of red knots resulting from the harvest of spawning horseshoe crabs for ponds that both harasses and harms red knots.

When an agency "allows or authorizes acts that exact a taking and that, but for the permitting process, could not take place," the agency too is liable under Section 9. *Strahan*, 127 F. 3d at 163–64 (Massachusetts liable for take of endangered right whales by authorizing state fishery that risked entangling them). It is undisputed that SCDNR authorizes containment ponds, which could not lawfully be used without an SCDNR permit. S.C. Code Ann. § 50-5-1330(A) ("Taking or possessing horseshoe crabs (Limulus polyphemus) is unlawful except under permit granted by the department.”). By authorizing activities that cause the incidental take of red knots, SCDNR is causing others to commit unauthorized incidental take.[21] *See, e.g., Loggerhead Turtle*, 148 F.3d at 1251–53 (county violated section 9 by authorizing beachfront lighting in turtle nesting areas that resulted in take of endangered turtles); *Red Wolf Coal. v. N.C. Wildlife Res. Comm’n*, No. 2:13–CV–60–BO, 2014 WL 1922234, at *7–9 (E.D.N.C. May 13, 2014) (state commission likely in violation of section 9 by authorizing hunting practices for coyotes that risked mistaken shootings of endangered red wolves); *Animal Welfare Inst. v. Martin*, 588 F. Supp. 2d 70, 98–100 (D. Me. 2008) (state agency likely violated Section 9 by approving traps that risked incidentally trapping threatened lynx); *United States v. Town of Plymouth*, 6 F. Supp. 2d 81, 90–92 (D. Mass. 1998) (town likely violated Section 9 by authorizing off-road vehicles on beach that risked harming piping plover’s nesting and feeding habitat).

In Section 9, Congress defined the “take” prohibition “‘in the broadest possible manner to include every conceivable way in which a person can ‘take’ or attempt to ‘take’ any fish or wildlife,’” including “indirect as well as deliberate takings.” *Babbitt*, 515 U.S. at 700, 704–05 (quoting S. Rep. No. 93–307, p. 7 (1973)). Under these standards, Plaintiffs are likely to succeed

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[21] SCDNR is well aware that [redacted]. *See* IC Ex. J ([redacted]. SCDNR is also [redacted] *See* IC Ex. C at 3–5. SCDNR did nothing to [redacted]. *See* IC Ex. J. Thus, SCDNR may not rely on its permit conditions as a defense when it has [redacted] for years. *See supra* at 3, 16–17; *see also, e.g., Animal Prot. Inst. v. Holsten*, 541 F. Supp. 2d 1073, 1076–80 (D. Minn. 2008) (concluding that state officers may be liable for lynx takings that are incidental to the trapping activities because “[i]n order to legally engage in trapping in Minnesota . . . one must obtain a license and follow all governmental regulations governing trapping activities”).

on their claims that Charles River's use and SCDNR's authorization of containment ponds cause the unauthorized incidental take of red knots via harm and harassment.

II. <u>Plaintiffs Are Likely to Suffer Irreparable Harm If a Preliminary Injunction is Not Granted.</u>

"Environmental injury, by its nature, can seldom be adequately remedied by money damages and is often permanent or at least of long duration, i.e., irreparable." *Amoco Prod. Co. v. Vill. of Gambell*, 480 U.S. 531, 545 (1987); *see also Nat'l Audubon Soc'y v. Dep't of Navy*, 422 F.3d 174, 201 (4th Cir. 2005). This case is no exception. In the absence of an injunction, the continued use and authorization of containment ponds will result in the unauthorized incidental take of listed red knots, thus causing irreparable harm to the birds and Plaintiffs' members.

"Irreparable harm should be determined by reference to the purposes of the statute being enforced." *Nat'l Wildlife Fed'n v. Nat'l Marine Fisheries Serv.*, 886 F.3d 803, 818 (9th Cir. 2018); *see also Amoco*, 480 U.S. at 544 (irreparable harm inquiry requires consideration of the "purpose" and "underlying substantive policy" of the act). For the ESA, that purpose is the protection of endangered and threatened species. *See TVA*, 437 U.S. at 184 (noting Congress' desire in ESA to "halt and reverse the trend toward species extinction, whatever the cost"). The threshold for establishing irreparable harm in ESA cases is low. *See Cottonwood,* 789 F.3d at 1091 ("In light of the stated purposes of the ESA in conserving endangered and threatened species and the ecosystems that support them, establishing irreparable injury should not be an onerous task for plaintiffs."), *cert. denied*, 137 S. Ct. 293 (2016); *see also Sierra Club,* 2017 WL 3480777, at *6 ("Generally, courts have found that there is a strong showing of irreparable harm in cases involving the ESA.").

Charles River's use, and SCDNR's authorization, of containment ponds violate the very purposes of the ESA to conserve listed species like the red knot, i.e., to ensure that the species recovers to the point that the ESA's protections are no longer needed, including by ensuring that any incidental take is lawfully authorized. Thus, the irreparable injury prong of the injunction analysis is easily satisfied.

a. Irreparable Harm to Red Knots.

An injunction is necessary to prevent irreparable injury to threatened red knots and Plaintiffs' members' interests in seeing and enjoying red knots. Absent an order preliminarily enjoining SCDNR's authorization and Charles River's use of containment ponds, red knots will again be deprived of the super-abundance of horseshoe crab eggs that they rely upon during the pendency of this litigation. Without this vital food supply, the red knots' survival rate along their migration will be significantly diminished and the individual birds will be less likely to survive and reproduce.

As this District has recognized, in the ESA context, interference with survival and reproductive capability is an irreparable harm. In 2017, Judge Norton held that a temporary sea wall caused irreparable harm because it blocked female sea turtles from reaching some nesting sites, reducing the chance of successful reproduction. *Sierra Club*, 2017 WL 3480777, at *7. Similarly, in *Red Wolf Coalition v. U.S. Fish & Wildlife Service*, another court in this Circuit found that an increased likelihood of mortality in a small, endangered population constitutes irreparable harm. 210 F. Supp. 3d at 805–06.

The harms to red knots imposed by the use and authorization of containment ponds to store spawning horseshoe crabs are similarly irreparable. Red knots are already in decline, and containment ponds remove the super-abundance of horseshoe crab eggs that red knots rely on from stopover beaches. Niles Decl. ¶ 59. Moreover, Charles River's harvest for containment ponds harasses red knots when horseshoe crabs are removed from stopover beaches at the precise moment that crabs are laying the eggs upon which red knots depend. Niles Decl. ¶ 58 The super-abundance of horseshoe crab eggs—eggs on which red knots have evolved to rely for their survival—will again be displaced from South Carolina's beaches, and therefore removed from vulnerable red knots. *Id.* ¶¶ 66–69; Brockmann Decl. ¶ 50–51. Without access to these vital eggs, red knots will be less likely to survive their migration and successfully breed at their destination. Niles Decl. ¶ 47. This is unquestionably irreparable harm to a listed species under the ESA.

b. Harm to Plaintiffs' Members' Interests.

A plaintiff's aesthetic and recreational interests are harmed by actions that impair his or her enjoyment of the environment. *Sierra Club v. U.S. Army Corps of Eng'rs*, 645 F.3d 978, 995–96 (8th Cir. 2011) (finding that construction of a power plant harmed plaintiffs by "interfer[ing] with [their] interests in studying and enjoying the environment"). Thus, a plaintiff is harmed by actions that impair his or her ability to enjoy wildlife in its natural environment. *See, e.g.*, *Humane Soc'y of the U.S. v. Hodel*, 840 F.2d 45, 52 (D.C. Cir. 1988) (finding harm where action would "deplet[e] the supply of animals and birds that refuge visitors seek to view"); *Fund for Animals, Inc. v. Lujan*, 962 F.2d 1391, 1396 (9th Cir. 1992) (finding that "the diminished opportunity of the Fund's members to view the northern bison herd in Yellowstone establishes standing to challenge the 1990 bison management plan").

The harms to red knots identified above will impair Plaintiffs' members' abilities to watch, enjoy, and, in the case of Defenders' member Chris Crolley, conduct a tourism business based on the species. For example, Mr. Crolley, the owner of Coastal Expeditions, used to run a charter trip to take birding photographers (including professionals) to see red knots gather during horseshoe crab spawning. *See* Crolley Decl. ¶ 16. In 2017, 2018, and 2019, Mr. Crolley ran the trip, but there were so few birds he has not offered this outing since then. *Id*. Defenders' members Crolley and Daniel Prohaska, and League member Dana Beach, all make special birding trips to look for red knots, including specific planned future trips to South Carolina stopover beaches. *See* Crolley Decl. ¶ 18, Prohaska Decl. ¶¶ 15–16, Beach Decl. ¶ 6. If red knots are no longer present and foraging on South Carolina beaches, or are present in even sparser numbers, these members will lose a special part of what it means to them to live in South Carolina and visit South Carolina beaches. *See* Prohaska Decl. ¶ 18; Crolley Decl. ¶ 19; Beach Decl. ¶ 8. Dana Beach photographs red knots and advocates for their protection. If containment ponds continue to result in unauthorized incidental take of red knots, it will interfere with Mr. Beach's ability to observe, enjoy and learn about these birds. Beach Decl. ¶¶ 7, 16.

These injuries to Plaintiffs' members' business, aesthetic, and research interests are irreparable. *See Red Wolf Coal.*, 2014 WL 1922234, at *9; *Red Wolf Coal.*, 210 F. Supp. 3d at 805; *Nat'l Wildlife Fed'n*, 886 F.3d at 822. Thus, if the Court does not enjoin this spring's use of horseshoe crab containment ponds, Plaintiffs' members will suffer irreparable harm.

III. **The Balance of Harms Tips in Plaintiffs' Favor.**

Once Plaintiffs have shown irreparable harm to an endangered or threatened species, the Court's inquiry is at an end: "[t]he equitable scales are always tipped in favor of the . . . species." *Red Wolf Coal.*, 210 F. Supp. 3d at 806 (quoting another source); *see TVA*, 437 U.S. at 194 (Congress "[made] it abundantly clear that the balance has been struck in favor of affording endangered species the highest of priorities"); *see also S.C. Dep't of Wildlife & Marine Res. v. Marsh*, 866 F.2d 97, 100 (4th Cir. 1989) (stating that if there is irreparable harm to the environment, the balance of harms usually favors an injunction).

Even in the absence of this standard, the balance of equities would still favor Plaintiffs. Allowing Charles River and SCDNR to continue the unauthorized incidental take of red knots while this suit is decided would undermine the protective purposes that animate the ESA. *See, e.g.*, *TVA*, 437 U.S. at 194. Any alleged harm Charles River and SCDNR might assert from the temporary deprivation of the "economic benefits" of containment ponds is "outweighed by . . . permanent harm to the environment." *Ohio Valley Envtl. Coal. v. U.S. Army Corps of Eng'rs*, 528 F. Supp. 2d 625, 632 (S.D.W. Va. 2007). And as shown above, Plaintiffs' harms are irreparable. *See supra* at 28–31.

On the other hand, SCDNR will not be harmed at all by an order enjoining it from permitting horseshoe crab storage ponds to be used before this litigation concludes. Any harm to Charles River from an injunction temporarily suspending the use of containment ponds in South Carolina during the pendency of this litigation does not outweigh the irreparable harm to red knots and Plaintiffs' members. Importantly, Plaintiffs do not seek to enjoin the harvest of horseshoe crabs from South Carolina beaches—only the use of containment ponds to store those crabs before they are taken for bleeding. Thus, Charles River may continue its harvest so long as it does not place horseshoe crabs in containment ponds—which are not even used in other states that have a robust and successful biomedical harvest.

Moreover, Charles River has already [redacted]. As noted *supra* at 11, [redacted], which it stated could "[redacted]." IC Ex. K. The company is "[redacted]," *see* IC Ex. L, and has received a permit to harvest, for the first time, up to 180,000 horseshoe crabs yearly from Virginia. Ex. N at 6. Given these facts, any harm to Charles River from a limited injunction is easily outweighed by the dire harm red knots and Plaintiffs' members will suffer if the use of storage ponds continues while this litigation proceeds. *See TVA*, 437 U.S. at 188 (in ESA cases, "[q]uite obviously, it would be difficult for a court to balance the loss of a sum certain–even $100 million–against a congressionally declared 'incalculable' value, even assuming we had the power to engage in such a weighing process, which we emphatically do not.").

IV. Preliminary Injunctive Relief Is in the Public Interest.

As noted, "the balancing and public interest prongs have been answered by Congress' determination that the balance of hardships and the public interest tips heavily in favor of protected species." *Strahan*, 127 F.3d at 160; *see also Leatherback Sea Turtle v. Flagler Cty. Bd. of Cty. Comm'rs*, 359 F. Supp. 2d 1209, 1212 (M.D. Fla.2004) (finding that, "given the monumental and important aims of the ESA, Congress effectively removed from the courts their traditional equitable discretion in injunction proceedings"); *see also S.C. Dep't of Wildlife & Marine Res.*, 866 F.2d at 100 (stating that if there is irreparable harm to the environment, the balance of harms usually favors an injunction). The protection of ESA-listed species is in the public interest because of their "'esthetic, ecological, educational, historical, recreational, and scientific value to the Nation and its people.'" *Gibbs*, 214 F.3d at 487 (quoting 16 U.S.C. § 1531(a)(3) (1994)).

Even if the balance had not been struck by Congress, the result would be the same. Because the red knot is a species "highly vulnerable to extinction," Niles Decl. ¶ 10, its protection through compliance with statutory requirements, including the requirement to comply with the statutory prohibitions against unauthorized incidental take, is of vital importance to the public's interest in ensuring the species' survival and ultimate recovery.

Temporarily enjoining SCDNR from authorizing, and Charles River from using, containment ponds for the pendency of this litigation is in the public interest. Charles River may continue to harvest horseshoe crabs so long as it does not place them into containment ponds, and thus, may continue to supply LAL to its customers. *See supra* at 32. Charles River has also taken steps [redacted], and to harvest and bleed even more crabs in other states. *Id*. Moreover, even during the height of the pandemic in 2021, Charles River repeatedly assured the public that it "can test the anticipated 5 billion doses of the most coveted COVID-19 vaccine" from "just a single day's production of LAL." Ex. O at 1. Thus, even a global pandemic "'place[d] no undue burden on the [lysate] supply chain.'" Ex. P at 3 (quoting Charles River official). In a letter co-authored in 2021, the company explained that "only a very small amount of LAL is needed to perform [endotoxin] tests;" that "[i]t takes roughly the same amount of LAL to test 1,000 doses as it does to test 100,000 doses;" and that "the demands of [] testing materials worldwide can and are being absorbed with available inventory and without a significant negative impact on the pharmaceutical industry or supply chain." Ex. Q at 4–5. Indeed, [redacted]. IC Ex. C at 3–4. A mere injunction on containment ponds, not even against the harvest, thus will have no impact on the public interest. By contrast, an injunction on containment ponds will have critically important public interest benefits for the ability of listed red knots to obtain the super-abundance of horseshoe crab eggs that they need to survive and reproduce.

Finally, the public interest cannot support allowing a company to continue practices that routinely violate state laws. As explained *supra*, Charles River's practices in harvesting, transporting, holding, and releasing crabs all violate South Carolina law and the Atlantic States Marine Fisheries Commission, a multi-state fisheries management cooperative, best management practices for handling horseshoe crabs. *See* Brockmann Decl. ¶¶ 21–51. Charles River also [redacted]

[redacted]. *Compare*, IC Ex. C at 5, *with* IC Ex. M ([redacted]"); *see also Defs. of Wildlife v. U.S. Fish & Wildlife Serv.*, 539 F. Supp. 3d 543, 559–60 (D.S.C. 2021) ("*Defenders*") (considering known illegal crab harvests by Charles River's agent), *appeal dismissed sub nom. Defs. of Wildlife v. Charles River Lab'ys Int'l, Inc.*, 2021 WL 6330714 (4th Cir. Aug. 24, 2021).[22]

V. No Bond Should Be Required.

Federal Rule of Civil Procedure 65(c) requires that Plaintiffs post security. But "where plaintiffs are public interest groups who might otherwise be barred from obtaining meaningful judicial review," a nominal bond suffices. *Red Wolf Coal.*, 210 F. Supp. 3d at 806–07; *see also Defenders*, 539 F. Supp. 3d at 560–61. Here no bond, or at most, a nominal bond, should be required consistent with these cases.

CONCLUSION

For the above reasons, Plaintiffs respectfully request that this Court grant this Motion for Preliminary Injunction of SCDNR's authorization of, and Charles River's use of, horseshoe crab containment ponds pursuant to the 2023 Possession Permit (or any other authorization).

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[22] In *Defenders*, Plaintiffs obtained a preliminary injunction of Charles River's unlawful harvest of horseshoe crabs from the Cape Romain National Wildlife Refuge without the special use permit required by federal law, and in violation of other provisions of federal law. While the Fourth Circuit ultimately stayed this ruling late in the horseshoe crab harvest season, the appellate court did not provide a rationale. In the end, Cape Romain remained closed, and that litigation was dismissed as moot when the Service made an announcement that the Refuge would remain closed to horseshoe crab harvesting absent a special use permit. All applications for such permits to this point have been denied, and the Service just issued a proposed finding that crab harvesting is incompatible with the Refuge's purposes to, *inter alia*, protect threatened red knots. *E.g.*, Draft CD for Cape Romain at 13–14 (citing, e.g., "reduction in food availability for the birds in the short and long term, [and] increased bird flushing" due to horseshoe crab harvesting).

Respectfully submitted this 15th day of March, 2023.

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EXHIBIT A

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH CAROLINA COASTAL CONSERVATION LEAGUE, Plaintiffs, v. ROBERT H. BOYLES, JR., in his official capacity as Director of the South Carolina Department of Natural Resources, and CHARLES RIVER LABORATORIES, INTERNATIONAL, INC., Defendants.	)	Civ. No. 2:22-CV-112-RMG

DECLARATION OF DR. LAWRENCE NILES

I, Lawrence Niles, declare the following to be true and correct, and to reflect my professional opinion and my best judgment on the matter at hand based on the body of scientific evidence.

PROFESSIONAL BACKGROUND AND QUALIFICATIONS

1. I am a wildlife biologist and my work has centered on endangered, threatened, and imperiled species since 1976.

2. I received my Ph.D. in Ecology and Evolution from Rutgers University in 1996, my Master of Science degree in Wildlife Management from Pennsylvania State University in 1976, and my Bachelor of Science degree in Zoology from Pennsylvania State University in 1973. Attached is my curriculum vitae. *See* Ex. A.

3. I am a partner at Wildlife Restoration Partnerships LLC, which I founded in 2015. From 2007 to 2015, I worked at L J Niles Associates, where I engaged in conservation and research projects on behalf of government agencies and non-profit organizations. Prior to 2007, I worked in the Endangered and Nongame Species Program at the New Jersey Department of

Environmental Protection for more than 20 years. I served as the program's Chief for more than a decade during that time.

4. At Wildlife Restoration Partnerships, my focus is primarily dedicated to researching and tracking Atlantic shorebirds, particularly the *rufa* red knot (*Calidris canutus rufa*), and conserving shorebirds by facilitating efforts to restore habitat conditions for feeding, sheltering, and breeding. Red knots consume the eggs of horseshoe crabs (*Limulus polyphemus*) from the beach, where the crabs spawn each spring. Horseshoe crab eggs are critical for the birds to survive their annual migration and reproduce.

5. I have authored and co-authored more than 70 studies, papers, and other works on these subjects, including 45 papers related to red knot migration, populations, habitat, and/or feeding behaviors, and 15 papers related to red knots' consumption of horseshoe crab eggs, horseshoe crab spawning, and horseshoe crab egg density. Red knots have been the primary focus of my research since 1997. In listing red knots as "threatened" under the Endangered Species Act, the United States Fish and Wildlife Service ("Service") relied on several of my studies.[1]

6. I am a former member of the Horseshoe Crab Adaptive Resource Management subcommittee of the Atlantic States Marine Fisheries Commission ("ASMFC"), a regulatory body composed of stakeholders and government officials from 16 states along the Atlantic Coast that was established to manage and coordinate fishery resources. On the ASMFC, I have also served on committees that assist the Commission in using data to develop horseshoe crab harvesting quotas and regulations to be adopted by member states.

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[1] *See, e.g.*, Endangered and Threatened Wildlife and Plants: Threatened Species Status for the Rufa Red Knot, 79 Fed. Reg. 73,706, 73,708, 73,725, 73,733, 73,742 (Dec. 11, 2014) ("Red Knot Listing").

7. I am a member of the U.S. Shorebird Council of the U.S. Fish and Wildlife Service and the Western Hemisphere Shorebird Reserve Network, both groups dedicated to the national and international conservation of shorebirds.

SUMMARY OF OPINIONS

8. I have reviewed documents produced in discovery during this litigation that have been provided by Plaintiffs' counsel. Based on these documents, the literature on red knots and horseshoe crabs, and my professional expertise and experience, it is my opinion that:

a. South Carolina beaches are major stopover sites for red knots along their nearly transpolar migration from Patagonia, and from northern South America and the Caribbean, to Arctic breeding grounds each spring;

b. Red knots that stop over in South Carolina rely on beach-laid horseshoe crab eggs as a critical food source to refuel and power an often-direct flight to the Arctic from South Carolina;

c. To survive their migration and breed successfully in the Arctic, red knots that stop over on South Carolina beaches require a super-abundance of horseshoe crab eggs on these beaches created by repeated spawning events of horseshoe crabs, as each red knot must quickly consume several hundred thousand horseshoe crab eggs during its stopover to replenish enough weight to survive its journey and then breed;

d. Since 2019 in South Carolina, around [redacted] horseshoe crabs have been harvested from beaches each spring as they come ashore to spawn, with roughly [redacted] of harvested crabs stored in containment ponds; crabs are stored in ponds during spawning season, laying eggs in ponds beyond the reach of red knots, rather than on beaches;

e. The use of containment ponds to store horseshoe crabs during spawning season results in the diminution of available eggs on beaches that are critical to red knots' ability to survive their migration to the Arctic, mate, and reproduce;

f. By depriving red knots of a critical food source, horseshoe crab containment ponds have likely caused the deaths of red knots along their migration and prevented others from reproducing. If they continue operating, containment ponds will continue to cause the death and impaired breeding of red knots.

BASIS FOR OPINIONS

I. The *Rufa* Red Knot

Figure 1. The beach-foraging *rufa* red knot.[2]

9. The *rufa* red knot is a medium-sized migratory shorebird (about 9 to 11 inches in length) whose numbers are in rapid decline.

10. Between the 1980s and 2000s, red knot populations declined from an estimated 100,000–150,000 to below 45,000. In their main wintering area in Tierra del Fuego, Chile, red knot numbers decreased from 56,000 in 1986 to 12,000 in 2011, and have not improved since. This has led researchers, including myself, to conclude that the species is "highly vulnerable to extinction." F.M. Smith et al., *Investigating Red Knot Migration Ecology Along the Georgia and South Carolina Coasts: Spring 2019 Season Summaries* at 3, CTR. FOR CONSERVATION BIOLOGY TECHNICAL REPORT SERIES (2019).[3]

11. As a result of these population declines, in 2015, the Service listed the *rufa* red knot as "threatened" under the Endangered Species Act. Red Knot Listing, 79 Fed. Reg. at 73,706.

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[2] Taken from On Nature Magazine, *Red Knot*, https://onnaturemagazine.com/red-knot.html (last visited Mar. 12, 2023), https://perma.cc/4NFX-59GY (permanent link).

[3] https://scholarworks.wm.edu/cgi/viewcontent.cgi?article=1587&context=ccb_reports, https://perma.cc/32ML-LLGU (permanent link).

A. Annual Spring Migration

12. Each spring, red knots embark on a nearly transpolar migration. From their main wintering area in the southernmost region of South America, red knots fly over 9,000 miles to reach their breeding grounds in the Canadian Arctic, a round-trip nearing 19,000 miles.

13. To prepare their bodies for migration, red knots undergo significant physiological changes:

> Before takeoff, the birds accumulate and store large amounts of fat to fuel migration and undergo substantial changes in metabolic rates. In addition, leg muscles, gizzard (a muscular organ used for grinding food), stomach, intestines, and liver all decrease in size, while pectoral (chest) muscles and heart increase in size.

Endangered and Threatened Wildlife and Plants; Proposed Threatened Status for the Rufa Red Knot (*Calidris canutus rufa*), 78 Fed. Reg. 60,024, 60,027 (Sept. 30, 2013).

B. Migratory Stopovers

14. Many red knots rely on only one or two stopovers between South America and their Arctic breeding grounds to refuel along their journey. After flying thousands of miles, red knots are often emaciated by the time they reach a stopover beach.

15. During a stopover, red knots must consume substantial amounts of food to: (1) recover fat and protein reserves used up on the prior leg of the migration; (2) fuel the remainder of the journey; and (3) build up energy to facilitate reproduction in frigid breeding habitat.

16. Red knots generally arrive at a stopover beach having lost significant weight and must increase their weight by roughly 50% to ensure that they can survive their migration and breed in the Arctic.[4]

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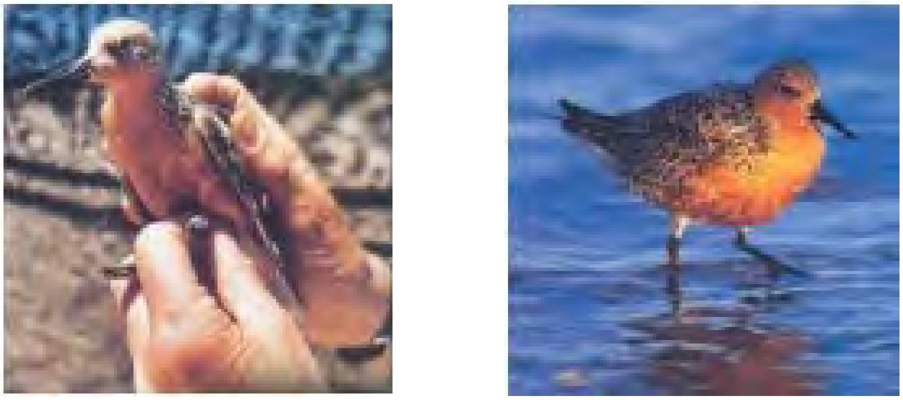
[4] *See, e.g.*, Allen J. Baker et al., *Rapid population decline in red knots: fitness consequences of decreased refuelling rates and late arrival in Delaware Bay*, 271 Proc R. Soc. Lond. B 875

17. If red knots do not consume enough food to gain the requisite weight during their stopover, they are significantly less likely to survive their migration and to reproduce in the Arctic.



Figures 2 and 3. Left: an emaciated red knot reaches a stopover beach in Delaware Bay. Right: a red knot prepares to depart a stopover having replenished its body mass.[5]

18. Red knots must reach the Arctic and breed before cold weather makes the region inhospitable. Because "stopovers are time-constrained," and due to the physiological changes to the birds' digestive systems before migration, red knots "require[] stopovers rich in easily digested food to achieve adequate weight gain that fuels the next migratory flight and, upon arrival in the Arctic, fuels a body transformation to breeding condition." 78 Fed. Reg. at 60,027 (citations omitted).

(2004), https://perma.cc/4T5E-R6C4 (permanent link); Sjoerd Duijns et al., *Body condition explains migratory performance of a long-distance migrant*, 284 Proc. R. Soc. B. 2 (2017), https://perma.cc/786X-MJST (permanent link); G. Michael Haramis et al., *Stable isotope and pen feeding trial studies confirm the value of horseshoe crab* Limulus polyphemus *eggs to spring migrant shorebirds in Delaware Bay*, 38 J. Avian Biol. 367 (2007), https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.0908-8857.2007.03898.x.

[5] Taken from N.J. Dep't of Env'tl Protection, Division of Fish & Wildlife, *Red Knot – An Imperiled Migratory Shorebird in New Jersey*, https://www.nj.gov/dep/fgw/ensp/redknot.htm (last visited Mar. 12, 2023), https://perma.cc/ZYV8-UEHP (permanent link).

19. The survival and propagation of the remaining population of red knots thus requires an abundance of fatty, nutrient-rich, easily-digested prey on stopover beaches at the precise time the birds arrive each spring.

C. South Carolina Stopovers are Critical for Both Individual Red Knots and the Species

20. Researchers from the Service, SCDNR, and other agencies have determined that the "Georgia and South Carolina Coasts are a major stopover area for *rufa* Red Knots in spring migration." Smith et al., *supra* ¶ 10, at 18; *see also* Mary M. Pelton et al., *Kiawah and Seabrook islands are a critical site for the* rufa *Red Knot (*Calidris canutus rufa), bioRxiv (Mar. 26, 2022).[6] Red knots stop over "by the thousands along South Carolina beaches," at times "form[ing] the largest flock [of the species] on the Atlantic Coast." SCDNR, *Shorebird research underscores the importance of South Carolina beaches* (June 7, 2018).[7]

21. South Carolina beaches are critical stopover sites for red knots that rely on them during spring migration. According to tagging research by SCDNR, "as many as two-thirds of the [red knots in South Carolina] fly directly to the Arctic after leaving our beaches." SCDNR, *supra* ¶ 20. This underscores the importance of food abundance on South Carolina beaches to the survival and breeding of red knots—for most red knots that stop over here, it is the last chance to refuel before a direct flight to Arctic breeding grounds.

22. The Service recently proposed a rule designating beaches across the South Carolina coast as red knot critical habitat. Proposed Rule, Endangered and Threatened Wildlife and Plants; Designation of Critical Habitat for Rufa Red Knot (*Calidris canutus rufa*), 86 Fed. Reg. 37,410,

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[6] https://www.biorxiv.org/content/10.1101/2022.03.21.485188v2.full.pdf, https://perma.cc/R93H-5GMN (permanent link).

[7] https://www.dnr.sc.gov/news/2018/jun/jun7_shorebirds.html, https://perma.cc/3DAT-ZUKP (permanent link).

37,425–27 (July 15, 2021). These beaches "contain[] one or more of the physical or biological features essential to the conservation of the species" and "serve[] as . . . important northbound migration stopover site[s] in South Carolina and on the Southeastern U.S. portion of the subspecies range." *Id.* at 37,453–62. Two of the State's beaches—Kiawah and Seabrook Islands––comprise "the most important known spring migration staging area in the Southeast." *Id.* at 37,458.

23. Food availability on South Carolina beaches in the spring carries species-level importance for red knots. Inadequate food availability on South Carolina beaches poses a significant threat to individual birds that stop over there and to the species as a whole.

D. Red Knots Require a Super-Abundance of Horseshoe Crab Eggs at South Carolina Stopover Beaches to Survive Their Migration and to Reproduce

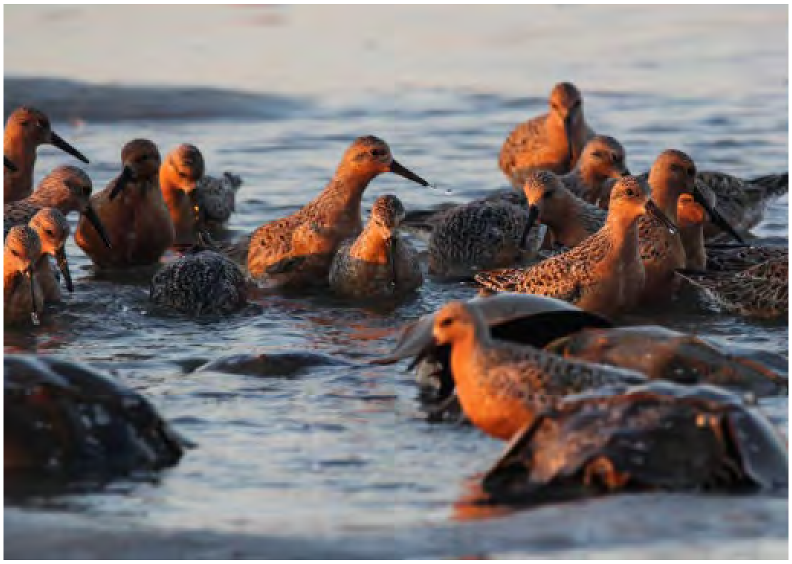

Figure 4. Red knots forage in the surf around horseshoe crabs on the coast of South Carolina.[8]

24. Horseshoe crabs live on the ocean floor in coastal areas.

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[8] Photo by Fletcher Smith. Taken from Bryan Watts, *CCB Team Spends Fifth Spring with Red Knots Along South Atlantic Coast*, CTR. FOR CONSERVATION BIOLOGY (Sept. 15, 2019), https://ccbbirds.org/2019/09/23/ccb-team-spends-fifth-spring-with-red-knots-along-south-atlantic-coast/, https://perma.cc/DY4H-UKWV (permanent link).

25. In the spring, horseshoe crabs come ashore to mate and lay eggs on South Carolina beaches between the mean low and high tide lines. Female crabs can lay over eighty thousand eggs in a spawning season, which runs from late spring to early summer.

26. Red knots have evolved to time their stopovers to coincide with horseshoe crab spawning season because horseshoe crab eggs are the only source of food available to red knots that allows the birds to reliably build up the fat and protein reserves needed to refuel from the initial stages of their migration, fly to the Arctic, mate, and reproduce.

27. As SCDNR researchers have confirmed, "[h]ighly nutritious horseshoe crab eggs and embryos . . . provide a critical food resource to the federally threatened *rufa* red knot . . . as it stops to feed in areas, such as . . . South Carolina during its annual migration from southern South America to the Arctic." Michael R. Kendrick et al., *Assessing the Viability of American Horseshoe Crab (*Limulus polyphemus) *Embryos in Salt Marsh and Sandy Beach Habitats*, 240 Biol. Bull. 145, 146 (2021) (attached as Ex. C to Plaintiffs' motion for preliminary injunction).

28. Horseshoe crab eggs are soft, fatty, energy-rich, and easily digestible. They allow red knots to gain about six grams of weight per day—a significantly higher rate than afforded by other prey that knots may consume during stopovers, like clams and mussels, which only allow the birds to gain between one and two grams per day. For these reasons, horseshoe crab eggs are an "ideal food source" for red knots. *See* SCDNR, *supra* ¶ 20; SCDNR, *Red Knot & Other Long-Distance Migrants* (last visited Feb. 17, 2023) ("Red Knot[s] spend time on spawning beaches and feed on the crabs' nutrient-rich eggs to fatten up before their migration to the breeding grounds in the Arctic.").[9]

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[9] https://www.dnr.sc.gov/wildlife/species/coastalbirds/shorebirds/RedKnot.html, https://perma.cc/4RCE-J97A (permanent link).

29. Studies show that red knots departing stopover locations with lower body mass are significantly less likely to survive migration and reproduce. In order to build the requisite body mass, red knots must consume several hundred thousand horseshoe crab eggs during their stopover. Red knots unable to consume their share of eggs are far less likely to survive their migration and breed.[10]

30. As noted, the red knot's Arctic breeding period is limited by weather and must end before frigid conditions take hold. *Supra* ¶ 18. As a result, birds arriving late at a stopover site must make up for lost time by rapidly gaining weight. Horseshoe crab eggs are the only resource that enables such rapid weight gain.

31. In modern times, the Georgia and South Carolina coasts and Delaware Bay were the only reliable locations for red knots to forage on horseshoe crab eggs during their northbound migration. These locations have therefore been the most-used spring stopover locations for the species in the last twenty years. Thus, red knots "'stop over at crab spawning sites on South Carolina beaches to gorge themselves on [horseshoe crab] eggs,'" which "play a critical role in the life cycles of . . . red knot[s]." SCDNR, *Horseshoe Crab spawning season a boon for shorebirds* (June 16, 2014) (quoting SCDNR wildlife biologist).[11]

32. Studies of South Carolina stopover sites confirm that horseshoe crab eggs are critical to red knots' diet and predict their foraging locations.

33. For example, in Cape Romain National Wildlife Refuge, a complex of barrier islands north of Charleston, South Carolina, ninety-five out of one hundred shorebird fecal samples,

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[10] *See, e.g.*, Haramis et al., *supra* note 4; Baker et al., *supra* note 4; Duijns et al., *supra* note 4.

[11] https://www.dnr.sc.gov/news/yr2014/june19/june19_horseshoecrab.html, https://perma.cc/VU6J-5VLJ (permanent link).

including red knots, taken between 2015 and 2016 tested positive for horseshoe crab DNA. Fumika Takahashi, *Shorebird Utilization of Horseshoe Crab (*Limulus polyphemus) *Eggs at Cape Romain National Wildlife Refuge, South Carolina*, CLEMSON UNIVERSITY TIGERPRINTS, ALL THESES, at 21, 61 (2016).[12] The same study found a significant positive correlation between horseshoe crab egg densities and the number of foraging red knots. *Id.* at 20, 59.

34. As noted, South Carolina at times hosts "the largest flock" of red knots on the Atlantic Coast, *see* SCDNR, *supra* ¶ 20, with birds from the flock observed on beaches "feeding on horseshoe crab eggs." Pelton et al., *supra* ¶ 20, at 3, 5.

35. Across the South Carolina coast, red knots disperse from larger flocks to search out horseshoe crab spawning sites and feast on crab eggs. *See* SCDNR, *supra* ¶ 20.

36. For a stopover site to be viable, it is not sufficient for horseshoe crabs merely to be present and spawning. Red knots require a super-abundance of crab eggs created by repeated spawning events. Female horseshoe crabs deposit their eggs several inches below the beach surface, where they are inaccessible to red knots due to the birds' size and small beak length. The crab eggs only become accessible to red knots when other horseshoe crabs are present and spawning at the same site, which pushes eggs to the surface. Without repeated horseshoe crab spawning events on beaches to create this accessible egg super-abundance, red knots cannot obtain the sustenance they need during stopovers.

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[12] https://tigerprints.clemson.edu/cgi/viewcontent.cgi?article=3583&context=all_theses, https://perma.cc/Z3PB-8BM8 (permanent link); *see also* Fumika Takahashi et al., *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*, 133 Wilson J. Ornithology 58 (2021), https://perma.cc/UR9M-D2GB (permanent link).

37. Red knots unable to obtain enough horseshoe crab eggs at a stopover site will be harmed in at least one of several ways. First, red knots may risk a dangerous flight to the Arctic directly from the egg-depleted site. Red knots that make such a flight may lack sufficient body weight and are significantly less likely to survive the trip and subsequently reproduce.

38. Second, red knots may forage at the egg-depleted stopover site for other, less effective food sources. This will likely delay or prevent birds from gaining the requisite body mass, causing birds not to fly to the Arctic to breed, to fly to the Arctic without enough weight to survive the flight and breed, and/or or to arrive late at the Arctic breeding grounds (which increases the risk of nest failure due to predation).

39. Third, red knots may search for an alternative stopover site. That search takes time and energy, further depleting fat and protein reserves needed to survive and reproduce, shortening the time left to breed in the Arctic before it is too cold there and/or increasing the risk of nest predation, and increasing the need to rapidly gain weight by consuming crab eggs. All of these factors reduce the birds' likelihood of survival as well as reproduction.

40. Beach-foraging red knots cannot access horseshoe crab eggs laid in crab containment ponds. Thus, when spawning horseshoe crabs are removed from beaches in South Carolina to containment ponds each spring, red knots suffer from a substantial diminution in their prey availability, significantly impairing their breeding and feeding and causing death and lower rates of reproduction.

II. <u>The Biomedical Harvest and Horseshoe Crab Containment Ponds</u>

A. Overview

41. Every spring, as red knots arrive and horseshoe crabs come ashore to spawn, Charles River's harvesters remove horseshoe crabs from beaches and place them into artificial

containment ponds. Since 2019, harvesters for Charles River have taken around [redacted] crabs from South Carolina beaches each spring, placing between [redacted] percent of crabs collected into containment ponds. IC Ex. C at 3 (exhibit to Plaintiffs' motion for preliminary injunction).

42. I understand that Charles River [redacted], to [redacted] horseshoe crabs per year, with [redacted] between [redacted]. IC Ex. H (exhibit to Plaintiffs' motion for preliminary injunction). A [redacted] of the [redacted] may be held in containment ponds.

43. In South Carolina, harvested crabs are removed from or near red knot foraging beaches and proposed critical habitat as they come ashore to spawn. The [redacted] are then stored in containment ponds during spawning season and red knot stopovers.

44. According to Dr. Brockmann, the use of containment ponds removes significant numbers of eggs from spawning beaches. *E.g.*, Brockmann Decl. ¶¶ 9(b)–(c), 62. The eggs of horseshoe crabs not present on beaches are not accessible to beach-foraging red knots. Thus, containment ponds remove [redacted] horseshoe crabs from spawning beaches, where eggs are accessible to red knots, to ponds beyond the reach of red knots.

45. [redacted]

46. According to Dr. Brockmann, releasing crabs at these release locations means that pond-held crabs are unlikely to spawn again for the entire season even after release, and particularly unlikely to spawn on the beaches from which they were harvested, Brockmann Decl. ¶¶ 45–51—

namely, on red knot foraging beaches. These post-release spawning impacts further deplete egg availability for red knots.

47. In my opinion, horseshoe crab containment ponds and release locations substantially deplete egg availability critical to the survival and reproduction of red knots that stop over in South Carolina. As a result, the use of containment ponds to hold horseshoe crabs harvested during spawning seasons likely causes the death of red knots and prevents others from reproducing.

B. The Harvest of Crabs for Pond Storage: Removal from Red Knot Feeding Areas

48. In South Carolina, horseshoe crabs are removed at an industrial scale from (or adjacent to) red knot foraging beaches—areas the Service has proposed as red knot critical habitat—and stored in containment ponds at the precise time red knots require a super-abundance of their eggs laid on beaches.[13]

49. In the last three years, there has been particularly heavy harvesting from on or near [redacted], [redacted], and the [redacted]. *See* IC Ex. D to Plaintiffs' motion for preliminary injunction. This heavy harvesting draws directly from red knot feeding beaches or adjacent areas. *See supra* note 13. I understand that a [redacted] of the crabs harvested from these beaches are [redacted]. *See* IC Ex. C

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[13] *Compare, e.g.*, 86 Fed. Reg. at 37,425–37,427, 37,581–37,582, 37,585–37,588 (proposed critical habitat designations for Turtle, Harbor, Hunting, Fripp, Hilton Head, and Seabrook Island beaches, and Deveaux Bank, among other locations), *with* IC Ex. D to Plaintiffs' motion for preliminary injunction (noting substantial harvest in recent years from proposed red knot critical habitat on [redacted] and [redacted]; [redacted], which abuts or encompasses proposed critical habitat on [redacted], [redacted], and [redacted]; [redacted], which abuts or encompasses proposed critical habitat on [redacted]; the [redacted] and [redacted], which abut or encompass proposed critical habitat on [redacted], [redacted], and [redacted]; and [redacted], which abuts or encompasses proposed critical habitat on [redacted], [redacted], and [redacted], among other locations).

(to Plaintiffs' motion for injunction) at 3 (between [redacted] percent of crabs collected were [redacted] since 2019).

50. Given the proximity of the harvest to stopover sites, harvesters are removing horseshoe crabs for pond storage that would otherwise be laying eggs on red knot foraging beaches. The removal of these crabs for pond storage deprives red knots of their eggs.

51. The below images from Turtle Island provide an example of what is occurring on red knot foraging beaches and proposed critical habitat across South Carolina. Turtle Island Beach has been proposed as critical habitat by the Service because, among other things, it "contains a high concentration of rufa red knots during the spring migration period, serving as an important northbound stopover site, particularly when horseshoe crabs are spawning." 86 Fed. Reg. at 37,461.

Figures 5, 6, and 7, below. Red knots, identifiable in the foreground of *Figures 6 and 7*, and other shorebirds forage in the surf around a few horseshoe crabs on the sound side of Turtle Island, while harvesters push their boat by the flock and pile in crabs in the background. PLTF045180, PLTF045182–183.


576 U33


576 U33

Figures 8, 9, and 10, below. Harvesters pile crabs in boats from the ocean-side of Turtle Island in 2019. Shorebirds, and potentially red knots, are visible in the background of *Figure 10*, foraging around horseshoe crabs in the surf. *See* ECF No. 67 at 13.

52. SCDNR and federal researchers observing the 2019 Turtle Island harvest, as well as harvesting in the adjacent Tybee Island National Wildlife Refuge, called the harvest at both sites "heavy" and "likely unsustainable," expressing concerns about impacts to red knots. Smith et al., *supra* ¶ 10, at 15, 17–18. In 2021, the Service reported that "intensive biomedical collections [on Turtle Island] in 2019" underscored "the risk of local extirpation." U.S. Fish & Wildlife Service, *Rufa Red Knot (Calidris canutus rufa), 5-Year Review: Summary and Evaluation*, at 21 (July 2021).[14] Data from SCDNR indicates that [redacted]. *See* IC Ex. D (from DNR000039–49).

53. SCDNR and federal researchers have identified "[t]he magnitude of the horseshoe crab harvest in critical shorebird foraging locations in South Carolina" as "likely the most significant

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[14] https://ecos.fws.gov/docs/tess/species_nonpublish/3624.pdf, https://perma.cc/S4TL-T4MT (permanent link).

shorebird conservation issue moving forward in the region." Smith et al., *supra* ¶ 10, at 17. I understand that the [redacted] of these crabs are harvested [redacted]. *See supra* ¶ 49.

54. Cape Romain National Wildlife Refuge provides another example. Historically, Cape Romain was known for providing an abundance of horseshoe crab eggs to red knots and other shorebirds. Large numbers of these birds therefore relied on the refuge to serve as their migratory stopover site.

55. For years, Charles River [redacted]. *See* IC Ex. D (from DNR000030–46). As noted, for a stopover to be viable for red knots, there must be repeated spawning events by horseshoe crabs to deposit a super-abundance of eggs that are accessible to red knots. *Supra* ¶ 36. After years of [redacted] in Cape Romain, mean egg densities measured in 2015–16 were far below levels associated with a 50% chance of red knot presence. Takahashi, *supra* ¶ 33, at 26–27, 53; *see also* IC Ex. A to this declaration (only submitted *in camera*) (noting [redacted]). Red knots unable to obtain sufficient crab eggs at a stopover beach are far less likely to return to that site in subsequent years.

56. The available evidence from South Carolina indicates that the harvest for ponds is a significant threat to red knots.

57. The fact that crabs are harvested from beaches, just as they come ashore to spawn and red knots need their eggs, increases the threat to the birds.

58. The time and place of the harvest-for-ponds presents an additional threat to red knots' feeding, breeding, and survival. The evidence I've reviewed, including figures 5 through 10 above, indicates that harvesters often take crabs for pond storage while red knots are present and

foraging for crab eggs. This presents threats in addition to food deprivation, likely disturbing and possibly flushing[15] the birds. Red knots disturbed in this manner will stop foraging for crab eggs, leave the perceived threat, and leave the beach, often until the disturbance stops. This will cause red knots to eat fewer crab eggs and also wastes critical time and energy during the brief period when red knots must build body mass, increasing the risk that red knots will not gain the weight they need to survive and breed.

59. In sum, the removal of [redacted] of spawning horseshoe crabs from red knot feeding areas for storage in containment ponds, at the precise time the birds require a super-abundance of their eggs to be laid on beaches, deprives knots of crab eggs critical to their survival and reproduction.

C. When Red Knots Most Need Their Eggs, Horseshoe Crabs are in Containment Ponds Instead of on Red Knot Beaches

60. Each spring, around [redacted]rseshoe crabs, including many females, are taken from South Carolina beaches, with [redacted]% stored in ponds during spawning season, IC Ex. C (to Plaintiffs' motion for injunction) at 3, the precise window when red knots critically need their eggs before departing for the Arctic. Harvesters move [redacted] crabs from spawning beaches, where eggs present in a super-abundance are accessible to red knots, to ponds beyond the reach of red knots.

61. The timing of the capture and holding of horseshoe crabs for bleeding in South Carolina shows the co-occurrence with red knot foraging in the same areas. Red knots feeding on horseshoe crab eggs in South Carolina build weight in late April and May, coinciding with peak horseshoe crab spawning. In my work with SCDNR to study movements of red knots, we trap in

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[15] "Flushing" means to cause a bird to fly away suddenly in response to a disturbance.

mid to late April because it is the peak of the red knot migration. [redacted].

62. By removing [redacted] of spawning crabs from red knot feeding beaches to inaccessible containment ponds, [redacted], ponds usage likely prevents significant numbers of red knots from gaining the requisite weight to survive their migration and breed in the Arctic.

D. Crab are Released [redacted] from Red Knot Stopover Beaches

63. It is my understanding that crabs that survive the biomedical process are released to coastal waters. I understand that release locations are based, at least in part, on [redacted] harvest beaches and proposed red knot critical habitat.

64. According to Dr. Brockmann, crabs released in these areas are not likely to spawn again for the rest of the season, and particularly unlikely to return all the way to their harvest beaches to spawn in the same season. Brockmann Decl. ¶¶ 47–51. As noted above, crabs are harvested from or near red knot foraging areas.

65. For red knots, this means that they are deprived of the eggs of pond-held crabs not only during the period of their transport to and storage in ponds, but also likely for the remainder of the season, even post-release. These post-release effects further deplete egg availability for red knots during their stopover.

III. <u>The Impacts of Containment Ponds on Red Knots.</u>

66. By removing significant numbers of horseshoe crabs from South Carolina spawning beaches each spring, containment ponds deprive red knots of vast numbers of crab eggs on their beach foraging grounds, precisely when the birds most need a super-abundance of crab eggs.

These harms are worsened by the fact that the use of ponds and [redacted] makes crabs unlikely to spawn again for the rest of the season even after release. *See* Brockmann Decl. ¶¶ 47–51. These harms will be greater beginning this season if Charles River [redacted] [redacted].

67. It is my opinion that removing [redacted] crabs—and vast numbers of crab eggs—from red knot stopover sites as a result of the harvest for containment ponds makes it highly likely that some red knots will not obtain the sustenance needed to survive their migration and breed in the Arctic.

68. Containment ponds preclude the super-abundant egg densities red knots need at South Carolina stopover sites to survive migration and reproduce.

69. Based on my professional experience, the literature, and the evidence I have reviewed in this case, it is highly likely that horseshoe crab containment ponds have caused the death of red knots and prevented others from reproducing by depriving the birds of a critical food source. If the use of containment ponds in South Carolina is allowed to continue, these harms to red knots will continue. If horseshoe crab harvesting [redacted], the harm to the birds will likely be more severe.

Pursuant to 28 U.S.C. § 1746, I declare under penalty of perjury that the foregoing is true and correct. Executed on March 13th, 2023.

Lawrence J. Niles

Lawrence Niles

EXHIBIT A

LAWRENCE J. NILES
109 Market Lane, Greenwich, NJ 08323
(856) 451-0465 office (908) 303-3843 cell

EDUCATION

Ph.D. Ecology and Evolution, Rutgers University. Stopover Ecology of Migratory Raptors. May 1996
M.S. Wildlife Management, Pennsylvania State University, August 1976.
B.S. Zoology, Pennsylvania State University, August 1973.

EMPLOYMENT

7/06 **:**present **Biologist/Mgt Partner, Wildlife Restoration Partnerships, Greenwich, NJ 08323**
I started my own business focusing on Wildlife Investigations, Habitat Conservation Planning, and Habitat Management in 2007. I conduct conservation and research projects for non-profit groups or agencies with foundation funding. I focus on four main tasks: 1. Research on Shorebirds in Delaware Bay, Gulf states, South America, and the Arctic, 2. New technology for tracking birds, including satellite transmitters, 3. Restoring habitat for wildlife, including breeding and migrant shorebirds and horseshoe crabs. I am now co-managing projects with the American Littoral Society to restore marsh and beach on Delaware Bay and a $13 million Project developing Living Shoreline structures to protect Bayshore communities. I continue to lead research in Chile, Brazil, Canada, and SC, MA, NY, and NJ on shorebird and horseshoe crab productivity and survival, the impact of offshore wind power, and a hemisphere-wide strategy for recovering shorebirds. In collaboration with NJ Audubon, I co-lead a new organization, the Horseshoe Crab Recovery Coalition, aiming to restore horseshoe crab populations throughout their east coast range as a primary strategy to restore coastal shorebird populations.

6/92 – 7/06: **Chief, Endangered and Nongame Species Program,** Division of Fish, Game, andWildlife, New Jersey Department of Environmental Protection, Trenton, NJ 08625.
I led 20 biologists, five technicians, and as many as 20 seasonal or part-time employees working on endangered and rare species research, management, wildlife recreation, and ecosystem-level projects. During my tenure, I developed a landscape-level conservation system now a major part of the State's land planning initiatives and conducted research and management the Bald Eagle, colonial waterbirds, migrant songbirds and raptors and Arctic nesting shorebirds. I led expeditions to the Canadian Arctic and the Argentine and Chilean portions of Tierra del Fuego to conduct shorebirds surveys and site protection.

11/82 - 5/92: **Principal Nongame Zoologist**; **Endangered and Nongame Species Program**; Division of Fish, Game, and Wildlife, New Jersey Department of Environmental Protection, Tuckahoe, Wildlife Management Area, Tuckahoe, NJ 08250

6/78 - 11/82: **Wildlife Biologist II**; **Georgia Department of Natural Resources**, Game and Fish Division, Route 1, Fitzgerald, GA 31750. As the regional game biologist in a twelve-county area of southeast Georgia, I conducted research and management on white-tailed deer and black bears.

8/76 - 6/78: **Forest Technician**; Belle Baruch Forest Science Institute, Clemson University, P.O. Box 596, Georgetown, SC 29440.

5/74 - 8/76: **Graduate Assistant and Research Technician**; School of Forest Resources, Pennsylvania State University, University Park, PA 16802.

OTHER ACTIVITIES

Undergraduate instructor, Rutgers University, Ecology and Environmental Science, 1994 – 1997, 2005
NJ Audubon Richard Kane Conservation Award 2008
Littoral Society Macmillian Award 2009
Penn State Natural Resources Hall of Fame 2018
Member National Shorebird Council
Member Western Hemisphere Shorebird Reserve Executive Committee

PUBLICATIONS Selected publications below, my full list can be provided upon request

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EXHIBIT B

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH	)	
CAROLINA COASTAL CONSERVATION	)	
LEAGUE,	)	
Plaintiffs,	)	
v.	)	
	)	
ROBERT H. BOYLES, JR., in his official	)	
capacity as Director of the South Carolina	)	Civ. No. 2:22-CV-112-RMG
Department of Natural Resources, and	)	
CHARLES RIVER LABORATORIES,	)	
INTERNATIONAL, INC.,	)	
Defendants.	)	

DECLARATION OF DR. H. JANE BROCKMANN

I, H. Jane Brockmann, declare the following to be true and correct, and to reflect my professional opinion and my best judgment on the matter at hand based on the body of scientific evidence.

PROFESSIONAL BACKGROUND AND QUALIFICATIONS

1. I am a biologist and animal behaviorist. Since the 1980s, I have spent the majority of my professional career studying horseshoe crabs, and their mating behavior and breeding aggregations.

2. I received my Master of Science in Zoology from the University of Wisconsin in 1972, and my Ph.D. in Zoology from the University of Wisconsin in 1976. I received my Bachelor of Science degree in Biology from Tufts University in 1967. Attached is my curriculum vitae. *See* Ex. A.

3. Since 2011, I have served as an Emeritus Professor in the Department of Biology at the University of Florida. Prior to this time, I was an Assistant Professor, Associate Professor, and

Professor in the Department of Zoology at the University of Florida from 1976-2011. During my tenure with the University of Florida, I chaired the Department of Zoology from 1997-2001.

4. From 2003 to 2004, I was the Program Director for the Animal Behavior Program at the National Science Foundation.

5. I have published dozens of articles in peer-reviewed journals and supervised numerous graduate students' research on horseshoe crab reproductive behavior. Since the early 1990s, I have published a vast amount of research on horseshoe crabs, including on the following topics: male mating tactics, nest site-selection, female nesting behavior, reproductive competition, conservation genetics, and multiple stressor interactions on the development of horseshoe crab embryos.

6. I have also published several studies on genetic differences between populations of horseshoe crabs, co-authored regional assessments on the conservation status of horseshoe crabs, and published research on trait differences between populations (e.g., size, sexual dimorphism, and behavior). These studies have included all populations of the American horseshoe crab. I was also co-author of *The American Horseshoe Crab* published by Harvard University Press in 2003.

7. Over the course of my decades of research, I have specifically studied the Southeast population of horseshoe crabs (which includes all South Carolina horseshoe crabs) – crabs which grow larger and very likely live longer than horseshoe crabs of any of the other genetically distinct populations.

8. I have managed numerous horseshoe crab surveys and volunteer trainings for horseshoe crab surveys in Florida in recent years. Working with the State of Florida Fish and Wildlife Conservation Commission and others, I organized and initiated (and continue to participate in) a

statewide tagging and breeding survey of Florida horseshoe crabs called the Florida Horseshoe Crab Watch.

SUMMARY OF OPINIONS

9. I have reviewed documents produced in discovery during this litigation that have been provided by Plaintiffs' counsel. Based on these documents, the literature on horseshoe crabs, and my professional expertise and experience, it is my opinion that:

   a. Holding ponds, and the practices associated with collection, transport, and release of crabs that are held in these ponds, negatively impact horseshoe crab spawning and survival;

   b. These impacts, combined with likely increased mortality from pond-held crabs during bleeding, and release in locations far from beaches where crabs were collected and normally breed, effectively remove these horseshoe crabs from the breeding population in South Carolina for the rest of the year;

   c. Horseshoe crabs held in artificial containment ponds are spawning in the ponds, far from natural beaches; any horseshoe crab eggs produced in ponds are not recruited into the horseshoe crab population in South Carolina;

   d. The terms put in place by South Carolina Department of Natural Resources ("SCDNR") in its 2022 holding ponds permits that were designed to minimize harm to horseshoe crabs and prevent female crabs from being stored in ponds were both impractical and wholly ineffective. As a result, [redacted]. A significant number of female horseshoe crabs (almost [redacted]) [redacted] in 2022 despite SCDNR's new permit conditions, and

[redacted].

BASIS FOR OPINIONS

A. Horseshoe Crabs and Containment Ponds Generally

10. Horseshoe crabs (*Limulus Polyphemus*) have existed for almost 450 million years; they are living fossils that are most closely related to arachnids like spiders.

11. Horseshoe crabs live on the continental shelf in coastal areas from Florida to Maine. Horseshoe crabs feed in coastal areas, spending colder weather in channels and deeper coastal regions.

12. Each spring, horseshoe crabs emerge on South Carolina’s beaches on the high tides associated with the new and full moon to mate and lay eggs. During these events in the spring, horseshoe crabs appear in large spawning aggregations on South Carolina beaches at the same time as shorebirds like the red knot migrate through South Carolina to feed on nutrient-rich horseshoe crab eggs to fuel their migration back to their Arctic breeding grounds.

13. Female horseshoe crabs lay eggs 10-20cm below the surface, but when there are large numbers of breeding females, they inadvertently excavate the eggs of females that bred earlier on that beach. Therefore, eggs are present on the beach surface only when very large aggregations of females have been nesting for a number of days. Spawning density directly affects the availability of eggs on the surface.

14. Each female horseshoe crab with a male attached to her shell can lay about 2,000-4,000 eggs in each nest which are fertilized by the attached male as the eggs are being laid. Studies conducted on Delaware Bay and mid-Atlantic female crabs show that they can lay over 80,000 eggs in total during a season. Because Southeastern female crabs are much larger, it is likely that

they lay even more eggs during a season, which typically runs from April into June in South Carolina.

15. After spawning is completed (which normally occurs during 1-5 high tides), the female returns to sea until the following year. This means that any female harvested on South Carolina's beaches has likely not yet completed her yearly spawning. Removing these spawning females from South Carolina's beaches into holding ponds results in female crabs continuing to spawn, if they are able, in the holding ponds. Regardless, it means that able crabs are not spawning on beaches.

16. The American horseshoe crab, the species present in South Carolina (and the only species currently existing in North America) is listed on the International Union for the Conservation of Nature's Red List of Threatened Species as "vulnerable," meaning there is a greater than 10% probability that horseshoe crabs will be extinct in the wild in 100 years.

17. The status of the Southeast population of horseshoe crabs (the population to which South Carolina crabs belong) is unclear but may be in decline. The Atlantic States Marine Fisheries Commission ("ASMFC") 2019 Stock Assessment Report classifies the South Carolina population of horseshoe crabs as stable because it could not demonstrate statistically that the population was either above or below a 1998 reference point. *See* Atlantic States Marine Fisheries Commission 2019 Horseshoe Crab Benchmark Stock Assessment Report at 15, 58, 197–201.[1] This conclusion is due in large part to the fact that there are so few data points that the statistical power to detect differences is very low (i.e., there would have to be a huge decline before it could be detected with these data). The chance of detecting a difference between years

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[1] *Available at* www.asmfc.org/uploads/file/5cd5d6f1HSCAssessment_PeerReviewReport_May2019.pdf (asmfc.org) (last visited March 6, 2023).

is low because very few horseshoe crabs are actually caught using their sampling methods – the two trawl surveys that were used for this analysis were designed for fish and crustaceans, not horseshoe crabs, and horseshoe crabs only rarely get into the trawl nets.

18. Any conclusion drawn from such limited information is likely to be highly unreliable. Until surveys are conducted that are properly designed to detect horseshoe crabs, it is impossible for anyone to estimate horseshoe crab populations with any degree of certainty.

19. Horseshoe crabs are highly susceptible to overharvesting. They have long lives of up to 20 years and take a long time to grow to sexual maturity – 9-10 years for males, 11-12 years for females (South Carolina horseshoe crabs are the largest along the entire coast and therefore are likely to take longer to develop than elsewhere). Because it takes young crabs so long to reach reproductive age, the population can take a decade or more to recover from high mortality events even under ideal conditions— i.e., with no further harvesting or mortality from transport to, holding in, and release from ponds and bleeding.

20. Harvesting of horseshoe crabs during their spawning events increases the risk of adversely affecting the population, as beach-mating exposes all spawning crabs to capture. The biomedical harvest in South Carolina takes the most important members of the population (large females and mature males) off of South Carolina beaches, by hand, during the most critical of their life phases from a population perspective (e.g., during spawning).

B. Charles River's Harvest of Horseshoe Crabs for Containment Ponds

21. Each year, Charles River's agents are permitted by SCDNR to harvest unlimited numbers of horseshoe crabs from South Carolina beaches, [redacted] [redacted]. *See* ECF No. 67—IC Ex. H at 3. [redacted]

[redacted].

22. Since 2019, Charles River's harvesters have taken roughly [redacted] crabs each spring from South Carolina beaches, storing roughly [redacted]% of their harvest in containment ponds. *Id.* For example, in 2021, Charles River harvested over [redacted] horseshoe crabs in South Carolina and over [redacted] of those crabs were placed in containment ponds; in 2022, Charles River harvested almost [redacted] horseshoe crabs in South Carolina and over [redacted] of those crabs were placed in containment ponds. *Id.*

Figure 1. Aerial view of a horseshoe crab containment pond near Beaufort, SC.

23. Containment ponds are, to state the obvious, not the natural habitat for horseshoe crabs. They are artificial, man-made holes in the ground, located away from coastal beaches. These ponds make for a very poor substitute for the natural environment of horseshoe crabs.

24. Horseshoe crabs are remarkably tolerant of a wide range of environmental conditions and [redacted], i.e., temperatures above 31° C, dissolved oxygen below 4.0 mg/L, and salinity less than 15 ppt. However, there is very good evidence (Hamilton et al. 2020) that horseshoe crabs held under containment pond conditions similar to Charles River's self-reported figures showed significant physiological deterioration as a consequence of prolonged holding, particularly at warmer water temperatures.

25. Of equal concern is that horseshoe crabs held in captivity for more than two weeks without food are starving, i.e., they are metabolizing their own tissues (Smith et al. 2013). Biomedical bleeding is known to be stressful for horseshoe crabs, with physiological effects that last for weeks (Anderson et al 2013; Kurz &James-Pirri 2002; Leschen & Correira 2010; Owings et al. 2019). The use of containment ponds greatly increases the stress that horseshoe crabs are subjected to prior to bleeding and therefore the use of these ponds is likely to have an even longer lasting effect on horseshoe crab behavior and reproduction.

26. Before Plaintiffs filed this case, SCDNR did not impose any restrictions on the length of time horseshoe crabs could be stored in ponds before bleeding. *See* ECF No. 1-1 at 23 (2021 permit). After Plaintiffs filed this case, SCDNR in 2022 added new conditions to its permits stating that "[h]orseshoe crabs may not be held longer than two weeks from the time of harvest to biomedical bleeding." ECF No. 42-1 at 4. Although the permits now limit pre-bleeding pond storage to two weeks, I have not seen evidence of successful measures implemented by pond permittees or SCDNR to determine when each of the tens of thousands of pond-held crabs were introduced to ponds or to ensure that they are removed and bled within two weeks or less. Thus, I

have not seen evidence that the two-week limit is being enforced, and crabs may be stored for much longer.

27. The added stress of placement in containment ponds is very likely to increase mortality after release and reduce future spawning on beaches. All biomedical bleeding operations cause some level of mortality and stress, but placement in containment ponds greatly increases that stress. This increased stress may not always result in immediate mortality, but it necessarily increases the risk of reduced breeding or mortality in the future.

28. There are no protections whatsoever for eggs laid or horseshoe crab larvae developing *in* holding ponds—SCDNR does not require Charles River to record their existence or return them to their natural habitat.

C. The Harvest for and Transport to Containment Ponds Harms Horseshoe Crabs

29. As noted above, Charles River's agents harvest large numbers of horseshoe crabs from South Carolina's beaches each spring, including beaches that are proposed as red knot critical habitat by the United States Fish and Wildlife Service. *See* Proposed Rule, Endangered and Threatened Wildlife and Plants; Designation of Critical Habitat for Rufa Red Knot (*Calidris canutus rufa*), 86 Fed. Reg. 37,410, 37,425–27 (July 15, 2021). South Carolina law, SCDNR permits, and best management practices from the ASMFC all prohibit the indiscriminate piling of crabs in boats. *See* S.C. Code Ann. § 50-5-1330(C) ("Horseshoe crabs . . . must be handled so as to minimize injury to the crab and "returned unharmed to state waters"); [redacted]; ECF No. 67– Ex. L at 2 (ASMFC best management practices for biomedical harvesting; requiring, inter alia, "[p]roper care and

handling of horseshoe crabs" and "avoiding over-stacking to minimize damage to other horseshoe crabs").

30. The photos below document the removal of spawning horseshoe crabs from Turtle Island in South Carolina and the piling of them indiscriminately on top of each other in boats in a manner that is likely to cause injury to these crabs.

Figures 2 and 3. Harvesters pile horseshoe crabs into boats off of Turtle Island in 2019.

31. These pictures also depict harvesters picking up crabs by the "telson," or tail. As SCDNR explains, "**[d]o not pick a horseshoe crab up by its tail—the tail is very sensitive and easily injured.**" SCDNR, *Horseshoe Crabs – Anatomy and Behavior* (2019) (emphasis in original).[2]

32. These photos are very concerning for the health and wellbeing of the horseshoe crabs being collected, and I have not seen any evidence from discovery that the above images are out of the ordinary. The crabs in South Carolina are particularly large and heavy. The manner of collection above (i.e., picking up heavy South Carolina crabs by the telson) is likely to damage the muscle at the base of the tail which is used by horseshoe crabs to turn themselves over when they get overturned in waves. Damage to the tail muscle will not result in immediate mortality but rather greatly increase mortality when they try to spawn in subsequent years.

33. Piling crabs indiscriminately on top of each other in massive numbers in boats likely causes additional injuries to crabs such as cracked shells, broken telsons, and injuries to the soft parts of the body. SCDNR's discovery additionally shows reports that harvesters then "walk[] across their boats, on top of a full load of crabs to get to the helm." DNR014067. The various injuries caused by these careless practices will likely increase mortality after release.

34. This sort of handling and transport to ponds has an impact on the status of horseshoe crabs that survive collection to make it to holding ponds. Documents obtained in discovery demonstrate that, independent of mortalities that occur in the holding ponds themselves, [redacted]. *See* ECF No. 67—IC Ex. H at 3–5. The crabs that [redacted]

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[2] *Available at* https://www.dnr.sc.gov/marine/pub/seascience/horseshoecrab.html (dnr.sc.gov) (last visited March 11,2023).

presumably result, at least in part, from rough handling, and they may be dead or lost during the transport and holding process.

35. Moreover, this sort of handling and transport likely adversely affects the health and wellbeing of horseshoe crabs after their release, assuming they survive holding ponds and the bleeding and release processes. Crabs with injured telsons cannot right themselves, and, if overturned by a wave when attempting to reach spawning beaches, they would likely die. Other injuries will also increase mortality through increased susceptibility to disease and predation, and through decreased ability to forage.

36. In sum, this treatment of horseshoe crabs during collection and transport to holding ponds is one more way Charles River's holding ponds are removing horseshoe crabs from the population and from spawning each year. These impacts are magnified by the fact that all crabs are handled multiple extra times (beyond what is normally done in biomedical bleeding facilities) – once into the boat, then into the ponds, then out of the ponds and into the bleeding facility, then back to the truck for release, then out of the truck into chutes or tossed back into release waters.

D. *The Unloading of Horseshoe Crabs into Holding Ponds Violates the Possession Permit and Further Harms Horseshoe Crabs.*

37. The 2022 Possession Permit further states that [redacted] [redacted].

38. But upon arrival at ponds, agents of Charles River also appear to [redacted] to unload crabs into ponds.

39. Crabs are [redacted] into holding ponds, often by the [redacted].

[redacted]

[redacted]

Figures 4 and 5. Horseshoe crabs [redacted]. Still images taken from undated videos disclosed by pond operator Jerry Gault in discovery. [redacted].

40. Again, this is a likely source of harm and mortality to many of the crabs entering holding ponds, and contributes to the negative impacts of holding ponds on the horseshoe crab population in South Carolina and on future spawning of horseshoe crabs.

E. Harm to Horseshoe Crabs from Spawning in Containment Ponds

41. Instead of spawning on South Carolina beaches, [redacted]% of harvested horseshoe crabs, [redacted], are stored in ponds each spring for weeks during spawning season, *See* ECF No. 67—IC Ex. H at 3–5. Any horseshoe crab eggs, larvae, and young horseshoe crabs in containment ponds are necessarily unable to be recruited to South Carolina's horseshoe crab population.

42. Although SCDNR permits do not require that horseshoe crab spawning in containment ponds be monitored or reported, evidence from discovery indicates that [redacted].

43. This is not surprising, as horseshoe crabs are harvested just as they come ashore to spawn in spawning aggregations and stored in ponds for weeks during their spawning season.

[redacted]

Figure 6. Horseshoe crabs [redacted]. Undated photo disclosed by pond operator Jerry Gault in discovery. [redacted].

44. The pond operators openly acknowledge that this spawning is occurring. For example, [redacted]. During a visit by reporters to one of his ponds, Mr. Gault dipped a water bottle into the pond and held it up, revealing "so many" of the "bab[y]" horseshoe crabs spawned in the pond. Radiolab, *Baby Blue Blood Drive* podcast at 38:00–39:15 (July 23, 2020).[3]

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[3] https://radiolab.org/episodes/baby-blue-blood-drive, https://perma.cc/53QU-Q4GD (permanent link).

F. Harm to Horseshoe Crabs from Release after Containment Ponds

45. Based upon information obtained in discovery, I understand that [redacted]. The ASMFC's Biomedical Best Management Practices provide that if horseshoe crabs are "not being returned to the area of capture, ensure that conditions (salinity, water temperature, etc.) are similar to those found at the harvest site." *See* Atlantic States Marine Fisheries Commission 2019 Horseshoe Crab Benchmark Stock Assessment Report at 239. Contrary to these Best Management Practices, [redacted].

46. The Possession Permit issued by SCDNR provides that [redacted].

47. Evidence produced in discovery indicates that [redacted].

48. Some (or all) release locations are apparently [redacted].

49. The manner of release is also injurious to crabs and appears to be in violation of the Possession Permit's mandate [redacted]

[redacted].

50. Not only are these release practices harming horseshoe crabs, but the [redacted].

51. This means that released crabs are unlikely to spawn again for the entire season, and particularly unlikely to travel miles back to the beaches from which they were taken to spawn again that season. Large numbers of eggs on the surface require very large aggregations of spawning crabs; reduced crab numbers means that crabs will spawn, but not enough for eggs to reach the surface. These post-release effects further deplete egg availability on beaches; it is likely that injured animals released far from familiar locations will take considerable time to resume normal breeding.

G. The Terms of the 2022 Possession Permits are Unworkable and Do Not Resolve These Harms

52. In 2022, SCDNR modified its Possession Permit to provide that [redacted].

53. SCDNR discovery responses indicate that the term of the 2022 permits [redacted]

[redacted]. *See* ECF No. 67—IC Ex. H at 3–5. [redacted]. *Id.* [redacted]. *Id.* at 5.

54. Moreover, Charles River pays pond operators [redacted]. Charles River continued to pay [redacted]. Female crabs are larger and Charles River can obtain more blood from them.

55. The term of the 2022 permits requiring [redacted], but it is unworkable in practice.

56. It is very difficult to separate male and female crabs during spawning, particularly the very large South Carolina crabs. Documents produced by Charles River in discovery corroborate this difficulty.

[redacted]

Figure 7. [redacted]. Image disclosed by Charles River in discovery. [redacted].

57. Males use a pair of specialized claws for holding onto a female's shell and if not removed carefully, a male may lose a claw rather than letting go. Not only will loss of a claw mean significant blood loss, but it will also mean that this male is no longer able to attach to females in the future.

58. Further, forcing a male's claw off the specialized spines (posterior opisthosomal spines) at the back of female's shell may damage the spines, and when a female has damaged spines, no male is able to hold onto her securely. Females with broken or damaged spines are unable to breed if a male cannot attach securely. Thus, any practice requiring separation of males and females will dramatically increase the risks that future spawning capacity will be diminished.

CONCLUSION

59. In sum, it is my opinion that the transport to, holding in, and release of horseshoe crabs from holding ponds is violating South Carolina statutory and permitting requirements and best practices defined by the ASMFC.

60. The South Carolina legislature requires that crabs harvested for biomedical bleeding "must be handled so as to minimize injury to the crab" and "must be returned unharmed to state waters of comparable salinity and water quality as soon as possible after bleeding," S.C. Code § 50-5-1330(C). The evidence that I have seen produced in discovery shows that crabs are not handled to minimize injury, returned unharmed, or returned to waters with comparable conditions; instead, there are harms to horseshoe crabs at every stage of the process related to containment ponds.

61. The harms to horseshoe crabs related to the harvest for, transport to, holding in, and release from containment ponds are significant enough to contribute to population declines, and to remove horseshoe crabs from spawning for an entire season.

62. The above practices also remove a significant number of eggs from South Carolina's beaches. There is clear evidence that horseshoe crabs held in holding ponds are spawning in those ponds, and that those eggs or baby horseshoe crabs are left to die in those ponds.

63. The 2022 Permit Terms did not prevent these harms and are unworkable in

practice. Significant harms to horseshoe crabs and removal of a substantial portion of horseshoe crab eggs from South Carolina's beaches will continue so long as Charles River and its agents are allowed to hold horseshoe crabs in artificial containment ponds.

Pursuant to 28 U.S.C. § 1746, I declare under penalty of perjury that the foregoing is true and correct. Executed on March 12th, 2023.

Jane Brockmann

H. Jane Brockmann

EXHIBIT A

September 2015

CURRICULUM VITAE

H. JANE BROCKMANN

Professor Emeritus of Biology
University of Florida, Gainesville, Florida 32611-8525

PERSONAL

Telephone: (352) 392-1297; 376-0473; FAX: 352-392-3704; email: HJB@ufl.edu

EDUCATION

1967	B.S., Tufts University, Department of Biology, Medford, Massachusetts
1972	University of Maryland, Department of Zoology (1968-1969)
	M.S., University of Wisconsin, Madison, Department of Zoology (advisor: Jack P. Hailman)
1976	Ph.D., University of Wisconsin, Madison, Department of Zoology (advisor: Jack P. Hailman)

PRESENT ACADEMIC POSITION

2011-present	Emeritus Professor, University of Florida, Department of Biology

POSITIONS HELD

1976-1981	Assistant Professor, University of Florida, Department of Zoology
1981-1989	Associate Professor, University of Florida, Department of Zoology
1989-2011	Professor, University of Florida, Department of Biology
1985-1986	Visiting Research Biologist, Princeton University, Department of Biology
1981-2004	Affiliate Professor, Department of Psychology
1997-2002	Affiliate Professor, Department of Physiology, College of Veterinary Medicine, UF
1980-2011	Affiliate Professor, Department of Entomology, University of Florida
2000-2011	Affiliate Professor, Center for Women's Studies, University of Florida
1997-2001	Chair, Department of Zoology, University of Florida
2003-2004	Program Director, Animal Behavior Program, IBN, National Science Foundation
2006-2007	Associate Chair, Department of Zoology

FELLOWSHIPS

1968-1969	Woodrow Wilson National Fellowship, University of Maryland, Dept. of Zoology
1970-1972	Federal Water Quality Training Grant Traineeship, University of Wisconsin, Dept. of Zoology
1977-1978	National Science Foundation, NATO Postdoctoral Fellowship, Animal Behaviour Research Group, Oxford University, England (mentor: Richard Dawkins)
1983-1984	Sabbatical, Department of Zoology, University of Florida
1984	Visiting Fellow, Wolfson College, Oxford, England
1985-1986	National Science Foundation Visiting Professorship for Women, Depart. of Biol., Princeton Univ.
1994-1995	Sabbatical, Department of Zoology, University of Florida
1994	Visiting Scholar, Dept. Evolution and Ecology, University of California, Davis, CA

HONORS

1967	T. H. Carmichael and Emily Leonard Carmichael Scholarship in Physiol Psychol. Tufts Univ.
1974	Elected member of Sigma Xi, University of Wisconsin
1978	American Men and Women of Science
1992	University of Helsinki Medal
1995	Animal Behavior Society Wm. C. Brown Animal Behavior Teaching Award
1995	Fellow of the Animal Behavior Society (elected)
1996	Teaching Incentive Program (TIP) Award, College of Liberal Arts and Sciences, Univ. Florida
2000	Animal Behavior Society, Distinguished Service Award
2000	STEP Award, University of Florida
2003	Distinguished Visiting Professor, University of Miami
2003	Sigma Xi Senior Research Award, University of Florida Chapter
2005	Doctoral Dissertation Advisor/Mentoring Award from UF Graduate School
2008	Fellow of AAAS (elected)
2011	Myrle E. and Verle D. Nietzel Visiting Distinguished Faculty Program Award, University of Kentucky

GRANTS

1979-81	Harry Frank Guggenheim Foundation Grant
1980-83	National Science Foundation (DEB 80-09334)
1985-86	National Science Foundation (RIT 85-03659)
1988-89	National Science Foundation (BNS 88-15345) (Int. Travel Grant to IEC Conference)
1990-94	National Science Foundation (OCE 90-06392)
	Division of Sponsored Research, University of Florida
1977, 1979, 1980	Seed Money Grants; Biomedical Research Grant, Faculty Research Award
1980-81, 1982-83, 1989-90, 1996	Graduate Research Assistantship Program
1983-84, 1990	Research Development Award
2000	CLAS Travel Award to attend International Ethological Conference
2002-2003	AAAS – WISC funded by the National Science Foundation
2003-2006	EPA Star Fellowship to my graduate student C.W. Gunnels
2006-2007	NSF Doctoral Dissertation Improvement Grant to my graduate student Hope Klug
2008-2012	PADI Foundation grant to my graduate student Daniel Sasson
2009-2011	NSF Doctoral Dissertation Improvement Grant to my graduate student Clare Ritschoff
2007-2011	National Science Foundation (IOB 06-41750)

EDITORSHIPS AND EDITORIAL BOARDS

1987-90	Associate Editor, Evolution
1979-80; 1988-1991	Consulting Editor, Animal Behaviour; Editorial Board, Animal Behaviour
1991-2001	Editor, Ethology
2002-2005, 2013-2015	Editor, Advances in the Study of Behavior
2005-2013	Editor-in-Chief, Advances in the Study of Behavior

INVITED LECTURES AT NATIONAL AND INTERNATIONAL MEETINGS AND WORKSHOPS

1978	Conference on Evolution and the Theory of Games, University of Bielefeld, Germany, Lecture
1979	XVI International Ethological Congress, Vancouver, B.C., Lecture
1981	XVII International Ethological Congress, Oxford, England, Symposium Lecture
	Nordic Council lecture series on Arthropod Behavioral Ecology, University of Uppsala, Uppsala, Sweden
1982	IX International Congress of the International Union for the Study of Social Insects, Boulder, Colorado
	NATO Workshop on Evolutionarily Stable Strategies, Queen's University, Kingston, Ontario, Lecture
1983	XVIII International Ethological Conference, Brisbane, Australia, Symposium Lecture
	Nordic Council lecture series on the Evolution of Behavior, Department of Entomology, University of Uppsala,
1985	XIX International Ethological Congress, Toulouse, France, Plenary Lecture
1987	XX International Ethological Conference, Madison, WI, Lecture
1989	XXI International Ethological Conference Utrecht, The Netherlands, Symposium Lecture
1990	XI Int. Congress of the Int. Union for the Study of Social Insects, Bangalore, India, Symposium Lecture
1991	Visiting Women Scholar's Program, Queen's University, Kingston, Ontario, Lecture
	XII International Ethological Conference, Kyoto Japan, Lecture.
	Behavior of Marine Animals Symposium, Okinawa, Japan, Lecture.
1992	Nordic Council lecture series on Reproductive Behavior, Dept. Zoology, Helsinki, Finland.
	IV International Behavioral Ecology Society, Princeton, NJ. Lecture.
1993	Frontiers of Science, Department of Physics, University of Florida, Public Lecture
1994	The Walton Lecture, University of Virginia Biological Station, Mt. Lake, VA
	V International Behavioral Ecology Society, Nottingham, England, Invited Lecture
1995	XIV International Ethological Conference, Honolulu, Hawaii, Invited Lecture
1997	XV International Ethological Conference, Vienna, Austria, Invited Lecture
1998	Fellows Lecture, Animal Behavior Society Annual Meeting, Carbondale, IL (Plenary Lecture)
1998	Assoc. for the Study of Animal Behavior & Soc. Italian Ethology, Urbino, Italy (Plenary Lecture)
1999	J.T. Emlen Endowed Lectureship, April 1999. University of Wisconsin, Madison, WI
1999	International Ethological Conference, Bangalore, India (Plenary Lecture)
2001	International Ethological Conference, Tübingen, Germany (Symposium Lecture)
2001	XIX Congress of Brazilian Ethology, Juiz de Fora , Brazil (Plenary Lecture)
2003	International Ethological Conference, Florianopolis, Brazil (Symposium Lecture)
2004	European Conference on Behavioral Biology, Groningen, The Netherlands (Symposium Lecture)
2005	International Ethological Conference, Budapest, Hungary (Symposium Co-Organizer)

2005 Biological Bases of Behavior, Tlaxcala, Mexico (Lecture at international course)
2005 Estuarine Research Federation, Norfolk, VA (Symposium Lecture)
2007 International Symposium on the Science and Conservation of Horseshoe Crabs, Oakdale, NY (Symp. Lecture)
2007 International Society of Invertebrate Reproduction, Panama City, Panama (Symposium Lecture)
2012 Animal Behavior Society Annual Meeting, Albuquerque, NM (Symposium Lecture)
2013 International Ethological Conference and Association for the Study of Animal Behavior, Newcastle, UK (symp lec)
2013 Coastal and Estuarine Research Federation (CERF), San Diego, CA (Symposium Lecture)
2014 8th International Crustacean Congress, Frankfurt, Germany (Symposium Lecture)
2015 International Symposium on the Science and Conservation of Horseshoe crabs, Sasebo-City, Nagasaki, Japan
2016 Animal Behavior Society Presidential Symposium, Columbia, Missouri (Symposium Lecture)

LECTURES AND POSTERS AT SCIENTIFIC MEETINGS

1972, 1975-76, 1979-81, 1983-84, 1987, 1989, 1993-99,
2001, 2003, 2004, 2006, 2008, 2009 Animal Behavior Society Annual Meeting
1980, 1988 Soc. for the Study of Evolution Annual Meeting
2000, 2002, 2008 International Behavioral Ecology Society
1982-1996, 2012 Winter Animal Behav. Conf. (Invited)
2002 American Fisheries Society Meeting
2007 International Ethological Conference, Halifax, Nova Scotia
2009, 2010, 2011 UF Marine Biology Meeting, St. Augustine, FL
2009 International Ethological Conference, Rennes, France
2011 International Ethological Conference, Bloomington, Indiana
2014 International Society for Behavioral Ecology Conference, New York
2015 International Ethological Conference, Cairns, Australia

INVITED SEMINARS (since 1986)

1986	University of Kansas, Lawrence, KS	American Museum of Natural History, New York
	University of Toronto, Toronto, NY	Brown University, Providence, RI
1987	SUNY-Binghamton, Binghamton, NY	University of Florida, Gainesville, FL
1989	Utah State University, Logan, Utah	Ohio State University, Columbus, OH
1990	University of Colorado, Boulder, CO	
1991	Queen's University, Kingston, Ontario	
1992	University of Helsinki, Helsinki, Finland	University of Jyvaskyla, Jyvaskyla, Finland.
	Florida International University, Boca Raton	Purdue University, Department of Zoology
	University of Chicago, Chicago, IL	
1993	Cornell Univ., Ithaca, NY	Kansas State Univ., Manhattan, KS
	Horseshoe Crab Festival, Cape May, NJ (public lecture)	
1994	U.C. Berkeley, CA	U.C. Davis, CA
1997	Univ. SW Louisiana, Lafayette, LA	Coastal Carolina College, Conway, SC
1998	Univ. Florida, Veterinary Medicine	Univ. Florida, Department Zoology
2001	University of Missouri, St. Louis	
2003	University of Miami, Department of Biology	Florida Institute of Technology, Melbourne, FL
2004	University of Maryland	ISPA, Lisbon, Portugal
2005	UNAM, Tlaxcala, Mexico	
2007	University of Montana, Missoula, MT	
2010	Florida State University, Tallahassee, FL	
	Institute of Ecology, National University of Mexico (UNAM), Mexico City, Mexico	
2011	University of Florida, Gainesville, FL	University of Kentucky, Lexington, KY
2014	Evening Public Lecture Series, Whitney Marine Laboratory for Bioscience	

PROFESSIONAL SOCIETIES: OFFICES HELD AND CONFERENCES/ SYMPOSIA ORGANIZED

1979-1981 Animal Behavior Society, Policy Committee Member
1998-2004 Animal Behavior Society, Latin American Affairs Committee Member
1982-1988 Animal Behavior Society, Treasurer (elected 2 terms)
1982-1988 American Soc. of Zoologists, Animal Behavior Section, Treasurer
1989-1991 Animal Behavior Society, Second President-Elect (elected); First President-Elect
1991-1992, 1992-1993 Animal Behavior Society, President; Animal Behavior Society, Past-President
1988-1989 Co-organizer Winter Animal Behavior Conference

1984-1991	International Council of Ethologists (elected U.S. representative)
1985-1990	U.S International Council of Ethologists Committee, Secretary
1995-1998	Delegate to write Evolutionary Biology White Paper (Evolution, Science and Society)
1998-2003	American Institute of Biological Sciences, Secretary/Treasurer (elected twice)
1998-2002	Councilor, International Society of Behavioral Ecology (elected)
1995-1999	International Ethological Conference, Vice-Secretary General
1999-2003	International Ethological Conference, Secretary General
2003-2007	International Ethological Conference, Past Secretary General; Corresponding Secretary
2005-2007	International Symposium on the Science and Conservation of Horseshoe Crabs (organizing committee)
2009	Animal Behavior Society symposium co-organizer and moderator
2009	International Ethological Conference symposium co-organizer and moderator
2012-present	Retired Faculty, University of Florida, Treasurer (elected 3X)
2013-2016	Commission Member IUCN SSC, Horseshoe crab Specialist Group

MAJOR PUBLIC OUTREACH (Broader Impacts)

1991-1995	Advisory Board, PBS series, "Natural History of the Senses". WETA, Washington, D.C.
2002, '04, '05, '06, ’08, 2010-2015	Green Eggs and Sand (workshop on Horseshoe crabs for middle-school teachers). Delaware, New Jersey and Maryland Divisions of Fish and Wildlife, Delaware Science Institute
2005, ’06, ’07, ’08, ‘10, '11, ’12, ’13, ’14, ‘15	Green Eggs and Sand workshop (3-day workshop for FL and GA middle school and high school teachers), Marine Institute, Savannah, GA
2008	Green Eggs and Sand workshop (3-day workshop for NY middle and high school teachers), Cornell University Extension Marine Camp, Peconic, NY
2006	Georgia Outdoors, Georgia Public Radio, "Georgia's Beaches" (major segment on horseshoe crabs)
2009-2011	Green Eggs and Sand curriculum revision team (in collaboration with teachers and State of MD, DE and NJ science educators)
2012	Green Eggs and Sand workshop (3-day workshop for MA teachers), Mass. Aud. Society, Wellfleet, MA
2012-2013	Board of Directors for ERDG; database entered in horseshoecrab.org
2013	Green Eggs & Sand Workshop, Boothbay Harbor, Maine (3-day workshop)
2013	Public Lecture at Cedar Key Library, Kathleen Tuck Memorial installation
2013	Public Lecture, Whitney Marine Lab, St. Augustine, FL
2013	Public Lecture, Marine Discovery Center, New Smyrna Beach, FL
2014	Green Eggs & Sand Workshop, Milford, Connecticut (3-day workshop)
2014	Institute of Learning in Retirement, Gainesville, FL, Lecture on horseshoe crab management
2015	Public Lecture, St. Petersburg College, St. Petersburg, FL

GRANT REVIEW PANELS AND EVALUATION COMMITTEES (since 1986)

1987, 1990, 1992	National Science Foundation N.A.T.O. Post-Doctoral Fellowships Panel
1991	National Research Council, Nat. Acad. of Sci. NSF Graduate Research Fellowships
1988	National Institutes of Mental Health Review Panel, Basic Behavioral Processes
1987-1992	Smithsonian Tropical Research Institute, Professional Evaluation and Review Committee
1988	Swedish National Research Council Review Panel, Invertebrate Zoology
1988	University of Chicago, Department of Evolution and Ecology, External Review Committee
1992	Nebraska EPSCoR Committee
1994	Swedish National Research Council Expert Committee, Evolutionary Ecology
1995-1999	Princeton University, Department of Ecology and Evolutionary Biology; Advisory Committee
1998	University of Virginia, Department of Biology, Program Review
1998	National Science Foundation Panel Member, IGERT
1998	National Science Foundation, Committee of Visitors, Animal Behavior Panel
1998	Atlantic States Marine Fisheries Commiss., Horseshoe Crab Management Plan, Peer Review Panel
2000	National Science Foundation, NEON Panel, Archbold Biological Station
2000, 2003	National Science Foundation, Animal Behavior Panel
2001	Oregon State University, Department of Zoology, External Review Committee
2003	Atlantic States Marine Fisheries Comm., Shorebird Management Plan, Peer Review Panel, Stone Harbor, NJ
2005	North Carolina State University, Department of Zoology, External Review Committee
2003-08	External Advisory Board, Montana PACE (NSF ADVANCE award)
2008	Purdue University, Department of Biology, External Review Committee
2009	Animal Behavior Society Student Grants Committee

2011	University of Oklahoma, Department of Zoology, External Program Review
2012	Simon Fraser University, Vancouver, B.C., External Examiner
2013	University of Western Australia, Perth, W.A., External Examiner
2014	Baker Fund Grant review, Ohio University, Miami, OH

MANUSCRIPT and GRANT REVIEWS

2012: PLOS One, PNAS, Animal Behaviour, Behavioral Ecology (2), Journal of Marine Biology, NSF
2013: PNAS, Animal Behaviour (4), Behavioral Ecology (2), Proc. Roy Soc. B, Biology Letters, PLOS One, Biol. Bull., NSF (2), Journal of Animal Ecol.,
2014. Animal Behaviour (2), Marine Biology, Behavioral Processes, Behavioral Ecology and Sociobiology, Proceedings of the National Academy of Sciences, Journal of Animal Ecology, Biological Bulletin, Behavioral Ecology, NSF (2)
2015. Estuaries and Coasts, Horseshoe Crab Conservation book (3), Animal Behaviour, NSF(2), Aquatic Biology, New Jersey Sea Grant,

TENURE AND PROMOTION REVIEWS

2014: UT Chattanooga, University of Manitoba, University of Georgia, University of Florida

MAJOR SERVICE TO COLLEGE OF LIBERAL ARTS AND SCIENCES and UNIVERSITY (since 2000)

2000-'03; 2006-11	University Senate; Faculty Senate (elected)
2007-2010	Senate Infrastructure Council (elected)
2008-2010	Chair, Senate Infrastructure Council and member of the Senate Steering Committee
2003-8	University of Florida Marine Laboratory at Seahorse Key Task Force Chair; SHK Advisory
1999-2001	Women's Studies and Gender Research Executive Board
2000-2002; 2004-2007	Facilities Planning and Land Use Committee
2000	Psychology Department Chair Search Committee
2000	University of Florida Presidential Search Committee (SAC)
2001	Associate Dean Search Committee, CLAS
2002	Town / Gown Task Force on Neighborhoods (Co-chair) University Senate Committee
2004-2005	UF Master Plan Steering Committee
2005-2008	Graduate Council
2006-2009	CLAS Finance Committee (elected)
2008-2010	Chair, Faculty Senate Infrastructure Council Member, ITAC-AT Committee
2008-2010	Member, Faculty Senate Steering Committee
2006-2011	Chair, United Faculty of Florida Grievance Team

COURSES TAUGHT

Introductory Zoology Winter 1977; Ecology Seminar (graduate) Winter 1977
Animal Behavior Spring 1977-present (each spring; 4 credit course with laboratory)
Evolution Fall 1979-81, 1987, 1993, 1994
Princeton University, Visiting Research Biologist: Animal Behavior Seminar (graduate) Fall 1985; Tropical Biology (Costa Rica field trip) Spring 1986
Behavioral Ecology: Fall 1995, 1999
Animal Behavior Seminar (graduate) 1977, 1979-81, 1983, 1989, 1991, 1995, 1997-2002, 2006, 2009, 2010, 2011
Ethology (graduate; 4 credit course with laboratory): Fall 1982-1996; 2002, 2005
Veterinary Ethology: Spring 1997, 1998
Integrative Principles Graduate Course: Fall 2006, 2007, 2008 (one 4-week module)
Biological Perspectives on Contemporary Social Issues (honors introductory level): Fall 2006.
Theory and Practice in Biology: Fall 2007, 2008, 2010
Animal Behavior Seminar (graduate) with St. Mary: spring 2012, 2013, 2015

POST-DOCTORAL SCIENTISTS SUPERVISED

Sheri Johnson (PhD 2007 from University of Maine; Post-doc UF 2007-2010). 2015-present, Assistant Professor, Department of Zoology, University of Otago, Dunedin, New Zealand

Mary Hart (PhD 2011 from University of Kentucky). 2014-present, adjunct assistant professor, Dept. Biology, University of Florida.

GRADUATE STUDENTS SUPERVISED

Jeff Lucas (PhD 1983). Dissertation: Feeding behavior and life history strategies of antlions. NATO post-doctoral fellow, Oxford, England, 1983-1984. Assistant Professor, College of William and Mary, 1984-1986 (temporary). Univ. of California at Redlands, Assistant Professor, 1986-1987. Purdue University, Assistant Professor 1987-1994, Associate Professor 1994-2001. Professor 2001-present.

Glenn Goodfriend (PhD 1983). Dissertation: Feeding behavior and the evolution of clinal variation in Jamaican land snails. Weizmann Institution of Science Post-doctoral Fellow, Rehovot, Israel, 1983-1994. Research Scientist, Smithsonian Institution, Washington, D.C. 1994-1995. Research Scientist, Geophysical Lab. Carnegie Institution of Washington, Washington, D.C. 1995-2002. Deceased 10/02

Steven Frank (MS 1983). Thesis: Sex ratios and reproductive behavior of Florida fig wasps. Univ. of Michigan Ph.D. 1987, Univ. of California-Irvine, Assistant Professor 1987-1993; Associate Professor 1994-1998; Professor 1998-present.

Martin Obin (MS 1983). Thesis: Territorial and marking behavior of a brood parasitic mud-daubing wasp. Assistant Professor, Tufts University Medical School, 1996-present.

Linda Fink (MS 1984, PhD 1989). Thesis: Maternal investment in the green lynx spider. Dissertation: Color polymorphism in sphingid caterpillars. Middlebury College. Assistant Professor, 1989-90. Sweet Briar College, Virginia, Assistant Professor, 1990-1995; Associate Professor 1995-2001; Professor and Chair 2000-2010. Duberg Professor of Biology 2010-present.

Giselle Mora-Bourgeois (MS 1987, PhD 1991). Thesis: Mating and nesting behavior of a tropical harvestman with paternal care. Dissertation: Site-based mating system in a tropical harvestman. Assistant Professor, University of Costa Rica, San Jose, 1992-1998; Associate Professor 1998-2002; National Park Service Center for Urban Ecology, Science Education Coordinator, Washington, D.C.

Karen Masters (MS 1989). Thesis: The adaptive significance of female-biased sex ratios in the neotropical treehopper *Umbonia ataliba*. Princeton University, Ph.D. 2000. Conducting research for Centro Científico Tropical (CCT), Monteverde, Costa Rica on the interactions between plants and insects.

Sharoni Shafir (MS 1991). Thesis: Honeybees foraging on artificial flowers - intransitivity of preferences and energetics. Stanford University Ph.D. 1994. Post-doctoral Fellowship, University of Ohio, Columbus, OH. 1995-1998; Lecturer 1998-2004, Associate Professor 2004-2010, Professor 2010-present, Department of Entomology, Hebrew University of Jerusalem and Director of the Triwaks Bee Research Center.

Richard Buchholz (MS 1989; Ph.D. 1994). MS Thesis: Singing behavior and ornamentation in the yellow-knobbed curassow (*Crax daubentoni*). Ph.D. Dissertation: Adaptive Functions of Fleshy Ornamentation in Wild Turkeys and Related Birds. University of Florida, Visiting Assistant Professor, 1994. University of Northeastern Louisiana, Monroe, LA Assistant Professor (tenure-track) 1995-1999; University of Mississippi, Oxford, MS. Assistant Professor (tenure-track) 1999-2003; Associate Professor 2003-present.

Bonnie Ploger (PhD 1992) Dissertation: Proximate and ultimate causes of brood reduction in brown pelicans (*Pelecanus_ occidentalis*). 1993-94, Visiting Assistant Professor, Grinnel College, Grinnel, Iowa; N.A.T.O post-doctoral fellowship, Queen's University Kingston, Ontario 1994-95. Hamline College, St. Paul, MN Assistant Professor, 1995-2001; Associate Professor 2001-2009; Professor 2009-present

Dustin Penn (MS 1992) Thesis: Nesting behavior of horseshoe crabs. University of Utah, Ph.D. 1993. Univ. Utah Post-doctoral Fellowship, 1997-2002. Director, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria. 2002-present.

Laurie Eberhardt (PhD 1994). Dissertation: Sap Feeding and its Consequences for Reproductive Success and Communication in Yellow-bellied Sapsuckers. Assistant Professor, Valparaiso University, Valparaiso, IN 1994-present (tenure-track); Associate Professor 2001-present.

Ron Clouse (MS 1994). Thesis: Nesting Decisions of the Social Paper Wasp *Myschocyttarus mexicanus.* Agriculture Department Chair, PATS, Pohnpei, FM. 1994-1995. Archbold Biological Station, Res. Sci. 1996. Res. Sci., Museum of the Everglades 1996-97. Economic Research, NYC (1998-2004). Ph.D., Harvard University 2004-2010. Post-doctoral research associate, American Museum, NY (2010-2013); post-doctoral fellow, Dept. Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, NC

Jose-Luis Osorno (PhD 1996) Dissertation: Male mate desertion in the magnificent frigate-bird. Research Associate, Instituto de Ecologia, Universidad National Autonoma Mexico, 1996-2004. Deceased October 2004.

Susan Chien (MS) 1994-2000. Paternity in the Pipe-organ Mud-daubing wasp. (did not complete thesis).

Cynthia Filgate (Hassler) (MS 1999) Thesis: Satellite male groups in horseshoe crabs (*Limulus polyphemus*): how and why are males choosing females? Computer firm in Austin, TX, 1999-2006. Senior Database Developer, QuickArrow, Inc., Austin, TX (2006-present).

Kavita Isvaran (PhD 2003) Dissertation: Mate choice in blackbuck. Gardiner Post-doctoral Fellowship from Cambridge University, UK. (Post-doc with T. Clutton-Brock, Department of Zoology) (2003-2007). Tenured Assistant Professor, Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560012 , India (2007-present).

Suhel Quader (PhD 2003) Dissertation: Mate choice in the baya weaverbird. Marie Curie European Union Post-doctoral Fellowship., Cambridge, UK (Post-doc in Department of Zoology, Cambridge University with N. Davies) 2003-2005; British Society for the Protection of Birds 2005-06. Scientist, National Centre for Biological Sciences, Bangalore, India. (2006-present)

Manuel Vélez (M.S 1999; Ph.D 2004) Thesis: Parental care strategies in the Central American cichlid *Aequidens coeruleopunctatus*: tradeoffs between present and future reproduction. Dissertation: Temporal variation in natural and sexual selection of calling behavior in the field cricket, *Gryllus rubens*. Boston University Law School (2003-2006). Law clerk for federal district court judge in the District of Puerto Rico (2006-2007); Intellectual property Associate Lawyer with Mayer Brown law firm, NY (2008-present).

Laura Sirot (MS 1999; PhD 2004) Thesis: Intersexual conflict and mating avoidance in the damselfly, *Ischnura ramburi*. PhD research: Sperm competition and mate conflict in *Diaprepes abbreviatus*, a weevil pest of citrus. NIH NRSA Post-doctoral Fellowship with Mariana Wolfner, Cornell University (2004-2010). Tenure-track Assistant Professor, College of Wooster, Wooster, OH (2010-present).

Billy Gunnels (Co-advisor with St. Mary) (PhD 2006) 2000-2006. Alternative strategies and nesting decisions in *Myschocyttarus mexicanus*, a primitively eusocial wasp. Teaching Post-doc, University of Florida (2006-2007); Assistant Professor, (2007-2014). Associate Professor, Florida Gulf Coast University, Dept. Biological Sciences (2014-present).

Dimitri Blondel (Co-advisor with Phelps) (MS 2006) Social behavior of Costa Rican singing mice. Ph.D. program UF (2006-2012); post-doctoral fellow, Department of Biology, Duke University (2012-2014)

Rachel Schwab (Co-advisor with St. Mary) (MS 2006) Thesis: Mating group formation in the horseshoe crab (*Limulus polyphemus*): are satellite males ideal-free distributed among females of variable fecundity and how do they assess fecundity? Naturalist, Amelia Island Plantation (2006-2007); graduate student College of Education, UF (2007-2009); teaching school in Jupiter, FL (2009-present).

Hope Klug (PhD 2007) 2006-2007. Filial cannibalism in gobies and flagfish. NSF International Post-doctoral Fellowship, University of Helsinki with Hanna Kokko (2008-2010). NSF post-doctoral fellowship with Dr. Suzanne Alonzo, Yale University (2010-2011); Tenure-track assistant professor, University of Tennessee at Chattanooga (2011-present).

Katharine Saunders (M.S. 2010) 2008-2010. Chemical cues are used by male horseshoe crabs, *Limulus polyphemus*, to locate and select mates. Field assistant, Farallon Islands 2011-2012; Copy editor, PLoSOne 2012-2013; Wildlife Technician, Bird Research Northwest, Burns, OR. (2013)

Clare Rittschof (PhD 2011). Environmental heterogeneity and phenotypic variation: the evolution of male body size in a golden orb-web spider. NSF Post-doctoral fellow, Department of Entomology and Carl R. Woese Institute for Genomic Biology, Dr. Gene Robinson, University of Illinois (2011-2014). Post-doctoral Associate, Department of Entomology, Pennsylvania State University, State College, PA (014-2015). Tenure-track Assistant Professor, Department of Entomology, University of Kentucky, Lexington, KY (2016-present).

Matthew D. Smith (PhD 2012). Evolution of body size and life-history patterns in horseshoe crabs. 2012-2013 Adjunct Lecturer, Dept of Biology, University of Florida, HHMI post-doctoral fellow with Dr. Martin Cohn, College of Medicine, UF

Daniel Sasson (PhD 2015). Evolution of sperm traits within and between populations. Post-doctoral Fellowship with Dr. Joe Ryan at the Whitney Laboratory for Marine Bioscience, St. Augustine, Florida.

Member of Committee (since 2007)

-Biology-

Charlotte Skov (PhD 2007)
Wendy Schelsky (PhD 2010)
Ondi Crino (MS 2008)
Samantha Hilber (PhD 2013)
Dimitri Blondel (PhD 2012)
Gustavo Londono (PhD 2013)
Alex Jahn (PhD 2013)
Ariel Zimmerman (MS 2010)
Scarlett Tudor (PhD)
Christina Vasquez (PhD 2014)
Joe Pfaller (PhD)
Patrick Norby (MS)

Other than biology -

Frank Bouchard (Ph.D. 2010, Genetics)
Evan Adams (MS 2007, Wildlife)
Julie Marischal (PhD 2009, Psychology)
Katherine Saulsgiver (PhD 2009, Psychology)
Erin McClure (PhD 2009, Psychology)
Mary Beth Manjerovic (PhD 2010, Biology Univ Central Florida)
Ashley King (MS 2009, Biology, University of Montana)
Rachelle Yankelevitz (PhD 2009, Psychology)
Edward Atkinson (PhD 2011, Entomology)
Monique Udell (PhD 2011, Psychology)
Frank Bouchard (PhD 2012, Genetics)
Natalie Williams (MS 2012, Wildlife)
Erik Blosser (PhD 2014, UF Entomology in Vero Beach)
Wendy Helmey-Hartman (PhD 2014, Entomology)
Erica Feuerbacher (MS 2011, PhD 2014, Psychology)
Mary Hart (Outside examiner, Ph.D. Bio. 2011, Univ.KY)
Lindsay Mehrkam (MS 2013, Ph.D. 2015, Psychology)
Alexandra Protopopova (MS 2012, Ph.D. 2015, Psychology
María Cristina Carrasquilla (PhD 2014, Entomol. in Vero Beach)
Nathaniel J. Hall (PhD 2015, Psychology)
Vanessa Simões Dias (PhD, Entomology)

EDITED BOOKS

2003 Shuster, C.S., R. B. Barlow and H. J. Brockmann. (eds.) The American Horseshoe Crab. Harvard University Press. Cambridge, MA. 427 pp.

2008 Oliveira, R., M. Taborsky and H.J.Brockmann (eds.) Alternative Reproductive Tactics – An Integrative Approach. Cambridge University Press. 507 pp.

PUBLICATIONS (peer reviewed journals or book chapters) *graduate students under my direction; ‡ post-doctoral students

1973 Brockmann, H. J. The function of poster-coloration in the beaugregory, *Eupomacentrus leucostictus* (Pisces: Pomacentridae). Zeitschrift für Tierpsychologie, 33: 13-34.

1975 Bayer, L. J. and H. J. Brockmann. Curculionidae and Chrysomelidae found in aquatic habitats in Wisconsin. Great Lakes Entomologist, 8: 219-226.

1976 Brockmann, H. J. and J. P. Hailman. Fish cleaning symbiosis: Notes on juvenile angelfishes (*Pomacanthus*, Chaetodontidae) and comparisons with other species. Zeitschrift für Tierpsychologie, 42: 129-138.

1978 Terborgh, J., J. Faaborg and H. J. Brockmann. Island colonization by lesser Antillean birds. Auk, 95: 59-72.

1979 Brockmann, H. J. and C. J. Barnard. Kleptoparasitism in birds. Animal Behaviour, 27: 487-514.

1979 Brockmann, H. J. Nest-site selection in the great golden digger wasp, *Sphex ichneumoneus* L. (Sphecidae). Ecological Entomology, 4: 211-224.

1979 Brockmann, H. J., A. Grafen and R. Dawkins. Evolutionarily stable nesting strategy in a digger wasp. Journal of Theoretical Biology, 77: 473-496.

1979 Brockmann, H. J. and R. Dawkins. Joint nesting in a digger wasp as an evolutionarily stable preadaptation to social life. Behaviour, 71: 203-245.

1980 Brockmann, H. J. The control of nest depth in a digger wasp (*Sphex ichneumoneus* L.). Animal Behaviour, 28: 426-445.

1980 Brockmann, H. J. Diversity in the nesting behavior of mud-daubers (*Trypoxylon politum* Say; Sphecidae). Florida Entomologist, 63: 53-64.

1980 Dawkins, R. and H. J. Brockmann. Do digger wasps commit the Concorde fallacy? Animal Behaviour, 28: 892-896.

1980 Brockmann, H. J. House sparrows kleptoparasitize digger wasps. Wilson Bulletin, 92: 394-398.

1981 Lucas*, J. and H. J. Brockmann. Predatory interactions between ants and antlions. Journal Kansas Entomological Society, 54: 228-232.

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1983 Cohen*, J. A. and H. J. Brockmann. Breeding activity and mate selection in the horseshoe crab, *Limulus polyphemus*. Bulletin Marine Science, 33: 274-281.

1984 Brockmann, H. J. The evolution of insect sociality. In J. Krebs and N. Davies (eds.) Behavioral Ecology. Sinauer Associates, Second Edition, pp. 340-361.

1985 Brockmann, H. J. Tool using in wasps. Psyche 92:309-329.

1985 Brockmann, H. J. Provisioning behavior of the great golden digger wasp. Journal Kansas Entomological Society, 58:631-655.

1986 Brockmann, H. J. Decision making in a variable environment: lessons from insects. In L. Drickamer (ed.) Behavior and the Dynamics of Populations. Privat Publ., Toulouse, France, pp. 95-111.

1987 Seger, J. and H. J. Brockmann. What is bet-hedging? In P. Harvey and L. Partridge (eds.) Oxford Surveys in Evolutionary Biology. Oxford Univ. Press, 4:182-211.

1988 Brockmann, H. J. Father of the brood (Conflict and cooperation in a mud-daubing wasp). Natural History Magazine. 97(7):32-37.

1989 Brockmann, H. J. and A. Grafen. Mate conflict and male behavior in a solitary wasp, *Trypoxylon politum*. Animal Behaviour 37:232-255.

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1992 Brockmann, H.J. and D. Penn*. Male mating tactics in horseshoe crabs, *Limulus polyphemus*. Animal. Behaviour 44:653-665.

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1994 Brockmann, H.J., T. Coleson and W. Potts. Sperm competition in horseshoe crabs (*Limulus polyphemus*). Behavioral Ecology and Sociobiology 35: 153-160.

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1996 Brockmann, H. J. Satellite male groups in horseshoe crabs. *Limulus polyphemus*. Ethology 102: 1-21.

1996 Arnold, S. and H. J. Brockmann. Evolution of behavior. Approaches to studying behavioral change IN Foundations of Ethology, L. D. Houck and L. C. Drickamer (eds.). University of Chicago Press. pp. 673-682.

1996 Dyer, F. C. and H. J. Brockmann. Sensory processes, orientation and communication: Biology of the *Umwelt*. IN Foundations of Ethology, L. D. Houck and L. C. Drickamer (eds.). University of Chicago Press. pp. 529-538.

1997 Brockmann, H. J. Cooperative Breeding in Wasps and Vertebrates: the Role of Ecological Constraints. IN Evolution of Social Behaviour in Insects and Arachnids, J. Choe and B. Crespi (eds.). Cornell University Press. pp. 348-371.

1997 Brockmann, H.J. Xiphosurida. McGraw-Hill Yearbook of Science and Technology 1997. McGraw Hill, NY. pp. 491-493.

1999 Harari‡, A. R. and H. J. Brockmann. Male beetles attracted by females mounting. Nature 401:763.

2000 Harrari‡, A. and H.J. Brockmann and P.J. Landolt. Intrasexual mounting in the beetle *Diaprepes abbreviatus*. Proceedings of the Royal Society of London B 267: 2071-2079.

2000 Brockmann, H.J., C. Nguyen and W. Potts. Paternity in horseshoe crabs when spawning in multiple male groups. Animal Behaviour 60:837-849.

2001 Sirot*, L. and H.J. Brockmann. Costs of sexual interactions to females in Rambur's forktail damselfly, *Ischnura ramburi* (Zygoptera: Coenagrionidae). Animal Behaviour. 61:415-424.

2001 Brockmann, H.J. The evolution of alternative strategies and tactics. Advances in the Study of Behavior. 30:1-51.

2001 Hassler*, C. and H.J. Brockmann, H.J. Evidence for use of chemical cues by male horseshoe crabs when locating nesting females (*Limulus polyphemus*). Journal of Chemical Ecology. 27: 2319-2335.

2002 Brockmann, H.J. An experimental approach to altering mating tactics in male horseshoe crabs (*Limulus polyphemus*). Behavioral Ecology 13:232-238.

2003 Harari‡, A.R., P.J. Landolt , C.W. O'Brien, H.J. Brockmann. Prolonged guarding behavior and sperm competition in the weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). Behavioral Ecology 14: 89-96.

2003 Sirot*, LK and HJ Brockmann, C. Marinis and G. Muschett. Maintenance of a female-limited polymorphism in *Ischnura ramburi* (Zygoptera: Coenagrionidae): evaluation of hypotheses and new insights. Animal Behaviour 66:763-775.

2003 Brockmann, H.J. Nesting behavior: a shoreline phenomenon. IN The American Horseshoe Crab. Ed. By C.S. Shuster, R.B Barlow and H. J. Brockmann. Harvard University Press, Cambridge, MA. Pp. 33-49.

2003 Brockmann, H.J. Male competition and satellite behavior. IN The American Horseshoe Crab. Ed. By C.S. Shuster, R.B Barlow and H. J. Brockmann. Harvard University Press, Cambridge, MA. Pp. 50-82.

2004 Brockmann, H.J. Variable Life-History Patterns in the Pipe-Organ Mud-daubing Wasp, *Trypoxylon politum* (Hymenoptera: Sphecidae). Journal of the Kansas Entomological Society (invited memorial volume for Howard E. Evans) 77: 503-527.

2005 King, T.L., M.S. Eackles, A.P. Spidle and H.J. Brockmann. Regional differentiation and sex-biased dispersal among populations of horseshoe crabs (*Limulus polyphemus*). Transactions of the American Fisheries Society 134:441-465.

2006 Duffy, E.E., D. Penn*, M.L. Botton, H. J. Brockmannn and R.E. Loveland. Eye and clasper damage influence male mating tactics in the horseshoe crab, *Limulus polyphemus*. Journal of Ethology 24: 67-74.

2006 Vélez*, M. and H.J. Brockmann. Seasonal variation in selection on male calling song in the field Cricket, *Gryllus rubens*. Animal Behaviour 72:439-448.

2006 Veléz*, M. and H.J. Brockmann. Seasonal variation in female response to male calling song of the field cricket *Gryllus rubens*. Ethology, 112:1041-1049.

2007 Sirot*, L.K., H.J. Brockmann and S.L. Lapointe. 2007. Male postcopulatory reproductive success in the beetle, *Diaprepes abbreviatus*. Animal Behaviour 74:143-152.

2007 Schwab*, R., H.J. Brockmann. The role of visual and chemical cues in the mating decisions of satellite male horseshoe crabs (*Limulus polyphemus*). Animal Behaviour 74:837-846.

2008 Brockmann, H.J. Alternative reproductive tactics in insects. IN: Alternative Reproductive Tactics – An Integrative Approach. R.F. Oliveira, M. Taborsky and H.J. Brockmann (eds.). Cambridge University Press. Pp. 177-223.

2008 Brockmann, H.J and M. Taborsky. Alternative reproductive tactics and the evolution of alternative allocation phenotypes. IN: Alternative Reproductive Tactics – An Integrative Approach. R.F. Oliveira, M. Taborsky and H.J. Brockmann (eds.). Cambridge University Press. Pp. 25-51.

2008 Taborsky, M., R. F. Oliveira and H. J. Brockmann. The evolution of alternative mating tactics: concepts and questions. IN: Alternative Reproductive Tactics – An Integrative Approach. R.F. Oliveira, M. Taborsky and H.J. Brockmann (eds.). Cambridge University Press. Pp. 1-21

2008 Brockmann, H.J., R.F. Oliveira and M. Taborsky. Integrating mechanisms and function: prospects for future research. IN: Alternative Reproductive Tactics – An Integrative Approach. R.F. Oliveira, M. Taborsky and H.J. Brockmann (eds.). Cambridge University Press. Pp. 471-489.

2009 Brockmann, H.J. and M.D. Smith*. Reproductive competition and sexual selection in horseshoe crabs. IN: Biology and Conservation of Horseshoe Crabs. J. Tanacredi, M. Botton, and D. Smith (eds.). Springer Science. Pp. 199-221.

2009 Zaldívar-Rae, J., R.E. Sapién-Silva, M. Rosales-Raya, and H. J. Brockmann. 2009. American horseshoe crabs, *Limulus polyphemus*, in Mexico: open possibilities. IN: Biology and Conservation of Horseshoe Crabs. J. Tanacredi, M. Botton, and D. Smith (eds.). Springer Science. Pp. 97-113.

2010 Taborsky, M. and H.J. Brockmann. Alternative reproductive tactics and life history phenotypes. IN: Animal Behaviour: Evolution and Mechanisms. P. Kappeler (ed.). Springer Verlag Publ. pp. 537-586.

2010 Pereira, R*., J. Sivinski, P. Teal and H.J. Brockmann. 2010. Enhancing male sexual success in a lekking fly (A*nanstrepha suspensa* (Loew; Tephritidae) through a juvenile hormone analog has no effect on adult mortality. Journal of Insect Physiology, 56: 1552-1557.

2010 Johnson, S.L. and H.J. Brockmann. Costs of multiple mates: an experimental study in horseshoe crabs Animal Behaviour 80: 773-782. Chosen as featured article of the month Animal Behaviour 80:771.

2010 Saunders, K*. and H.J. Brockmann, W. Watson and S.J. Jury. Male horseshoe crabs, *Limulus polyphemus*, use multiple sensory cues to locate mates. Current Zoology, 56: 485-498.

2011 Brockmann, H.J. and S.L. Johnson. A long-term study of spawning activity in a Florida Gulf coast population of horseshoe crabs (*Limulus polyphemus*). Estuaries and Coasts. 34: 1049-1067. doi: 10.1007/s12237-011-9419-1

2012 Johnson, S.L. and H.J. Brockmann. Alternative reproductive tactics in female horseshoe crabs. Behavioral Ecology 23: 999-1008.

2012 Sasson, D.A., S.L. Johnson and H.J. Brockmann. The role of age on sperm traits in the American horseshoe crab, *Limulus polyphemus* Animal Behaviour 84: 975–981.

2013 Smith, M.D., H.E. Schrank and H.J. Brockmann. 2012. Measuring the costs of alternative reproductive tactics in horseshoe crabs, *Limulus polyphemus*. Animal Behaviour 85: 165-173. 10.1016/j.anbehav.2012.10.021.

2013 Johnson, S.L. and H.J. Brockmann. Parental effects on early development: testing for indirect benefits of polyandry. Behavioral Ecology 24: 1218-1228. DOI: **10.**1093/beheco/art056

2014 Nossa, C., P. Havlak, J-X Yue, K. Vincent, J. Lv, N. Lee, H.J. Brockmann and N. H. Putnam. Joint assembly and genetic mapping of the Atlantic horseshoe crab genome reveals ancient whole genome duplication. accepted GigaScience 3:1-21. doi:10.1186/2047-217X-3-9

2014 Smith, M.D. and H.J. Brockmann. The evolution and maintenance of sexual size dimorphism in horseshoe crabs: an evaluation of six functional hypotheses. Animal Behaviour 96:127-139.

2014 Vasquez, M.C., S.L. Johnson, H.J. Brockmann and D. Julian. Nest site selection minimizes environmental stressor exposure in the American horseshoe crab, *Limulus polyphemus*. Journal of Experimental Marine Biology and Ecology 463: 105-114. doi: 10.1016/j.jembe.2014.10.028

2015 Brockmann, H.J., T. Black and Tim L. King. Florida horseshoe crabs: Populations, genetics and the marine life harvest. IN R.H. Carmichael, Mark L. Botton, P.K.S. Shin and S.G. Cheung, editors. Changing Global Perspectives on Biology, Conservation and Management of Horseshoe Crabs. Springer Scientific (in press)

2015 Brockmann, H.J., S.L. Johnson, M.D. Smith and D. Sasson. Mating tactics of the American Horseshoe crab. IN R.H. Carmichael, Mark L. Botton, P.K.S. Shin and S.G. Cheung, editors. Changing Global Perspectives on Biology, Conservation and Management of Horseshoe Crabs. Springer Scientific (in press)

2015 King, T., M. Eackles, A.W. Aunins, H.J. Brockmann, E.M. Hallerman and B.B.Brown. Conservation genetics of the horseshoe crab (*Limulus polyphemus*): allelic diversity, zones of genetic discontinuity, and regional differentiation. IN R.H. Carmichael, Mark L. Botton, P.K.S. Shin and S.G. Cheung, editors. Changing Global Perspectives on Biology, Conservation and Management of Horseshoe Crabs. Springer Scientific (in press)

2015 Sasson, D., Johnson, S.L. and H.J. Brockmann. Sperm traits in a system with condition-dependent alternative reproductive tactics. Behavioral Ecology and Sociobiology (in press)

2015 Vasquez, M.C., A. Murillo, H.J. Brockmann and D. Julian. Multiple stressor interactions influence embryo development rate in the American horseshoe crab, *Limulus polyphemus*. Journal of Experimental Biology. (in press)

SUBMITTED AND IN PREPARATION

Brockmann, H.J., S.L. Johnson, D.A. Sasson and M.D. Smith. Seasonal variation in horseshoe crab spawning (Limulus polyphemus). Being revised

Sasson, D., Johnson, S.L. and H.J. Brockmann. The role of sexual selection on sperm trait variation across populations of the horseshoe crab, *Limulus polyphemus*. J. Evol. Biol.

Smith, M.D. and H.J. Brockmann. The interaction between temperature and season length predicts a rare non-linear ecogeographic cline in body size.

Keegan**, L., M.D.Smith* and H.J. Brockmann. Life-history patterns of juvenile horseshoe crabs from Florida.

Keegan**, L, S.L. Johnson and H.J. Brockmann. Juvenile behavior

Ponciano, J.M. and H.J. Brockmann. Spawning survey methods.

St. Mary, C. and H.J. Brockmann. Model of the horseshoe crab mating system.

Hart, M.K. and H.J. Brockmann. Mate choice in horseshoe crabs

THESIS AND DISSERTATION

1972 The function of poster-coloration in the Beau-Gregory, *Eupomacentrus leucostictus* (Pisces; Pomacentridae). (Jack P. Hailman - adviser)

1976 The control of nesting behavior in the great golden digger wasp, *Sphex ichneumoneus* (L.) (Hymenoptera, Sphecidae). Dissertation Abstracts International B, 37(5): 2705. (Jack P. Hailman-major professor)

ABSTRACTS

1983 Brockmann, H. J. Nest Sharing in Solitary Wasps: Mutualism or Parasitism? In Breed, M. D., C. D. Michener and H. E. Evans. The Biology of Social Insects. Westview Press, Boulder, CO. p. 180.

1990 Brockmann, H. J. Primitive eusociality: Comparisons between Hymenoptera and vertebrates. In Veeresh, G. K., B. Mallik and C. A. Viraktanuth, editors. Social Insects and the Environment. Proc. 11th. Int. Congress of IUSSI, 1990. Oxford & IBH Publ. Co., New Delhi.

1992 Brockmann, H.J. The effects of age on male behavior in *Limulus polyphemus*. Galaxea 11: 61-62.

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BOOK REVIEWS AND MISCELLANEOUS

1977 Brockmann, H. J. Review of: *Territory* by A. W. Stokes. Animal Behaviour, 25: 252-253.

1984 Brockmann, H. J. Review of: *The Evolution of Insect Mating Systems* by R. Thornhill and J. Alcock. Florida Entomologist 67:180-182.

1984 Brockmann, H. J. Review of: *The Biology of Social Insects* by M. Breed, C. Michener and H. Evans (eds.). Amer. Sci. 72:298.

1988 Brockmann, H. J. Review of: *The Natural History and Evolution of North American Beewolves* by H.E. Evans and K. O'Neill. Trends in Ecology & Evolution, 3: 313-314.

1988 Brockmann, H. J. Why I became a scientist. Amer. Scientist. (requested for 75th anniversary issue) 76: 456.

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1992 Brockmann, H. J. Review of: *The Social Biology of Wasps* by K. Ross and R. Matthews. Trends in Ecology and Evolution 7:140-141.

2001 Brockmann, H.J. 50 years of International Ethological Conferences. Advances in Ethology 36: I-II.

2006 Brockmann, H.J. Preface. Advances in the Study of Behavior 36: xi-xii.

2006 Brockmann, H.J. Review of: *The Evolution of Animal Communication* by W. Searcy and S. Nowicki. Bioscience 56: 849-851.

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CURRENT RESEARCH

I am an animal behaviorist interested in the evolution of alternative strategies and tactics and the economics and mechanisms of decision making. In particular, I am studying the evolution of mating and nesting behavior of horseshoe crabs. My current focus is on the evolution of multiple mating in female and group spawning by male horseshoe crabs. I am also interested in sexual selection, mate choice, and paternal care, the evolution of sex ratios, the evolution of life-history patterns and the evolution of social behavior (e.g. solitary and social wasps).

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EXHIBIT B

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Short communication

Physiological impacts of time in holding ponds, biomedical bleeding, and recovery on the Atlantic horseshoe crab, *Limulus polyphemus*

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ABSTRACT

Atlantic horseshoe crabs, *Limulus polyphemus* (HSC), are commercially harvested along the eastern U.S. coast and bled for hemolymph used in pharmaceutical safety testing. In South Carolina, some HSCs are held in outdoor ponds before transport to facilities where they are bled and then released to the wild. This study examined whether the time HSCs are held before bleeding, bleeding itself, or the duration of the recovery after bleeding affects HSC mortality and physiological condition. Female HSCs were collected from Coffin Point Beach, South Carolina (April 22–24, 2016), held in ponds for 2, 4, 6, or 8 weeks, then bled or held as controls. Body weights, hemocyanin concentrations, and hemocyte densities were measured prior to treatment (bled/control) and at 2, 6, and 12 days of recovery. Hemocyanin concentrations declined significantly in HSCs held in ponds for 8 weeks prior to bleeding and were excluded from further analyses. Compared to some studies, HSC mortalities were low (11%). Impacts of time in holding ponds, bleeding, and recovery from bleeding on physiological measures were assessed using 3-way fixed-effects ANOVA. While duration of recovery had main effects on physiological measures, significant interactions were also present. There was an interaction of treatment and recovery duration, with control crabs having higher hemocyte densities than bled animals at days 2 and 6 of recovery. There were two significant two-way interactions influencing hemocyanin concentration: pond time and recovery, and treatment and recovery. Our study suggests both main and synergistic effects are important when assessing the physiology and mortality of HSCs harvested for biomedical purposes.

The Atlantic horseshoe crab (HSC), *Limulus polyphemus*, is harvested and bled to produce *Limulus* Amebocyte Lysate (LAL), a cellular extract derived from the hemolymph used to screen pharmaceutical drugs and medical instruments for contamination by bacterial endotoxins. A 67% increase in biomedical harvest (2004–2017) and lethal harvest of HSCs for bait likely contributed to the recent declines documented for HSCs in parts of the United States (Eyler et al., 2018). The Atlantic States Marine Fisheries Commission (ASMFC) assumes a 15% mortality rate for biomedical bleeding in HSCs (Eyler et al., 2018), but estimates range from 8% (Walls and Berkson, 2003) to 30% or higher (Hurton and Berkson, 2006). Sublethal effects also have been associated with biomedical bleeding, and some evidence supports the idea that holding conditions between initial harvest and hemolymph extraction can be detrimental alone and in combination with bleeding (James-Pirri et al., 2012; Anderson et al., 2013; Smith et al., 2017).

The steps involved in the biomedical harvest of HSCs to produce LAL vary. Animals collected by licensed harvesters may be placed temporarily in ponds or immediately transported to extraction facilities. After bleeding, HSCs are often returned to the ocean. The South Carolina (SC) fishery is distinct in that HSCs can be held in ponds from 1 day to multiple weeks between mid-April and mid-June before biomedical bleeding. In SC, ponds used to hold HSCs must be aerated, mortalities must be reported, and all HSCs are required to be released by the end of the harvest season, per state-issued permits. It is well

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Abbreviations: ASMFC, Atlantic States Marine Fisheries Commission; HSC, horseshoe crab; LAL, *Limulus* Amebocyte Lysate; SC, South Carolina

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known, however, that changes in water quality (temperature, dissolved oxygen, salinity) can be harmful to marine organisms (reviewed by Burnett and Stickle, 2001; Fotedar and Evans, 2011) and HSCs in particular (Crabtree and Page, 1974; Hurton and Berkson, 2006; Coates et al., 2012).

Here, we examined whether the length of time HSCs are kept in outdoor holding ponds prior to bleeding, biomedical bleeding, and recovery after bleeding can separately or synergistically affect HSC physiological condition and mortality. The physiological condition of individual animals was monitored by measuring body weight, hemocyte density, and hemocyanin concentration in the hemolymph. These hemolymph variables have been used to monitor the physiological condition of many marine species, including HSCs (Coates et al., 2012; James-Pirri et al., 2012; Anderson et al., 2013; Kwan et al., 2014).

Between April 22 and 24, 2016, 101 female HSCs were hand-collected from Coffin Point Beach, SC (32.433802, −80.473438) and transported to the Waddell Mariculture Center in Bluffton, SC in open air trucks covered by tarpaulins. HSCs were placed randomly in three outdoor, aerated, open-bottom ponds (each approximately 1000 m^2) filled with unfiltered, natural seawater to approximately 1-m depth. These ponds simulated the environmental conditions of commercial HSC holding ponds. All HSCs were introduced into ponds at the same time and were divided between the three ponds (~34 HSCs per 1000 m^2). HSCs were not fed, and pond water was not changed, but continually aerated. Data loggers measured salinity, temperature, dissolved oxygen and pH in each pond every 15 min over the duration of the experiment (Table S1). Ponds were checked daily for mortality.

Every two weeks over an eight-week period, eight HSCs were selected randomly from each of the three experimental ponds ($n = 24$) and processed as described below. At each time point, the first animals to be processed were the biomedically bled treatment group. Four animals were removed from each of the three ponds and processed as a set; individual HSCs in each set were weighed and prosoma width was measured. Next, 1 mL of hemolymph was sampled from the large cardiac sinus for measurements of hemocyte density and hemocyanin concentration. To do this, the surface of the arthrodial membrane was disinfected with 10% betadine, a sterile 21-gauge needle was inserted through the arthrodial membrane, and 1 mL of hemolymph was drawn with a syringe. Following the 1 mL hemolymph sampling, HSCs in the biomedically bled treatment group were left exposed to air for 2 h to mimic the time out of water during transport to the bleeding facility. Afterwards, HSCs were biomedically bled simulating the commercial bleeding process used in SC. This was achieved by placing HSCs individually in a bleeding harness consisting of a board with a rubber strap used to stabilize and position each animal to expose its arthrodial membrane. The surface of the arthrodial membrane was disinfected with 10% betadine, and a sterile 14-gauge, 2-inch trocar needle was inserted through the arthrodial membrane into the cardiac sinus. The pressure in the cardiac sinus pushed hemolymph through the needle and into a beaker. Following industry standards, animals were bled for 8 min. To simulate aerial exposure during transport from the bleeding facility back to the ocean, HSCs in the treatment group were held out of water for 2 h after hemolymph extraction and then placed in the same recovery tank (described below) as the control crabs.

After the biomedically bled HSCs had been sampled and aerial exposure had begun, four animals were removed from each of the three ponds to constitute the unbled control group. Individual HSCs were weighed and prosoma width was measured. One millilitre of hemolymph was sampled from the large cardiac sinus with a syringe, as described above, for measurements of hemocyte density and hemocyanin concentration. These animals did not undergo a period of aerial exposure but were placed immediately in the recovery tank.

The recovery tank was a shaded outdoor 4.6-m circular tank (≈16,000 L) of aerated natural flow-through seawater under ambient temperature and salinity regimes. The recovery tank was monitored continuously for salinity, temperature, dissolved oxygen, and pH. In the recovery tank, however, unlike in the experimental ponds, HSCs were fed thawed shrimp once every other day to satiation. On recovery days 0, 2, 6, and 12, both treatment and control HSCs ($n = 24$ total) were weighed, and 1 mL of hemolymph was sampled as above and analyzed for hemocyte density and hemocyanin concentration. On recovery day 12 after hemolymph sampling, these 24 HSCs were returned to the ocean. This process was repeated every two weeks for a total time of eight weeks, until all HSCs were removed from experimental ponds (total bled $n = 48$; total control $n = 48$).

Variables (body weight, hemocyte density, hemocyanin concentration) were measured in all HSCs immediately after removal from experimental ponds and prior to treatment (day 0) and then after 2, 6, and 12 days of recovery. Each 1 mL hemolymph sample was divided into two aliquots. Total circulating hemocytes were enumerated by diluting 0.1 mL of hemolymph with 0.9 mL anticoagulant solution (1.74% NaCl, 100 mM glucose, 34 mM trisodium citrate, 26 mM citric acid monohydrate, 4 mM EDTA, pH 4.5; Jill Arnold, Directory of Laboratory Services, National Aquarium, Baltimore, Maryland, USA, pers. comm.) and then fixing the diluted sample with 0.1 mL 10% neutral buffered formalin. The hemocytes per volume of hemolymph were counted with a hemocytometer (Hausser Scientific; Mix and Sparks, 1980; $n = 3$ technical replicates) and hemocyte densities expressed as total hemocytes mL^{-1} hemolymph. Total hemocyte densities in HSCs at day 0 prior to biomedical bleeding ranged from about 7 to 21 × 10^6 mL^{-1}. These values are slightly lower but within an order of magnitude of values reported by others (Yeager and Tauber, 1935; Levin and Bang, 1968). Similarly, Coates et al. (2012) reported hemocyte densities of 26 to 46 × 10^6 hemocytes mL^{-1} for animals acclimated to 15 °C, but hemocyte densities declined from a mean of 29 ± 1.7 SD × 10^6 to 10 ± 1.4 SD × 10^6 hemocytes mL^{-1} hemolymph in animals shifted from 15 °C to 23 °C, overlapping values reported in the present study.

To quantify hemocyanin concentration, the second hemolymph aliquot was allowed to clot; the clot was homogenized and pelleted by centrifugation for 6 min at 600 ×g, 4 °C; supernatant was stored at 4 °C. A volume of hemolymph supernatant was diluted 1:100 in 10 mM EDTA, 2.5% NaCl (pH 10), and absorbance was measured at 338 nm on a UV-VIS spectrophotometer (Eppendorf; $n = 2$ technical replicates). Hemocyanin concentration was calculated using an extinction coefficient of 2.23 (Nickerson and Van Holde, 1971) for a 1 g 100 mL^{-1} dilution of HSC hemocyanin. In the present study, conducted at approximately 26 °C, hemocyanin concentrations of HSCs at day 0 prior to biomedical bleeding ranged from an average of 2.12 to 6.92 g 100 mL^{-1} (Table 1). Using the same assay method, Mangum (1976) measured generally higher mean hemocyanin values (9–12 g 100 mL^{-1}) in adult HSCs held at slightly cooler temperatures (22–24 °C), while Coates et al. (2012), using a different method, found higher average hemocyanin concentrations (8.0 ± 2.4 SD g 100 mL^{-1}) in smaller (~1.0 kg) HSCs held at 15 °C.

Differences in variables were tested across pond holding times using a one-way ANOVA on animals before they were biomedically bled; at this stage there was no difference in the way the animals in the bleeding treatment and the unbled control treatment were handled, so these data were combined and only the factor of time in the ponds was considered. Holding time did not significantly affect body weight ($P = .125$, ANOVA) or hemocyte densities ($H = 6.940$, $P = .079$, Kruskal-Wallis one-way ANOVA on ranks) (Table 1). These results for HSCs held at mean temperatures of 25.8–27.5 °C stand in contrast to Coates et al. (2012) who reported a 65–71% decline in hemocyte densities of HSCs held at 23 °C for eight weeks, with smaller but significant declines in animals held at colder temperatures over the same period. Their results after six weeks also showed significant reductions in hemocyte density, but only at 23 °C and not at the lower temperatures. This lower temperature of collection and acclimation compared with the present study may be an important difference. Furthermore, the declines in hemocyte densities observed by Coates et al. (2012) may be related to the high stocking density of HSCs employed in their study (i.e., ≤3 m^{-2})

Table 1

Hemocyte densities and hemocyanin concentration of female horseshoe crabs sampled at 0, 2, 6, and 12 days from the recovery tank, after having been held in experimental ponds for 2, 4, 6, or 8 weeks prior to biomedical bleeding (biomedically bled HSCs) or maintained as unbled controls. Horseshoe crabs that died before hemolymph sampling on recovery day 12 were excluded.

	Hemocyte Density (hemocytes × 10^6 mL^{-1} hemolymph)								Hemocyanin Concentration (g 100 mL^{-1})							
	Held in Ponds for 2 Weeks								Held in Ponds for 2 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	12.79	11.23	11.14	10.32	20.71	21.38	15.52	9.96	5.26	3.50	3.41	3.73	6.11	4.94	5.29	6.16
SE	1.42	2.41	2.01	2.87	3.11	3.07	3.33	2.37	0.92	0.51	0.58	0.56	0.77	0.79	0.86	0.99
n	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	Held in Ponds for 4 Weeks								Held in Ponds for 4 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	19.84	12.85	9.18	8.25	18.46	14.52	11.92	7.89	6.92	4.29	4.79	3.92	5.08	4.07	3.61	3.05
SE	2.48	2.78	1.85	2.79	3.50	3.10	1.64	2.82	1.02	0.67	0.76	0.63	0.72	0.61	0.52	0.46
n	12	12	12	12	11	11	11	11	12	12	12	12	11	11	11	11
	Held in Ponds for 6 Weeks								Held in Ponds for 6 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	17.77	8.95	8.87	9.88	13.60	13.94	13.43	10.74	5.44	3.79	2.99	3.75	5.49	4.95	3.90	4.54
SE	3.29	1.61	1.43	2.07	3.11	2.28	1.78	3.00	0.67	0.52	0.51	0.48	1.00	0.87	0.77	0.76
n	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
	Held in Ponds for 8 Weeks								Held in Ponds for 8 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	16.97	11.06	10.85	7.40	7.44	11.09	9.52	7.02	4.55	4.04	2.34	4.14	2.12	3.04	2.61	2.99
SE	1.99	2.80	1.52	1.12	1.16	1.70	1.55	1.41	0.91	0.63	0.57	0.64	0.52	0.51	0.43	0.53
n	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11

compared with the present study (≤ 0.034 m^{-2}).

Time held in ponds had a significant effect on hemocyanin concentration ($H = 12.862$, $P = .005$, Kruskal-Wallis one-way ANOVA on ranks) (Table 1); pairwise comparisons using Dunn's Method revealed significant differences between weeks 2 and 8 ($P = .020$), 4 and 8 ($P = .011$), and 6 and 8 ($P = .037$). Hemocyanin concentrations after 8 weeks were 55% to 61% of the values in weeks 2, 4, and 6. These observations were consistent with the findings of Coates et al. (2012), who showed that HSC hemocyanin concentrations negatively correlated with holding time and temperature, with greatest declines of 69.3% and 65% in HSCs held at the warmest test temperature (23 °C) for 56 days. These results suggest that the physiological condition of HSCs deteriorated as a consequence of prolonged holding in captivity, especially at warmer water temperatures.

Since the physiological status of HSCs at week 8 was different from those at weeks 2, 4, and 6 before treatment began, week 8 animals were excluded from the analyses of the effects of the biomedical bleeding treatment and recovery. To examine the effects of the time HSCs were held in the ponds up to 6 weeks, biomedical bleeding, and time in the recovery tanks (0, 2, 6, and 12 days) on hemocyte densities, hemocyanin concentrations, and body weight, three-way fixed-effects ANOVA were performed, with recovery time treated as the repeated measure. Statistically significant three-way interactions ($P < .05$) were further assessed for two-way interactions using Bonferroni-adjusted alpha values ($P < .0125$). In the absence of a significant three-way interaction, data were assessed for simple two-way interactions and where present, simple main effects were tested in SPSS. Outliers were not included in statistical analyses. Statistical tests on hemocyanin concentrations were performed on raw data (as summarized in Table 1) and in the case of hemocyte densities, data were square root-transformed prior to analysis. To illustrate physiological change, hemocyte densities and hemocyanin concentrations of individual animals (Table 1) were normalized to their values on recovery day 0 (Fig. 1).

Hemocyte density declined during the recovery period ($P < .001$; Table 1; Fig. 1A), with higher values on day 2 and lower values on day 12. Neither holding time up to 6 weeks nor treatment had a significant effect on hemocyte density. There were no significant interactive effects of the three factors on hemocyte densities (($F_{6,\ 174}$) = 1.038, $P = .402$), however, there was a significant two-way interaction of treatment and days of recovery (($F_{3,174}$) = 3.168, $P = .026$). At all three pond holding times, controls had higher average hemocyte densities than bled animals at days 2 and 6 of recovery (Table 1). This pattern was not evident at 0 or 12 days of recovery, at which times unbled control animals from only one of the three pond holding times had lower average hemocyte densities than bled animals. All other interactions were not significant ($P > .05$).

Similar to hemocyte density, hemocyanin concentration declined over recovery days ($P < .001$; Table 1; Fig. 1B), but neither time in the experimental holding ponds up to 6 weeks nor treatment had a significant overall effect on hemocyanin concentration. There was no significant three-way interaction on hemocyanin concentration ($F_{4,122} = 1.353$, $P = .254$). There were, however, two significant two-way interactions that influenced hemocyanin concentration: holding time up to 6 weeks and days of recovery ($F_{4,\ 122} = 7.876$, $P < .001$), and treatment and days of recovery ($F_{2,\ 122} = 9.111$, $P < .001$). There were no significant simple main effects associated with either of the two-way interactions ($P > 0.05$). Compared to the present study, others have reported more substantial effects of biomedical bleeding on hemocyanin concentration. Anderson et al. (2013) found a rapid (one-week post-bleeding) and sustained decline (up to six weeks post-bleeding) in hemocyanin concentration of HSCs after biomedical bleeding compared to control animals under laboratory conditions. Additionally, no indication of hemolymph protein recovery has been observed at 17 days (James-Pirri et al., 2012) or even six weeks (Anderson et al., 2013) after biomedical bleeding when compared to control animals. Unlike the present study, however, previous studies did not use ponds to mimic pre-bleeding holding conditions and most conducted bleeding experiments using HSCs recently collected from the wild.

There was an overall, experiment-wide mortality rate of 11% ($n = 11$, of 101 crabs), with 5% mortality occurring in the ponds before and 6% mortality after the bleeding treatments. The same number of mortalities occurred among bled ($n = 2$) and control ($n = 2$) HSCs held in ponds for up to 6 weeks, with two additional mortalities among

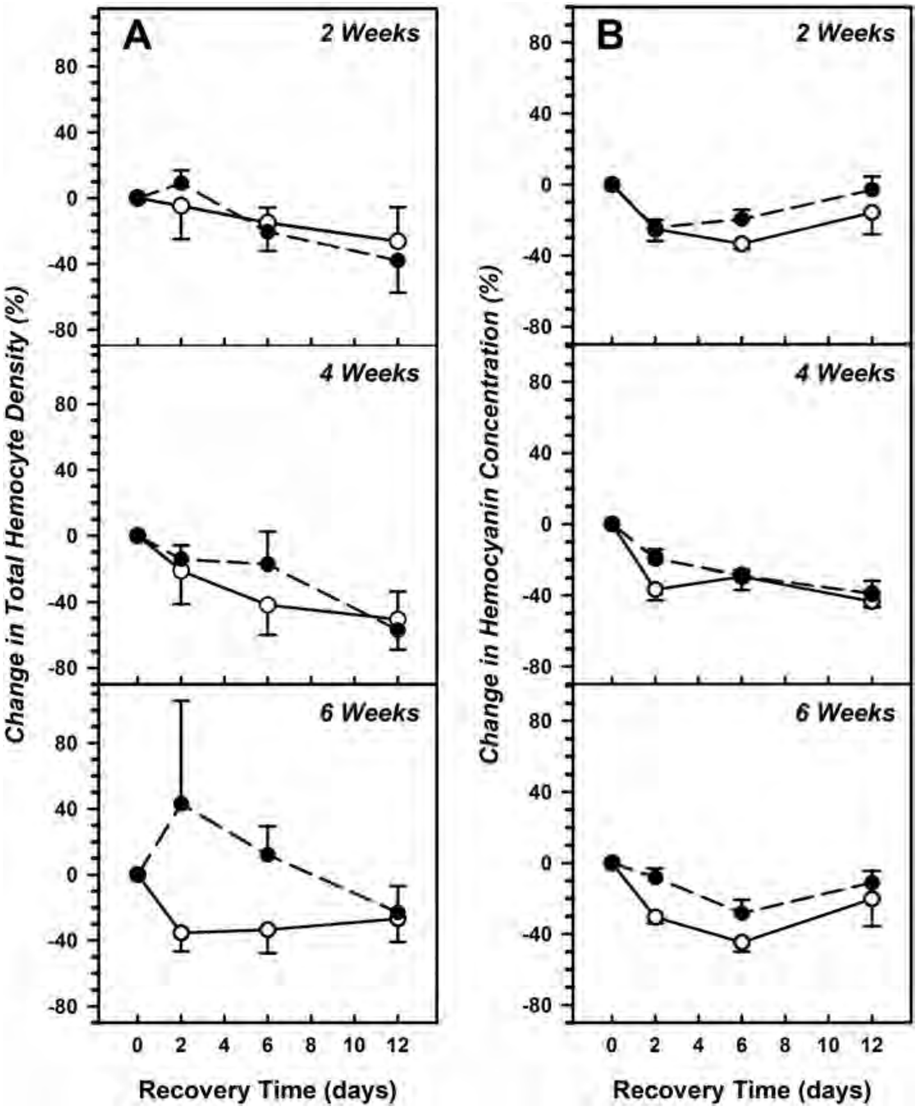

Fig. 1. Mean percent change (± SE) of hemocyte densities (A) and hemocyanin concentration (B) in biomedically bled (open circles, solid lines) and control (closed circles, dashed lines) female horseshoe crabs sampled at 0, 2, 6, and 12 days from the recovery tank, after being removed from the experimental ponds. Horseshoe crabs were held for 2, 4, or 6 weeks in experimental ponds prior to biomedical bleeding or maintained as unbled controls in May and June 2016. Horseshoe crabs that died before hemolymph sampling on recovery day 12 were excluded.

animals held for 8 weeks. The overall mortality rate was lower than the ASMFC's assumed mortality rate (15%) for HSCs undergoing biomedical bleeding (Eyler et al., 2018). Although the present study is unique in that it incorporates holding ponds representative of industry prebleeding conditions, this mortality rate was still well-within the range of rates (~8–30%) found in other studies of biomedical bleeding (Walls and Berkson, 2003; Hurton and Berkson, 2006; Leschen and Correia, 2010).

The initial body weights of HSCs held in ponds for 2, 4, and 6 weeks ranged from 2.35 to 4.65 kg ($\overline{x}$ = 3.30, $n = 70$, SE = 0.066). Body weight was positively correlated with prosoma width (linear regression: log body weight (kg) = −6.296 + (2.75 × log prosoma width (mm)), $r^2 = 0.78$; $n = 70$); Fig. S1). Body weight was analyzed using a three-way fixed effects ANOVA with recovery time treated as a repeated measure. There was a main effect of recovery day on body weight ($P < .001$), but there was no significant three-way interaction of the test factors on body weight. Pond holding time and days of recovery had a significant two-way interaction (($F_{1,54}$) = 2.641, $P = .018$). This analysis supported the observed decrease in the average body weight of animals (controls and bled) with longer pond holding times at each recovery day tested. Average body weights for animals held in ponds for 2, 4, and 6 weeks respectively were 3.33, 3.24, and 3.20 kg at day 0 of recovery, 3.29, 3.21, and 3.21 kg at day 2 of recovery, 3.32, 3.25, and 3.19 kg at day 6 of recovery, and 3.32, 3.25, and 3.19 kg at day 12 of recovery. These differences were small however, and all pair-wise interactions between pond holding time and day of recovery were not statistically significant.

There was a positive relationship between HSC body weight and the volume of hemolymph biomedically extracted for 8 min, with heavier animals yielding more hemolymph. This held true when HSCs that died after bleeding were included in the analysis (linear regression: hemolymph volume extracted (g) = −70.007 + (0.104 × body weight (kg)); $r^2 = 0.34$, $F_{1,33} = 17$, $P < .001$) and when they were not included (linear regression: hemolymph volume extracted (g) = −94.794 + (0.1092 × body weight (kg)); $r^2 = 0.40$, $F_{1,31} = 20.35$, $P < .001$). The total volume of hemolymph in an HSC is approximately 25% of its body weight (Hurton et al., 2005; i.e., a 3000 g HSC would have 750 mL hemolymph). Total hemolymph volumes were calculated based on body weight (Hurton et al., 2005), and volumes extracted were converted to percentages of the calculated total hemolymph volumes. The average volume of hemolymph extracted from individual HSCs was 263.2 mL (32% of the total hemolymph volume), ranging from 18.6 to 437.1 mL (3–51% of total hemolymph volume). Novitsky (1984) previously estimated that 30% of HSC blood volume could be safely extracted during the biomedical bleeding process, while Hurton and Berkson (2006) found that mortalities increased with extraction volumes up to 40% of total hemolymph. Indeed, in this study the two bled HSCs that died had 47.2% and 50.9% of their total hemolymph volume extracted. Our results indicate that as much as half of an individual's total hemolymph volume might be extracted during a standardized eight-minute biomedical bleeding, a substantial loss of hemolymph during an already stressful process (Leschen and Correia, 2010; James-Pirri et al., 2012). Current protocols for biomedical bleeding, however, do not measure the total or extracted volume of hemolymph in individual animals.

To our knowledge, this is the first published study to explicitly incorporate holding ponds into mortality and physiological assessments of HSCs used for biomedical bleeding. Hemocyte densities and hemocyanin concentrations of HSCs were not significantly affected by being held in ponds up to six weeks, but reduced physiological status (i.e., hemocyanin concentration) was documented at eight weeks. While duration of recovery had significant main effects on physiological measures, the interactive effects reported here indicate that synergistic impacts of the biomedical harvest on HSC health are important to consider. Overall, this study supports previous findings that HSC physiological status may be impacted by biomedical harvest.

Ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.110554.

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Measuring the costs of alternative reproductive tactics in horseshoe crabs, *Limulus polyphemus*

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Trade-offs are inherent to alternative reproductive tactics (ARTs), and identifying the costs and benefits of tactics is essential to understanding their evolution and maintenance within a population. Male horseshoe crabs exhibit two condition-dependent ARTs: males that are in better condition arrive on spawning beaches attached to a female, while males in poorer condition join spawning pairs as satellites and engage in sperm competition. Previous research has identified several benefits to the attached tactic, but the costs are less well understood. We examined a previously uninvestigated potential cost to the attached male tactic: nutritional stress caused by a restricted ability to feed. We found that field-caught attached males produced 57% less faeces in a 12 h period than satellite males, and had 2.5 times emptier digestive tracts than satellite males. We further examined this cost using stable isotopes because nutritionally stressed animals are predicted to have higher δ^{15}N levels. We found that field-caught attached males had higher δ^{15}N values than satellite males. However, higher δ^{15}N values could result from nutritional stress or from feeding on higher trophic levels. We tested this experimentally and found that starved animals had higher post-treatment δ^{15}N values compared to animals that were fed. Furthermore, the digestive tracts of field-caught attached males contained three times more sea grass (lower tropic levels have lower δ^{15}N values) than satellite males. These findings mean that the higher δ^{15}N values of field-caught attached males likely result from fasting rather than differences in diet. Taken together, our results indicate that a period of nutritional stress caused by reduced food consumption is a novel cost of the attached tactic. This study provides a key piece of information to explain why ARTs in horseshoe crabs take the form they do and provides a novel method for studying costs associated with ARTs in other species.

Alternative reproductive tactics (ARTs) by males are common in competitive mating systems (Taborsky et al. 2008). For example, in some anurans, ‘sneaker’ males sit silently near larger vocalizing males and either intercept females that are attracted to the callers, or wait to take over a resident male’s calling site or territory (Wells 1977a, b; Robertson 1986a, b). The evolution and maintenance of such discrete alternative phenotypes is puzzling because we generally expect that if one phenotype is only slightly less successful than the other, it would be eliminated by selection (Brockmann 2001). In some cases, ARTs are maintained as a genetic polymorphism, but in most cases they depend on the individual’s phenotype (e.g. body size, condition) and the circumstances in which they live (e.g. population density, sex ratio; Gross 1996; Zamudio & Chan 2008). In order for alternative tactics to be maintained at high frequencies, conditions must exist under which each tactic is more successful than the other (i.e. fitness curves must cross; Brockmann & Taborsky 2008). This means that trade-offs are inherent to ARTs. For example, an animal simply cannot simultaneously call and sneak, or maintain a territory and disperse widely in search of females. As a consequence, the phenotypes that maximize fitness for one tactic are different from those that maximize fitness for the other tactic. Therefore, the decision about which tactic to follow is based not only on an individual’s phenotype and the circumstances in which it lives, but also on the costs and benefits of the alternative tactics. Understanding the nature of the trade-offs for each tactic is vital to understanding why particular tactics take the form they do, and to understanding the evolution and maintenance of alternative mating tactics within populations (Brockmann et al. 2008). In this study, we examine the trade-offs associated with ARTs of male horseshoe crabs.

Horseshoe crabs have a highly competitive, explosive mating system (Brockmann 1990) in which males show two condition-dependent, alternative phenotypes (Brockmann & Penn 1992; Brockmann 2002). Younger males in better condition (based on visual inspection of the carapace) attach to females at sea and arrive on spawning beaches paired in amplexus with females. The attached

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male remains with the female until she has completed egg laying for the season, and then detaches and seeks another female. Unattached, older males in poorer condition roam the shoreline, join spawning pairs as satellites on the beach, and engage in sperm competition with attached males and other satellite males (Brockmann & Penn 1992; Brockmann et al. 1994; Brockmann 1996, 2002). These behavioural differences are not just a consequence of a male being unable to locate or hold onto a female, but instead result from an evolved decision rule based on age or condition (Brockmann 2002). That is, individuals maximize fitness by switching tactics at a given age or condition (i.e. fitness curves of the two tactics cross with condition).

Several trade-offs involving differences in paternity and righting behaviour have been identified for each tactic. During each 1-week spawning cycle (Cohen & Brockmann 1983; Barlow et al. 1986; Sekiguchi 1988; Smith et al. 2002), attached males normally mate with only one female, whereas satellite males may join several pairs. During each mating bout in a spawning cycle, satellite males have similar paternity success compared to attached males (Brockmann et al. 2000), but attached males do not always compete for paternity with satellites (depending on the number of unattached males present). In contrast, satellite males must always engage in sperm competition (Brockmann et al. 1994, 2000). Satellites appear at the beach to mate more often than attached males (Brockmann & Penn 1992), but they do not always find a mating pair. Lastly, horseshoe crabs are often overturned on the beach, leaving them vulnerable to desiccation and predation (Botton & Loveland 1989, 1993). When this happens, attached males are better able to right themselves than satellite males because righting ability is related to condition (Penn & Brockmann 1995). But also, the risk of coming ashore may be greater for unattached males because they may overturn more easily than attached males since they do not have a large female to act as an 'anchor' and stabilize them against wave action. Taken together, attached males have higher mating success overall (Brockmann et al. 2000) and are less likely to become stranded than unattached (satellite) males. However, if an age or condition threshold for switching tactics has evolved that maximizes fitness, then we would expect some compensating costs associated with the attached tactic. In this study we investigate a previously unexplored, potential cost to the attached male tactic: nutritional stress caused by a reduced ability to feed while attached to a female (Brockmann 2003).

Adult horseshoe crabs feed on a variety of items (e.g. bivalves, polychaetes, crustaceans) by digging into the substrate, stirring up sediment with their walking legs, and grasping food and directing it to their ventral mouth with their chelae and chelicerae. Gnathobases (leg bases) that surround the mouth macerate the food and also help to manipulate food into the mouth where it is then drawn into the esophagus (Manton 1964; Wyse & Dwyer 1973; Botton 1984; Botton et al. 2003). During the breeding season, attached males hold onto the posterior opisthosomal spines of the female using a modified pair of pedipalps. As a result of amplexus, the attached male's mouth is dorsal to and covered by the female's telson (Brockmann 2003); moreover, attached males cannot bury themselves in the substrate to feed in their normal manner. Thus, our first hypothesis is that the attached tactic inhibits feeding ('reduced feeding hypothesis'). In Florida, males typically remain attached for a 1-week spawning cycle (mean ± SD length of attachment is 3.7 ± 6.1 days; Brockmann & Penn 1992), but occasionally may stay attached up to 51 days (Brockmann 2003). In other populations, they can remain attached for much longer (Shuster 1954); for example, in New England, attached pairs have been observed overwintering together (Barlow et al. 1987; Moore 2004). If attaching to females inhibits feeding, then our second hypothesis is that fasting is costly; that is, it results in a period of nutritional stress for males adopting the attached tactic ('nutritional stress hypothesis'). Since attached males do not spend energy locating spawning pairs as satellite males do, and since attached males are 'carried' along by females (females are the ones exerting energy in locomotion and digging while spawning and during circatidal movements to and from the beach), it is possible that fasting is not nutritionally stressful for attached males.

Reduced Feeding Hypothesis

The first prediction of this hypothesis is that if the attached tactic inhibits feeding, then attached males will not defecate at all, or will produce less faecal material than satellite males (Table 1). We tested this prediction with a waste production experiment that compared the amount of faeces produced by attached and satellite males. Alternatively, if physical condition affects assimilation efficiency and subsequently the amount of faeces defecated, then differences in waste production may be due to the differences in condition between attached and satellite males. We tested this possibility by analysing the relationship between condition and waste production in both males and females. Additionally, our ability to detect differences in waste production assumes that food transit times (the latency for an indigestible marker to first appear in faeces; Karasov & del Rio 2007; Barboza et al. 2009) are the same for attached males and satellite males. Transit time can be influenced by the size of the animal's digestive tract, by the amount, type and quality of food intake (Barboza et al. 2009), and possibly by an animal's condition. Thus, differences in waste production between attached and satellite males may be due to differences in transit time. We tested this with an experiment by hand-feeding crabs with an indigestible marker to determine food passage time (Table 1). This experiment also allowed us to test a second

Table 1
Summary of the hypotheses, predictions and assumptions regarding a cost to feeding for the attached male reproductive tactic in horseshoe crabs, and the methods we used to test them

Hypothesis	Prediction	Method
Reduced feeding	(1) Attached males defecate less than satellite males	Measure waste production over 12 h in wild animals
	(a) Assumes condition does not influence defecation	Correlate condition and faeces in males and females
	(b) Assumes equal gut transit times	Measure gut transit times in hand-fed, wild animals
	(2) Attached males more motivated to feed than satellites	Measure food consumption during hand feeding
	(3) Attached males eat less food than satellite males	Examine gut fullness of wild animals
Nutritional stress	(4) Higher δ^{15}N values in attached males	Measure δ^{15}N values of faeces of wild animals
	(c) Assumes starved animals will have higher δ^{15}N	Experimentally starve animals, measure δ^{15}N of faeces
	(d) Assumes no differences in diet between tactics, or, if differences exist, attached males are feeding at a lower tropic level (lower δ^{15}N)	Examine gut contents of wild animals

The reduced feeding hypothesis addresses whether attached males eat less than satellite males; the nutritional stress hypothesis addresses whether this reduction in feeding is costly.

prediction of the reduced feeding hypothesis: if the attached tactic inhibits feeding, then attached males should be more motivated to eat than satellite males (Table 1). A third, more direct prediction is that if the attached tactic inhibits feeding, then the amount of food in the digestive tract should be lower for attached males compared to satellite males (Table 1). We tested this by examining the gut fullness of attached and satellite males.

Nutritional Stress Hypothesis

Nutritional stress due to a period of fasting (i.e. when feeding is forgone in favour of other activities; McCue 2010) or starvation (i.e. when feeding is prevented due to some extrinsic limitation; McCue 2010) can be inferred from stable isotope values of animal tissues (Hobson et al. 1993; Gannes et al. 1997, 1998; del Rio & Wolf 2005; Castillo & Hatch 2007; McCue 2007; McCue & Pollock 2008; del Rio et al. 2009). If an animal is in a negative energetic balance, ^{15}N is preferentially retained, while ^{14}N is excreted. As a result, $\delta^{15}N$ values increase in tissues over time as the animals 'feed on themselves' (McCue & Pollock 2008). Increased $\delta^{15}N$ values during fasting or starvation occurs in a wide variety of taxa and tissue types (reviewed in McCue & Pollock 2008), including in the excreta of lizards (Castillo & Hatch 2007) and rattlesnakes (McCue 2007). Thus, the first prediction of the nutritional stress hypothesis is that values of $\delta^{15}N$ from faeces of attached males will be higher compared to satellite males and females (Table 1). We tested this prediction by examining the stable isotope signatures of faeces from attached males, satellite males and females.

Alternatively, variation in $\delta^{15}N$ values can reflect differences in the trophic level at which animals are feeding: as animals feed at higher trophic levels, the value of $\delta^{15}N$ in their tissues increases (Deniro & Epstein 1981; Michener & Schell 1994). Hence, changes in $\delta^{15}N$ values may reflect either nutritional stress or differences in diet. Inferring that differences in $\delta^{15}N$ values are due to nutritional stress, rather than differences in diet, requires demonstrating that: (1) fasting or starving does indeed cause an increase in the $\delta^{15}N$ values of faeces in horseshoe crabs, and (2) any differences in diet between attached and satellite males cannot explain the differences in $\delta^{15}N$ values. We tested whether differences in $\delta^{15}N$ values were due to fasting in two ways. First, we conducted a 4-week experiment in which we starved some animals, but fed others. If differences in $\delta^{15}N$ values are due to nutritional stress rather than differences in diet, then $\delta^{15}N$ values should be higher in animals that were starved compared to those that were fed (Table 1). Second, we compared the gut contents of attached and satellite males. If gut contents analysis revealed that attached males fed at a higher tropic level than satellite males, then we would not be able to tell whether high $\delta^{15}N$ values were due to nutritional stress or diet. However, if gut content analysis revealed that diets of attached males were at a lower trophic level than those of satellite males, then high $\delta^{15}N$ values would be due to nutritional stress.

METHODS

We conducted this study during 2008–2011 at the University of Florida Seahorse Key Marine Laboratory. Seahorse Key is a 67-ha island that is part of the Cedar Keys National Wildlife Refuge along the northwestern Gulf coast of Florida. We collected adult horseshoe crabs as they initially came to the beach to spawn during evening high tides. Each animal was marked uniquely with a numbered thumb tack, placed immediately into a clean bucket, transported to the laboratory on the island, and placed into separate, randomly assigned holding tanks (one crab per tank). The holding tanks were 61 × 61 × 20 cm deep, and fed by a flow-through, running sea water system. For all animals used in our experiments, we measured their body size (maximum carapace width in cm) and assessed their condition using an index based on visual inspection of their carapace. Each individual was assigned a condition score based on (1) carapace colour, which darkens as the carapace erodes, (2) the amount of mucus present, which deters fouling organisms, and (3) the degree of pitting of the carapace, which is caused by chitinoclastic bacteria (modified from previous studies; for complete methods see Brockmann & Penn 1992; Brockmann 1996; Brockmann 2002). Each of the three criteria had a maximum score of 5 points, thus a maximum of 15 points was possible and represented the highest condition. Previous studies have shown that attached males are in better condition than satellite males, but there are no differences in carapace width (CW) between males that show different mating status (attached or satellite; Brockmann & Penn 1992; Brockmann et al. 1994; Brockmann 1996, 2002); these patterns were also supported for the animals used in this study.

Measuring Waste Production

During 4–17 October 2008 and 10–14 March 2009, we conducted an experiment to test whether attached males produced less faecal waste than satellite males (Table 1: prediction 1). Each experimental replicate consisted of three animals collected from the beach at the same time: a female, her attached male, and one satellite male associated with this pair ($N = 27$ replicates and 81 individual horseshoe crabs). In the running sea water system where we conducted this experiment, sea water is pumped into a large holding tank before entering the individual tanks, and water runs out of each individual tank through an overflow pipe. Hence, the relatively heavy packet of waste produced by horseshoe crabs did not flow out of the individual tanks. However, some debris did flow in, which could affect our measure of waste produced. So, in addition to the three tanks that each housed an attached male, a satellite male and a female, we added a fourth empty tank as a control for each replicate. We left all individuals in their individual holding tanks without food for 12 h before collecting waste (i.e. attached males and females were not coupled during the experiment). The average food passage time of horseshoe crabs was not known, and so we chose a 12 h time period (a priori) based on our best estimate of what it might be; this time period turned out to be reasonable because all animals had produced waste within 12 h. After the crabs had been returned to the ocean, we siphoned out all visible waste and debris from each holding tank through a fine-mesh plastic filter. We then rinsed off this plastic filter and collected the sample remaining on a piece of filter paper. Each sample was dried for 4 h in an oven at 60 °C and weighed (in g, minus the weight of the filter paper).

Body size was positively correlated with the amount of faeces produced (linear regression: $r^2 = 0.25$, $F_{1,78} = 26.0$, $P < 0.0001$). Therefore, we applied a size correction to our measure of waste production ((log waste − control)/log CW × 100) and compared the amount of waste (minus the amount of debris found in the control tanks) that was produced among the groups using paired *t* tests. If differences in waste production were due to the differences in physical condition between attached and satellite males (Table 1: assumption a), then we would expect a relationship between condition and the amount of faeces produced for both males and females. We analysed the influence of condition on the amount of faeces produced with two ANOVAs: one for males and a second for females.

Measuring Food Transit Time

We tested the assumption of equal transit times (Table 1: assumption b) by conducting an experiment during 28–30 March and 12–27 April 2010 that compared transit time between male tactics (females were also tested for comparison). We collected animals for this experiment ($N = 16$ for attached males, 15 for satellite males, and 14 for females), and then left them in the holding tanks for 12 h before the experiment began so that all had defecated prior to the start of the experiment. We cut a large, fresh shrimp into 10 equal pieces (1.07 ± 0.08 g) and soaked it for 10–30 min in a solution of 2.1 g carmine red dye (an inert digestion marker) and 1–2 ml of water. We fed each animal by taking it out of the holding tank, turning it ventral side up on a table and placing individual pieces of shrimp in its mouth, ad libitum for 20 min. We then checked the animals every 3 h until we observed the red dye in their faeces. We chose this 3 h interval based on observations from the waste production experiment.

We wanted to compare the transit time among our three groups, but we also wanted to know whether the amount of shrimp consumed or the animal's physical condition influenced transit time. Therefore, we conducted an ANCOVA with transit time as the response variable, status (attached, satellite or female) as the explanatory variable, and the amount of shrimp consumed and the crab's physical condition as covariates. Additionally, the reduced feeding hypothesis predicts that attached males will eat more than satellite males when given an opportunity to feed (Table 1: prediction 2). We compared the amount of shrimp eaten among the three groups with an ANOVA, and then conducted least squares means contrasts to identify specific differences between groups.

Stable Isotope Analysis of Faeces

To test whether values of $\delta^{15}N$ of faeces were higher for attached males compared to satellite males (Table 1: prediction 4), we collected faeces from the waste production experiment (spring 2008 samples only, $N = 19$ for each group). We removed any sand present in the samples, and then each faecal sample was ground to a homogenous, fine powder using a mortar and pestle. All samples were analysed by the Stable Isotope Mass Spectrometry Lab in the Department of Geological Sciences at the University of Florida to determine values of $\delta^{15}N$ (‰ normalized to air). We compared stable isotope values among the three groups with an ANOVA, and then conducted least squares means contrasts to identify specific differences between groups.

Effect of Starvation on Faecal Stable Isotope Values

We first tested whether differences in $\delta^{15}N$ values between attached and satellite males are due to nutritional stress (Table 1: assumption c) by conducting an experiment where we fed some crabs and starved others. On 27 March 2011, we collected 20 satellite males from Seahorse Key and brought them to the lab at the University of Florida in Gainesville. Crabs were kept on a 12:12 h light:dark cycle in a 1360-litre tank of filtered sea water with a salinity of 28–30 ‰, at room temperature (approximately 21 °C). Partial water changes were conducted every third day to alleviate nitrate build-up. We hand-fed all crabs a diet of freshly frozen bay scallops (*Chlamys patagonica*) that were purchased from a local grocery store (Publix brand). Every other day for 4 weeks, each crab was fed ad libitum for 15 min. After this acclimation period, we placed each crab in a 22-litre container with an oxygen bubbler for 48 h. We checked the containers every 3 h and collected any faeces that were produced. The faeces collected at this time were used to obtain the pretreatment $\delta^{15}N$ values. We then randomly chose 10 crabs to receive a feeding treatment, and another 10 crabs for a starving treatment. For animals in the feeding treatment, we continued the same feeding schedule as before. Animals in the starving treatment received no food, but were handled exactly as in the feeding treatment to simulate the feeding process. The experiment ran for 4 weeks, and on the last day we feed all crabs once ad libitum for 15 min. We then placed animals back into individual containers for 48 h and collected all faeces to obtain post-treatment $\delta^{15}N$ values. Each faecal sample was placed onto a coffee filter and dried for 4 h in an oven at 60 °C. The samples were then removed from the filter, ground to a homogenous powder, and analysed at the University of Florida Stable Isotope Mass Spectrometry Lab. All horseshoe crabs were then fed ad libitum for 1 week and returned to the beach from which they had been collected.

We conducted paired t tests on the feeding and starving groups separately to test whether the mean difference between pre- and post-treatment values was different from zero. We then conducted a matched-pairs analysis of grouped data with 'treatment' (feeding or starving) as a grouping variable. This analysis performs two F tests that evaluate whether the values across treatment groups differ: (1) the 'mean difference' tested whether the change across the pair of responses (pre- and post-treatment values) differed in the feeding and starving groups; (2) and the 'mean mean' tested whether the average response for a subject differed in the feeding and starving groups (SAS Institute 2007).

Gut Contents Analysis

The above experiments reflect our best efforts to measure the feeding habits of attached and satellite males without sacrificing animals. While these experiments can show support (or not) for the reduced feeding and nutritional stress hypotheses, they are indirect. Therefore, a measure of gut contents was needed to confirm the waste production experiment and to fully interpret the isotope results. We attempted to use a nonlethal lavage technique, but this failed. Thus, in order to measure directly what and how much males were eating, we decided to sacrifice a limited number of animals and examine their gut contents. We collected 10 attached males and 10 satellite males on 20 April 2011 while they were spawning on the evening tide. We collected these animals on the first day of that particular week-long spawning cycle to maximize the likelihood that attached males would have some food in their gut. This also allowed us to compare directly (and conservatively) the amount of food in the gut between attached and satellite males (Table 1: prediction 3). The animals were euthanized by immediately placing them in a freezer for 24 h (Botton & Ropes 1989). The euthanized crabs were fixed in 10% formalin for 4–5 days; we then dissected out the digestive tract and stored it in 90% ethanol for 2 weeks (Botton & Ropes 1989). We first cut open the digestive tract (esophagus, proventriculus, mid gut and hindgut) to estimate gut fullness (e.g. a score of 100% was assigned if the entire length and width of the gut was filled). Gut contents were then removed by hand and placed in vials with 90% ethanol (Botton & Ropes 1989).

During the removal of gut contents, we specifically separated sea grass that was found in the esophagus and proventriculus (but not the lower digestive tract) from other materials because it represents a low trophic-level food source. Therefore, differences in sea grass consumption between attached and satellite males may inform whether any differences in $\delta^{15}N$ values are the result of nutritional stress, or of feeding on different trophic levels (Table 1: assumption d). All sea grass was then dried in an oven at 60 °C for 4 h and weighed (mg). We used t tests to compare (1) gut fullness and (2) amount of grass in the esophagus and proventriculus between attached and satellite males.

To meet the assumptions of normality and homogeneity of variance, we log-transformed the values of (1) the amount of faeces defecated, (2) transit times, (3) weight of shrimp consumed and (4)

Table 2

Values of various measures for three groups of horseshoe crabs (attached males, satellite males, and females) collected from Seahorse Key, FL, U.S.A., in 2008–2010

	Attached males			Satellite males			Females		
	Mean±SD	*N*	95% CI	Mean±SD	*N*	95% CI	Mean±SD	*N*	95% CI
Carapace width (cm)	16.2±0.85	43	16–16.5	16.1±1.0	43	15.8–16.4	21.4±2.1	43	20.8–22.1
Defecation (g, dry weight) [*]	0.79±1.1	27	0.34–1.24	1.24±1.4	27	0.70–1.78	3.8±3	27	2.61–4.94
Shrimp consumed (g, wet weight)	0.48±0.3	16	0.31–0.65	0.44±0.3	16	0.28–0.59	0.29±0.2	16	0.16–0.42
Transit time (h)	18.9±9.1	16	14.0–24.0	17.5±6.7	15	13.8–21.2	22.8±10.7	14	16.3–29.0
Faeces δ^{15}N (‰)	5.0±1.3	19	4.4–5.6	4.2±1.1	19	3.7–4.7	4.0±1.6	19	3.3–4.8
Gut fullness (%)	12.4 ± 1.2	10	8.9–17.4	31.0±1.2	10	22.2–43.4	—	—	—
Foregut sea grass (mg)	9.4 ± 2.3	10	4.2–14.5	3.1±1.4	10	0–6.3	—	—	—
Median condition[†]	8	43	7–10[‡]	7	43	5–9[‡]	7	43	6–9[‡]

[*] Waste minus debris in control tanks. Control tanks had an average of 0.13 ± 0.3 g of material present (95% CI = 0.01–0.26).

[†] Based on carapace colour, amount of mucus present and degree of pitting of the carapace (5 points each, 15 points denotes best possible condition).

[‡] 25% and 75% quartiles.

sea grass weight in the gut prior to all analyses. All tests were two tailed, and all variation is reported as standard error, except where noted. Statistical tests were performed with JMP v.8 (SAS Institute, Inc., Cary, NC, U.S.A.), and all figures were created using Sigma Plot (SYSTAT Software 2008) and Adobe Illustrator (Adobe Systems 2007).

RESULTS

Waste Production

The mean amount of waste produced (controlled for body size) differed between the three groups (Table 2, Fig. 1). In support of prediction 1, satellite males produced 57% more waste than attached males (paired *t* test: $t_{25} = 2.7$, $P = 0.012$); females also produced more waste than either attached males ($t_{25} = 6.1$, $P < 0.0001$) or satellite males ($t_{25} = 5.4$, $P < 0.0001$). Condition was not related to the amount of faeces produced for males (ANOVA: $F_{7,\ 45} = 0.8$, $P = 0.569$) or females ($F_{7,\ 19} = 0.6$, $P = 0.722$), which verifies assumption a.

Transit Time

We found verification for assumption b: the whole model ANCOVA was not significant ($F_{10,\ 34} = 1.0$, $P = 0.440$), and transit time was not influenced by mating status ($F_2 = 1.3$, $P = 0.284$), carapace condition ($F_7 = 1.0$, $P = 0.421$), or the amount of food consumed ($F_1 = 0.2$, $P = 0.669$; Table 2, Fig. 1). In contrast to prediction 2, attached and satellite males did not differ in the amount of food eaten during the 20 min feeding period (contrasts: $F_{1,\ 45} = 0.2$, $P = 0.679$); however, females ate more (contrasts: $F_{1,\ 45} = 4.1$, $P = 0.048$) than both attached and satellite males (Table 2).

Stable Isotope Analysis of Faeces

In accordance with prediction 4, the mean δ^{15}N values for attached males were slightly higher than those for satellite males (difference = 0.82 ± 0.4‰; contrasts: $F_{1,\ 54} = 3.6$, $P = 0.063$) and females (difference = 0.99 ± 0.4‰; contrasts: $F_{2,\ 54} = 3.1$, $P = 0.024$; Table 2, Fig. 2). We found no difference between satellite males and females (difference = 0.18 ± 0.4‰; contrasts: $F_{1,54} = 0.2$, $P = 0.677$; Table 2, Fig. 2).

Experimental Starvation

The mean difference between pre- and post-treatment was not greater than zero for the fed group (mean = −0.54 ± 0.4‰; paired *t* test: $t_8 = -1.4$, $P = 0.208$), but was greater than zero for the starved group (mean = 1.06 ± 0.2‰; paired *t* test: $t_9 = 6.3$, $P = 0.0001$), confirming assumption c. Analysis of grouped data showed

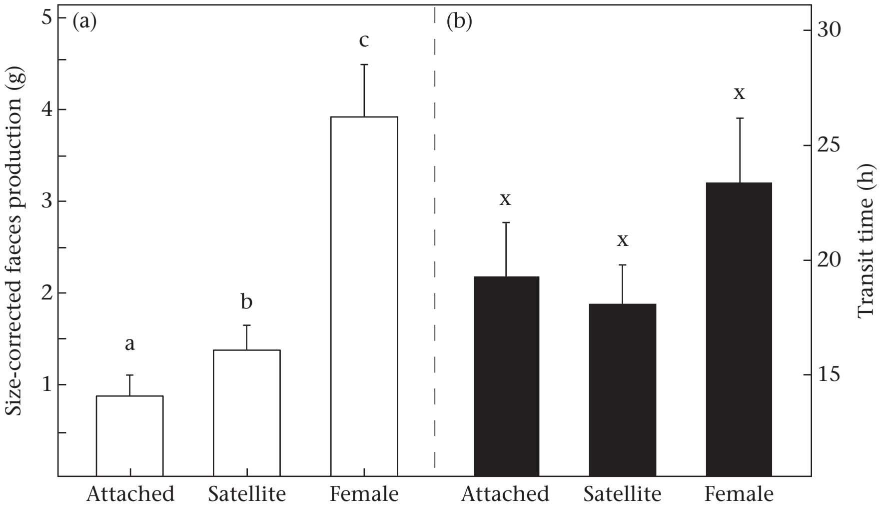

Figure 1. (a) Mean ± SE faecal mass produced in 12 h by wild-caught horseshoe crabs ($N = 19$/group), corrected for the amount of waste found in control tanks and the body size (carapace width, CW) of animals: ((waste − control)/CW × 100). (b) Mean ± SE transit time of food through the gut in horseshoe crabs that were experimentally fed carmine-red-dyed shrimp ($N = 16$ attached males, $N = 15$ satellite males, $N = 14$ females). All individuals from the three groups were collected from Seahorse Key, FL, U.S.A. Different letters above bars denote significant differences ($P = 0.05$) between groups based on least squares means contrasts.

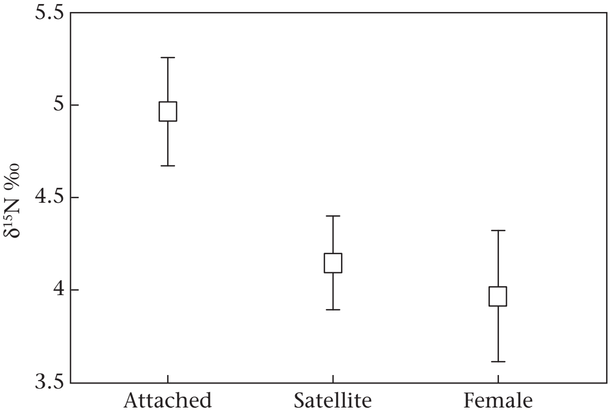

Figure 2. Mean ± SE values of $\delta^{15}N$ stable isotopes in faeces produced by three groups of horseshoe crabs ($N = 19$ each for each group) collected from Seahorse Key, FL, U.S.A. Values for attached males were higher than those of satellite males ($P = 0.063$) and females ($P = 0.024$).

differences in the response (pre- and post-treatment) of $\delta^{15}N$ values across the two treatment groups (fed and starved) for both the among-pairs 'mean mean' (matched pairs: $F = 9.1$, $P = 0.008$) and the within-pairs 'mean difference' (matched pairs: $F = 14.9$, $P = 0.001$; Fig. 3).

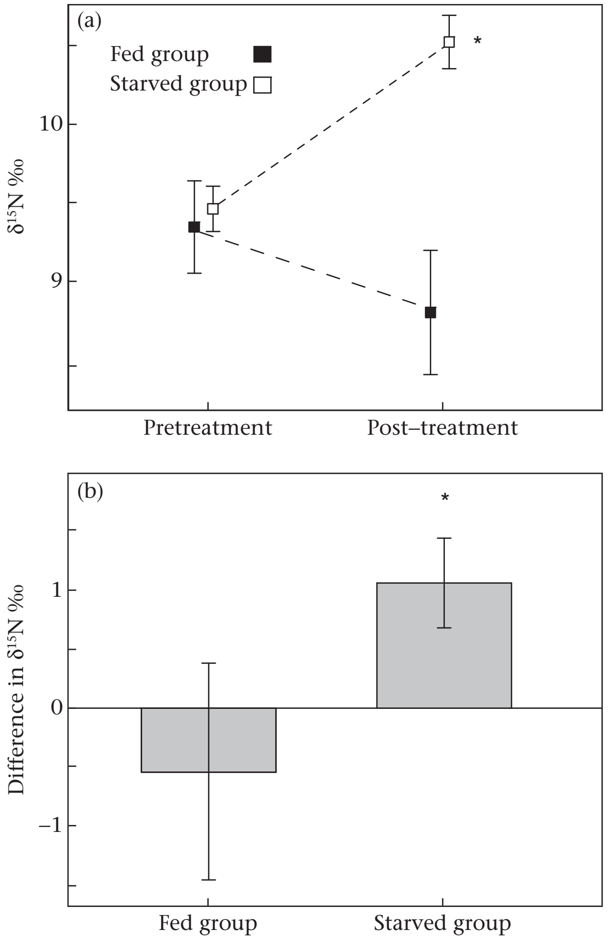

Figure 3. (a) Mean ± SE pre- and post-treatment values of $\delta^{15}N$ from two groups of satellite male horseshoe crabs: one group was starved for 4 weeks and the other group was fed scallops ad libitum for 4 weeks ($N = 10$ each). (b) Mean ± 95% CI difference in $\delta^{15}N$ values for the two experimental groups (post-treatment minus pretreatment). An asterisk indicates a significant difference ($P \leq 0.001$) between pre- and post-treatment values.

Gut Contents

In accordance with prediction 3, gut fullness (all contents) was 150% greater (t test: $t_{18} = 4.0$, $P = 0.0008$) for satellite males compared to attached males (Table 2, Fig. 4). Additionally, we found confirmation for assumption d: attached males had 200% more sea grass in the esophagus and proventriculus (t test: $t_{15} = 2.3$, $P = 0.035$) than did satellite males (Table 2, Fig. 4).

DISCUSSION

The maintenance of condition-dependent ARTs in a population depends on there being conditions under which each tactic is more successful (Gross 1996; Brockmann & Taborsky 2008). Until this study, the trade-offs for the attached tactic in male horseshoe crabs have not been obvious. Satellite males produced more faeces than attached males, had higher gut fullness and had slightly lower $\delta^{15}N$ values than attached males. Thus, our results support the hypothesis that advantages of the attached tactic come at a cost of reduced feeding and nutritional stress.

Food transit times (range 6–39 h) were well within the time that most males remain attached to females (mean ± SD = 3.7 ± 6.1 days; Brockmann & Penn 1992). Coupled with the fact that attached males produced some faeces during the waste production experiment, it appears that males and females probably feed while paired. However, attached males only produced approximately half as much waste on average as satellite males. In addition, there was no difference in transit time between attached and satellite males, and condition did not influence the amount of faeces produced. Thus, the results from the waste production experiment demonstrate support for the reduced feeding hypothesis. The gut content analysis showing that the guts of satellite males were 2.5 times fuller than those of attached males also strongly supports this hypothesis.

Lower faecal production and an emptier gut could have been due to attached males being less motivated to feed, as opposed to being due to the physical constraint of being attached. But, this appears to be unlikely because the amount of food eaten by attached and satellite males in the transit time experiment did not differ. In fact, the reduced feeding hypothesis makes the opposite prediction: that attached males should be more motivated to eat. We found no support for this prediction, although our measure of consumption might not accurately reflect motivation to feed due to the rather artificial feeding conditions. Alternatively, our results may have been due to satellite males eating more recently. For example, perhaps attached males do not feed at all after attaching, and that the waste we saw was what remained of their intake prior to attaching. Nevertheless, both possibilities suggest reduced food consumption for attached males in this population. Furthermore, we show that this reduced feeding is costly for males that are using the attached tactic, even though attached males may have lower energetic requirements than satellite males, because the female 'carries' them and because they are not required to spend energy locating multiple mating groups on each tide. Additionally, given the trade-off between food passage time and digestive efficiency (Penry 1993), males may slow their food passage time to increase assimilation efficiency when attached, thereby compensating for not eating as much as the satellite males. Our study was not designed to test this, but passage times did not differ for attached and satellite males, and evidence of increased $\delta^{15}N$ values for attached males refutes this hypothesis and demonstrates nutritional stress in attached males.

A model proposed by del Rio & Wolf (2005) predicts that $\delta^{15}N$ values will increase with fasting time. It seems paradoxical that $\delta^{15}N$ should increase in excreta of starving animals, even though ^{15}N is preferentially retained and ^{14}N is excreted. However, the increase in

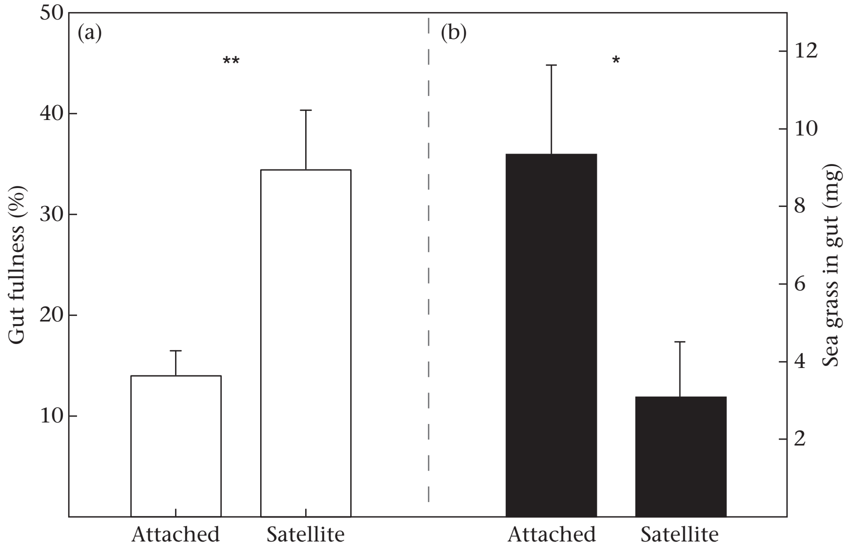

Figure 4. Mean ± SE (a) percentage of gut fullness and (b) mass of sea grass found in the gut of attached and satellite male horseshoe crabs ($N = 10$ each). Asterisks indicate significant differences ($^*P \leq 0.05$; $^{**}P \leq 0.001$) between attached and satellite males.

$\delta^{15}N$ values of excreta during starvation is thought to result from the breakdown of structural proteins (that tend to have higher $\delta^{15}N$ values) that progressively contribute to the pool of labile proteins (i.e. those most readily metabolized to nitrogenous waste), thus becoming the primary source of nitrogen in the excreta (Castillo & Hatch 2007; McCue 2007). Additionally, in horseshoe crabs, nitrogenous waste is converted to ammonia and dumped via their book gills and coxal gland (Towle et al. 1982) rather than in their faeces.

Our observations of horseshoe crabs showed that the faeces of attached males had $\delta^{15}N$ values that were 0.82 ± 0.4‰ higher than satellite males. While this difference was not statistically significant, the effect size was nearly 20% higher for attached males, and lack of significance may be due to our relatively small sample sizes. In order to attribute this difference to nutritional stress we first had to show that starvation produces an increase in $\delta^{15}N$ values in faeces. Experimentally, we found that a 4-week starvation increased $\delta^{15}N$ values by 1.06 ± 0.2‰ from the initial measurement, whereas the values did not change for animals that were fed. The degree of enrichment that we found observationally and experimentally is comparable to the results from other studies. For example, in quail that were fed a reduced food intake, blood $\delta^{15}N$ values increased by 0.8‰ compared to controls (Hobson et al. 1993); in lizards, uric acid $\delta^{15}N$ values increased by 2.2‰ after 14 days of starving (Castillo & Hatch 2007); in *Daphnia*, whole body tissue $\delta^{15}N$ values increased by 0.4‰ after 5 days of starving compared with controls (Adams & Sterner 2000); and in spider hatchlings, whole body tissue $\delta^{15}N$ values increased by 1.3‰ after 12 days of starving compared with initial values (Oelbermann & Scheu 2002).

We found little evidence that differences in $\delta^{15}N$ values between attached and satellite males reflects a difference in the trophic level at which they are feeding or a difference in their diet. Horseshoe crabs create a slurry of sediment and food when they feed (Botton et al. 2003), and it seems likely that attached males are able to grab food particles missed by the females. However, we found that attached males are less similar to females in $\delta^{15}N$ values than are satellite males. Because attached males are physically associated with females, we would have expected the opposite outcome. Perhaps attached males selectively feed on animal tissue (which has higher $\delta^{15}N$ values) rather than on plant matter and detritus (which is a typical food source that has low $\delta^{15}N$ values; Carmichael et al. 2004). If attached males ate at a higher trophic level (e.g. ate less organic material) than satellite males or females, it could explain why attached males had higher $\delta^{15}N$ values, and also why they differed more from females than from satellite males. However, we found the opposite pattern: attached males actually consumed three times more plant material than did satellite males. This result, along with the finding that experimental starving increased $\delta^{15}N$ values, indicates that the increase in $\delta^{15}N$ values of naturally occurring attached males are the result of a period of fasting, as opposed to differences in diet.

The pattern of more plant material in the diets of attached males may further explain how amplexus disrupts feeding. In addition to blocking the mouth and preventing attached males from burying into the substrate, amplexus may also interfere with the processing of food. Horseshoe crabs grab food items using terminal pincers located on their chelicerae, pedipalps and prosomal legs 2–4 (sensu Botton et al. 2003), which then direct the food towards the mouth for processing (Manton 1964; Wyse & Dwyer 1973; Botton 1984; Botton et al. 2003). Like many Chelicerata, horseshoe crabs use pairs of biting or chewing coxal gnathobases to process food (Manton 1964). Coxal feeding is performed by repeated transverse abduction and adduction of the proximal segments of the walking legs (i.e. gnathobases and coxa). Spines on each gnathobase are directed inward and serve to hold and macerate food. During the rhythmic movements of feeding, successive pairs of legs move out of phase (phase difference = 0.5 s), resulting in food being partially shredded and pushed towards the mouth (Manton 1964; Wyse & Dwyer 1973). Once drawn into the mouth, food passes through the esophagus into the proventriculus, which is a muscular organ that further fractures food into a pulp by muscle action (Botton et al. 2003).

Larger food items are processed by gnathobases of legs 3 and 4, which shred out a strand of tissue and draw it forward towards the mouth. Hard food items are gripped between the chilaria (highly reduced, spine-covered appendages) and are frequently held in position by extending the genital operculum 90° from the normally flat position. Once in position, hard food items are cracked by the 6th coxae before being passed to gnathobases of legs 2–4. In contrast, soft food is manipulated by limbs 1–4 and can be placed directly into the mouth (Manton 1964). Amplexus may inhibit the grasping of food with chelicerae (as they are in close contact with a female's opisthosoma when attached) or interfere with the rhythmic movements of coxal feeding that are needed to process larger, tougher food items, and may also prevent attached males from extending their genital operculum to hold harder food items in place. Thus, attached males may be restricted to feeding on soft food items that can be grasped by the pincers of their more posterior legs and placed directly in the mouth, and then macerated by the proventriculus. Sea grass may be an easily accessible food item that could fulfil such requirements.

Taken together, our results demonstrate that reduced consumption of food and a period of nutritional stress are costs of the attached tactic not previously considered. There is evidence that male horseshoe crabs in other populations remain attached longer than those in the Seahorse Key population used in our study (Shuster 1954; Barlow et al. 1987; Moore 2004), and that males in other horseshoe crab species are more firmly attached and remain attached for longer periods (Botton et al. 1996; Brockmann & Smith 2009), suggesting even greater costs of attaching in those populations and species.

In conclusion, this is one of the first studies to use stable isotopes to investigate a predicted period of nutritional stress in a natural population of animals. Moreover, this study furthers our understanding of the trade-offs in this system and provides a key piece of information that may potentially explain why these alternative tactics in horseshoe crabs take the form that they do. Our findings show that the satellite tactic has specific benefits in that these males are able to feed, whereas feeding is restricted for attached males. Low-energy alternative phenotypes or behaviours often evolve as a release from the energetic demands of 'preferred' phenotypes (Taborsky 1998; Widemo 1998; Cummings & Gelineau-Kattner 2009), and in some systems, a male's success for a given tactic may partially depend on energy reserves (McCauley et al. 2000). Therefore, older males in poorer condition may not be able to afford the cost of reproduction (i.e. periodic fasting) that accompanies being attached to a female during breeding. Consequently, the satellite tactic may allow males to maintain (or regain) a positive energy balance while still obtaining reproductive success. Investigating this hypothesis is the next step to fully understanding the evolution and maintenance of alternative tactics in horseshoe crabs. Finally, the feeding costs associated with reproduction that we found, and the use of stable isotopes techniques to measure such costs, has implications in other systems with similar ARTs (Wells 1977a, b; Robertson 1986a, b) and in systems with extended periods of mate guarding (Alberts et al. 1996; Sparkes et al. 1996; Saeki et al. 2005).

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Sub-lethal behavioral and physiological effects of the biomedical bleeding process on the American horseshoe crab, *Limulus polyphemus*

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Abstract

The hemolymph of the American horseshoe crab, *Limulus polyphemus*, is harvested from over 500,000 animals annually to produce Limulus Amebocyte Lysate, a medically important product used to detect pathogenic bacteria. Declining abundance of spawning *Limulus* females in heavily harvested regions suggests deleterious effects of this activity and, while mortality rates of the harvest process are known to be 10–30%, sub-lethal behavioral and physiological effects are not known. In this study, we determined the impact of the harvest process on locomotion and hemocyanin levels of 28 female horseshoe crabs. While mortality rates after bleeding (18%) were similar to previous studies, we found significant decreases in the linear and angular velocity of freely moving animals, as well as changes in their activity levels and expression of circatidal behavioral rhythms. Further, we found reductions in hemocyanin levels, which may alter immune function and cuticle integrity. These previously unrecognized behavioral and physiological deficits suggest that the harvest of Limulus Amebocyte Lysate may decrease female fitness, and thus may contribute to the current population decline.

Keywords

American horseshoe crabs; *Limulus polyphemus*; circadian; circatidal; LAL; bleeding; activity

Introduction

The American horseshoe crab, *Limulus polyphemus*, is valued for both its ecological and economic importance. Ecologically, *L. polyphemus* is a keystone species in marine ecosystems of the Atlantic and Gulf coasts of North America (Botton, 1984b), serving as a bioturbator (Krauter and Fegley, 1994; Lee, 2010), a food source for shorebirds, fish and crustaceans (Botton 1984a), and a predator of mollusks and polychaete worms (Botton, 1984b). Commercially, *L. polyphemus* is the preferred bait source for the whelk (*Buscyon carica*, *Buccinum undatum*, and *Busycotypus canaliculatus*; ASMFC, 2012) and eel (*Anguilla rostra* and *Anguilla Anguilla*: ASMFC, 1998) fisheries, and its hemolymph provides the raw material for Limulus Amebocyte Lysate (LAL), the industry standard for

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detection of bacterial endotoxin in pharmaceuticals, vaccines, and medical devices (Novitsky, 2009). The magnitude of the LAL harvest, principally composed of female horseshoe crabs (50–77%; Rutecki *et al*., 2004; ASFMC, 2012), has increased 76% since 2006, during which time New England populations of *L. polyphemus* have declined, despite a 45% bait harvest reduction (ASMFC, 2012). Further, population trends in heavily harvested Pleasant Bay, MA, have evoked concern over possible effects of the LAL harvest process (Malkoski, 2010; ASFMC, 2012; James-Pirri, 2012). In this region, where horseshoe crabs have been harvested for LAL production for over 30 years, but closed to the bait fishery since 2006 (Rutecki *et al*., 2004; Leschen and Correia, 2010), the proportion of females appearing at spawning beaches has declined from 30% (Carmichael *et al*., 2003) to 10% (Malkoski, 2010; James-Pirri, 2012) and egg abundances at spawning beaches have significantly decreased (James-Pirri, 2012). These trends have prompted researchers and environmental managers to suggest that the LAL harvest process, while causing moderate mortality rates of 8–15% in males (Walls and Berkson, 2003; Hurton and Berkson, 2006) and 10– 29% in females (Hurton and Berkson, 2006; Leschen and Correia, 2010), may also induce behavioral and physiological effects in *L. polyphemus*, which could lead to alterations in spawning activity (Malkoski, 2010; James-Pirri, 2012). Moreover, these effects could be exacerbated by the high level (50%) of the annual LAL harvest that occurs during the spawning season (Leschen and Correia, 2010).

The LAL harvest procedure incorporates multiple stressors, several of which have been shown to alter both the behavior and physiology of marine species. Briefly, hemolymph is obtained in a 24–72 h process that includes trawl or hand-harvest capture, transport to, and time spent in, containment at a biomedical facility, a 30% blood extraction, and return to the point of capture (ASMFC, 1998; Leschen and Correia, 2010). Interestingly, numerous crustaceans exhibit both transient and long-term (1–4 week) behavioral (Harris and Andrews, 2005; Parsons and Eggleston, 2005; Haupt *et al*., 2006) and physiological (Vermeer, 1987; Bergmann *et al*., 2001; Ridgway *et al*., 2006; Patterson *et al*., 2007) alterations in response to similar capture stressors, with effects including altered hemolymph biochemistry (Vermeer, 1987; Ridgway *et al*., 2006), reduced immune function (Ridgway *et al*., 2006), decreased predator avoidance behaviors (Brown and Caputi, 1983: Vermeer, 1987), altered responses to stimuli (Parsons and Eggleston, 2005), reduced locomotion (Davis *et al*., 1978), and diminished or altered spawning behaviors (Smith and Ritar, 2005). The LAL harvest process compounds typical capture through an extended (up to 72 h) period of aerial exposure and substantial blood loss, and thus has the potential to effect analogous behavioral and physiological changes in *L. polyphemus*.

Immediate and long-term behavioral responses of *L. polyphemus* to the biomedical bleeding process have been little studied. Both activity levels (Rudloe, 1983) and movement velocity (Kurz and James-Pirri, 2002) have been reported to be unaffected by a hemolymph extraction performed with a minimal (30 min to 3 h) amount of aerial exposure. Interestingly, a high-stress hemolymph extraction process, which includes 48 h of aerial exposure combined with thermal stress, causes the highest mortality (Hurton and Berkson, 2006), although neither activity nor velocity alterations in response to such a process have been investigated. In addition, a salient behavioral feature of *L. polyphemus* is the expression of tidal activity rhythms, driven by a circatidal clock (Chabot *et al*., 2004), which facilitates successful spawning and foraging activities in the wild (Cohen and Brockmann, 1983; Barlow *et al*., 1986; Watson and Chabot, 2010). In other species, disruptions of behavioral and physiological circadian rhythms occur in response to a variety of environmental stressors, including alterations in light intensities (*Procambarus clarkii* and *Procambarus digueti*; Fanjul-Moles *et al*., 1998), decreased water quality (*Astacus astacus*; Styrishave *et al*., 1995), and trawl capture (*Nephrops norvegicus*; Aguzzi *et al*., 2005), and disruptions in circatidal rhythms occur in response to osmotic (*Ruditapes philippanarum*; Kim *et al*., 2001) or thermal stress (*Chthamalus bisinuatus*; Kasten and Flores, 2013). However, to date, the ramifications of the high-stress biomedical bleeding process on behavior and physiology of horseshoe crabs have not been well characterized.

The effects of the harvest process on hemolymph properties also have not been clearly elucidated. While *L. polyphemus* regains its blood volume within three to 30 days after being bled (Rudloe, 1983; Novitsky, 1984), restitution of amebocytes takes up to four months (Novitsky, 1984), and the length of time required for recovery of additional hemolymph constituents is unclear. Capture stress alone decreases total hemolymph protein concentration in some crustaceans (Ridgway *et al*., 2006), and this decline is correlated with reduced immune system functioning and increased susceptibility to infection in lobsters, *Homarus americanus* (Theriault *et al*., 2008). The biomedical bleeding process combines capture stress with substantial hemolymph loss, and *L. polyphemus* exhibits significantly decreased hemolymph protein concentration for at least two weeks after extraction (James-Pirri *et al*., 2012). Over 90% of *L. polyphemus* hemolymph protein is the respiratory pigment hemocyanin (Ding *et al*., 2005). In addition to aiding in the circulation of oxygen, hemocyanin may also participate in the primary immune response (Coates *et al*., 2011), and cuticle hardening and wound repair (Adachi *et al*., 2005). Poor environmental conditions, including thermal and captivity stress, accelerate hemocyanin decline in *L. polyphemus* (Coates *et al*., 2012), and therefore the combination of all these stressors is likely to produce a more dramatic decline than either of them alone.

The overall goal of this investigation was to determine whether the biomedical bleeding process induces sub-lethal physiological and behavioral effects in female horseshoe crabs during their spawning season. Our specific aims were to evaluate: (1) overall activity, linear and angular velocities of their movements, and expression of tidal rhythms for two weeks before and four weeks after a 52 h bleeding process, and (2) hemocyanin concentrations immediately prior to, and six weeks after, a 52 h bleeding process. We evaluated these parameters in *L. polyphemus* from Great Bay, NH. While this population is genetically distinct from the harvested mid-Atlantic populations, the genetic distance is low (King *et al*., 2005); further, this population has not historically been harvested for biomedical bleeding (ASFMC, 2012), and so animals were presumably naïve to the process. We performed both laboratory studies, which allowed continuous monitoring of animal activities, and an outdoor study, which enabled evaluation of bleeding impacts in a quasi-natural environment. Our results suggest that *L. polyphemus* experiences sub-lethal behavioral (reduced activity, velocity of movements, and expression of circatidal rhythms) and physiological (chronic hemocyanin loss) alterations in response to the bleeding process, and these should be considered when assessing the impact of this procedure on horseshoe crab populations.

Materials and Methods

2.1 Animals- Treatment Groups and Conditions

Fifty-six female horseshoe crabs were collected during high tide at spawning beaches on Adams Point, Durham, New Hampshire, from May 15–23, 2012, and their prosomal width was measured. Animals were distributed by size into four experimental groups; this distribution by size was necessary because of constraints of experimental equipment. The largest 14 animals (prosomal width: 18–23 cm) were assigned to the outdoor unrestrained (OU) group, and they were placed in tanks outside of the University of New Hampshire Jackson Estuarine Laboratory (JEL). These outdoor tanks could accommodate larger animals than could our indoor laboratory tanks. The remaining 42 animals were transported by van to Plymouth State University (PSU; trip duration was 2 h) in polyurethane coolers. The smallest 14 animals (prosomal width: 16–18 cm) were assigned to the laboratory running wheel (LRW) group; the smallest animals were selected for this group because pre-constructed running wheels could only fit animals of prosomal width less than 18.5 cm. The remaining 28 animals were equally divided between the laboratory unrestrained (LU) and laboratory communal tank (LCT) groups. Because smaller animals were selected for the LRW group, prosomal width varied significantly (F(3,52) = 6.05, $P = 0.002$; Table 1) across the four groups, with prosomal width in the OU and LU groups greater than that of the LRW group ($P < 0.05$), while prosomal width in the LCT group was not significantly different from any of the other groups.

2.1.1 Outdoor Unrestrained Group—The purpose of the OU group ($n = 14$; prosomal width: 19.1 ± 0.4 cm, mean ± SEM) was to monitor activity in animals exposed to a natural photoperiod and constantly replenished estuarine water. Accelerometers (Onset Computer, Pocasset, MA), set to measure acceleration (g) in the three orthogonal axes, were affixed to the prosoma of each animal using cable ties, duct tape, and cyanoacrylate (Schaller *et al*., 2010). Animals were placed in separate cylindrical wire enclosures (70 cm diameter × 48 cm height) within seven 850-L tanks (183 cm × 92 cm × 50 cm) containing approximately 15 cm of sand so animals had the opportunity to bury. Water from the estuary continuously flowed (~4 L/min) through the tanks, keeping salinity and temperature consistent with that of Great Bay, NH, and the tanks remained uncovered and exposed to the natural light/dark cycle (approximate sunrise: 5:01–5:15 am; sunset: 8:00–8:28 pm). The animals were allowed access to 2% of their body weight in diced quahogs three times a week by placing the food in the bottom of the tanks. Activity was logged via the accelerometers for two weeks prior to the biomedical bleeding process.

2.1.2 Indoor Groups at Plymouth State University—To simulate the summer photoperiod, all indoor groups were maintained under a 14:10 light/dark (LD) cycle with instantaneous photic transitions, salinity between 25 and 30 psu, and temperature between 18–21°C. Temperature and lighting conditions were continuously recorded using Vernier Labquest handheld data collection units connected to a light sensor and temperature probe

(Vernier Software and Technology LCC, Beaverton, OR). Activity was measured in two separate laboratory groups via two different monitoring systems, enabling comparison between the two techniques for validation of results.

Laboratory Running Wheel Group: The activity of this group of 14 animals (prosomal width: 17.5 ± 0.8 cm) was monitored using "running wheels," constructed as described in Chabot *et al*. (2004, 2007). The animal was secured within the wheel with its telson sticking out through a slit. Then a polypropylene plastic golf ball was placed on the telson to prevent it from being drawn into the wheel. Cable ties were used to attach the front of the carapace to the frame. After all animals were prepared, the running wheels were distributed among four custom-made acrylic recirculating open top tanks (80 cm L × 65 cm W × 32 cm D). Wheel rotations were recorded with ClockLab Data Collection Software (Actimetrics, Wilmette, IL).

Laboratory Unrestrained Group: The activity of this group of 14 freely moving animals (prosomal width: 18.9 ± 0.3 cm) was monitored using video recording. The animals were distributed between two large (1.7 m L × .9 m W × .75 m D) tanks, each with a separate filter system. The tanks were subdivided by plastic egg grating (1 cm × 1 cm) into a total of eight arenas per tank (each 21 cm × 45 cm), and a 4 m length of waterproof red LED Ribbon Flex strip lighting ($\lambda = 630–660$ nm; LED Liquidators, Inc., Westlake Village, CA) was threaded through the egg grating in each tank to provide continuous illumination for video recording. One animal was placed into each of seven arenas per tank (one arena in each tank was excluded to house the filtration system). An infrared video camera was suspended 1 m above the tanks, and digital video recordings were obtained at a rate of one frame per 20 s using Gawker software (Piwonka, Seattle, WA). The videos were then analyzed for total distance moved, linear velocity, and angular velocity using Ethovision XT software (Noldus Information Technology Inc., Wageningen, Netherlands).

Laboratory Communal Tank Group: The 14 animals in this group were kept in one acrylic recirculating open top tank (80 cm L × 65 cm W × 32 cm D). Activity of this group was not measured; hemocyanin concentrations in control and bled horseshoe crabs of the LCT group ($n = 14$; prosomal width: 18.4 ± 0.4 cm) were measured from 1–2 mL blood samples taken each week, using the "Hemolymph Sampling" process detailed below.

2.2 Biomedical Bleeding Procedure

After collecting behavioral data for two weeks, 28 animals were bled using a process that approximated the standard biomedical bleeding procedure (high stress: Hurton and Berkson, 2006).

2.2.1 Laboratory Groups—The bleeding process for these groups was completed from June 1–3, 2012. Half of the horseshoe crabs from each of the LRW, LCT, and LU groups ($n = 7$ each) were randomly selected to undergo the bleeding process and distributed among three 50-gallon plastic barrels; the remaining 21 remained in their treatment conditions as controls. The temperature within the barrels was monitored using a Vernier Labquest with thermometer attachment during the 52 h process.

Pre-Bleeding Procedure: To replicate the capture and transportation during a typical biomedical bleeding process, the barrels were placed on the roof of Boyd Hall at PSU for 8 h. For the first 4 h, the barrels were kept in direct sunlight to simulate time spent on the deck of a boat; during this time, temperatures reached 37°C (mean ± SD: 32.0 ± 2.9°C). For the next 4 h, the barrels were covered with cardboard and moved into shade to simulate time spent in a truck en route to a bleeding facility (26.1 ± 1.1°C). Then the covered barrels were moved indoors (20.8 ± 0.9°C) for 16 h to simulate time spent overnight at the bleeding facility and then hemolymph was withdrawn.

Bleeding Procedure: Hemolymph was extracted using the procedure of Armstrong and Conrad (2008). One person held the animal in the abdominal flexure position, exposing the arthrodial membrane of the medial dorsal surface at the joint between the prosoma and the opisthosoma, while a second person withdrew the hemolymph. The arthrodial membrane was sterilized with 70% ethanol and punctured with a 14 gauge needle. Hemolymph was collected in 50 mL conical tubes, pre-chilled on ice, until the flow stopped or the estimated 30% volume had been reached. The equation of Hurton *et al*. (2005) was used to estimate total hemolymph volume for each horseshoe crab:

$$\mathrm{H}=21.6\,\mathrm{e}^{0.1234\mathrm{P}}\;[\mathrm{H}\;=\;\mathrm{hemolymph\ volume\ (mL)};\mathrm{P}\;=\;\mathrm{prosonal\ width\ (cm)}].$$

Extraction volumes ranged from 30 to 75 mL (mean ± SE: 35.8 ± 4.6 mL) and were generally less than the calculated 30% volume (17.3 ± 2.0%; Table 1). The hemolymph was kept on ice until processed further.

Post Bleeding Procedure: Animals were returned to their barrels and held indoors for 24 h (19.8 ± 0.9°C) to simulate a second overnight at a bleeding facility. The barrels were placed next to a heater (24.0 ± 1.3°C) and shaken periodically for 4 h to simulate transportation by truck back to the ocean. Finally, they were returned to their treatment conditions after a total of 52 h out of water. Activity in the LU and LRW groups was recorded for the next six weeks, and weekly hemolymph samples were taken from the LCT group. Tanks were checked daily for mortalities.

2.2.2 Outdoor group—The bleeding process for the OU group took place from June 6–8 and used a treatment paradigm similar to that used on the laboratory groups, though with minor modifications to adjust for poor weather conditions. Seven horseshoe crabs (one per tank) were selected to undergo the bleeding process; their accelerometers were detached, and they were distributed between two 50-gallon plastic barrels. The barrels were first kept under a heat lamp for 4 h to simulate time spent on the deck of a boat; during this time, temperature reached 28°C (26.1 ± 2.1°C). Then the barrels were transported by van to PSU (2 h; 21.3 ± 2.7°C) and placed indoors (18.2 ± 1.7°C) overnight (16 h). Then hemolymph was extracted as described for the indoor groups and the animals were returned to their barrels and kept indoors, uncovered, for 24 h (18.0 ± 0.5°C). The barrels were transported by van back to JEL (2 h; 25.6 ± 0.3°C) and placed outdoors, covered, for the final 4 h to complete the simulation of transport back to the ocean (24.2 ± 1.1°C). Finally, the accelerometers were reattached and the horseshoe crabs were returned to their original tanks, after being out of water for 52 h, and their activity was recorded for the next six weeks.

The percent of hemolymph extracted varied significantly ($F(3,24) = 7.2$, $P = 0.001$; Table 1) across the four groups. Percent extracted in the OU group significantly exceeded that of the LRW and LCT groups ($P < 0.05$), while percent extracted in the laboratory unrestrained group was not significantly different from the other groups.

2.3 Hemolymph Sampling

Weekly 1–2 mL hemolymph samples were taken from all animals in the LCT group. Samples were taken from all control animals at the time of the bleeding process for the bled groups, and from all animals six weeks after the bleeding process. Hemolymph was extracted following the procedure used for the bled group, except it was collected with 25-gauge needles in 2.0-mL microcentrifuge tubes. During this process, each crab was kept out of water for no longer than 5 min.

2.4 Determination of Hemocyanin Concentrations

Hemocyanin concentrations were determined using the procedure of Coates *et al*. (2012). Samples were centrifuged for 10 min at 3000 g and 4°C, and then the supernatant (cell-free hemolymph) was stored at 4°C until analysis (1–2 days). An aliquot of hemolymph was diluted 1:100 in 0.1 M Tris-HCl buffer (pH- 7.5), and absorbance was measured at 280 nm on a UV-160 spectrophotometer (Shimadzu, Columbia, MD). Hemocyanin concentration was calculated using an absorbance of 1.39 for a 1 mg/mL hemocyanin solution in a quartz cuvette (pathlength of 1 cm; Coates *et al*., 2012).

2.5 Data Analysis

Laboratory Running Wheel Activity: The ClockLab collection system recorded the number of wheel revolutions per minute, and these data were used to generate actograms and Lomb-Scargle periodograms. The number of wheel revolutions was multiplied by the circumference of the wheel to obtain distance moved per minute, and these distances were summed to obtain distance moved per day. The daily sums were averaged over seven day intervals to obtain average daily distance during the two weeks before bleeding and the three weeks after bleeding.

Laboratory Unrestrained Activity: Video files were analyzed for distance moved (cm), linear velocity (cm/s), and angular velocity (degrees/s) at 20-s intervals using Ethovision XT software (Noldus Information Technology Inc., Leesburg, VA). Distance was summed per minute, and these values were used to generate actograms and Lomb-Scargle periodograms using ClockLab. Distance moved per day was calculated, and these sums were averaged over seven day intervals to obtain average daily distance for the two weeks before and the three weeks after the bleeding process. Linear velocity and angular velocity during periods of movement (distance moved > 5 cm per 20 seconds) were averaged for the two weeks prior to and the four weeks after the bleeding process. Linear velocity measured locomotive speed in one direction (net distance moved divided by 20 s between sampling intervals), while angular velocity measured rate of directional change (change in direction of movement between two consecutive samples divided by 20 s; Ethovision XT).

Outdoor Unrestrained Activity: The accelerometers provided acceleration in the three orthogonal axes each minute. The difference between successive x, y, and z coordinates was calculated, and these differences were used to calculate the net acceleration vector. These values were used to generate actograms and Lomb-Scargle periodograms. The daily percent of time active was determined by summing the number of minutes per day with a net vector exceeding 0.02 (determined by frequency histogram analysis to be the threshold value for background noise). The daily percentages were averaged over seven day intervals to obtain weekly values for the two weeks before and the three weeks after the bleeding process.

Hemocyanin Concentration: The percent of original hemocyanin remaining for each animal was calculated as the ratio of final hemocyanin concentration (six weeks post bleeding) to the original hemocyanin concentration (day of bleeding). To distinguish hemocyanin loss due to hemolymph extraction from hemocyanin decline due to other factors, the amount of hemocyanin extracted (volume of hemolymph extracted multiplied by initial hemocyanin concentration) was subtracted from the estimated total hemocyanin (initial hemocyanin concentration multiplied by estimated hemolymph volume); this value was compared to final total hemocyanin (hemocyanin concentration six weeks post-bleeding multiplied by estimated hemolymph volume). In the LCT group, rate of hemocyanin concentration decline ($mg*mL^{-1}*week^{-1}$) was calculated as the ratio of concentration difference between successive hemolymph samples to the number of weeks between samples.

2.6 Statistical Analyses

Repeated measures ANOVAs, two-way ANOVAs, mixed model ANOVAs, or Student's T-tests ($P < 0.05$) were performed using Minitab (Minitab Inc., State College, PA) to examine the effects of bleeding on physiological and behavioral parameters. Tukey's HSD post-hoc analyses ($P < 0.05$) were used to examine differences between means. Lomb-Scargle periodogram analyses were used to determine whether animals expressed significant circatidal (~12.4 h) or daily (~24 h) rhythms each week (peaks exceeding $P = 0.001$; tidal: 10–14 h range; daily: 22–26 h range; arrhythmic: no significant peaks). Mixed model ANOVAs with repeated measures in one factor were used to compare percentages of animals expressing tidal rhythms in the three activity groups and the rates of hemocyanin decline in the LCT group. Two-way ANOVAs were used to test for effects of environmental conditions (LRW, LU, and OU) and treatment (bled/control) on percent of animals expressing tidal rhythms and percent of hemocyanin remaining in the four treatment groups. Repeated measures ANOVAs were used to compare pre-bleeding activity, linear velocity, and angular velocity to that post-bleeding. Correlational and single linear regression analyses were used to determine relationships between hemocyanin decline and activity, and immediate activity to second week post-bleeding activity.

Results

3.1 Alterations in Behavior: Biological Rhythms, Activity, and Velocity

The bleeding process affected both activity levels and expression of tidal rhythms (Figs. 1–3; Table 2). Bled animals (LRW and OU) decreased their expression of tidal rhythms during the second week post- bleeding (Table 2, Fig. 1). There was a significant interaction of the bleeding treatment by time ($F(3, 23) = 9.55$, $P = 0.005$) on the percent of animals expressing tidal rhythms. Specifically, expression of tidal rhythms in bled animals during the second week after bleeding was significantly less ($P < 0.05$) than it was in bled and control animals before bleeding, during the first week after bleeding, and during the third week after bleeding. During this second week, the percent of animals expressing tidal rhythms decreased by 83% in the OU group and by 60% in the LRW and LU groups. Significant differences between treatment conditions on the expression of tidal behavioral rhythms were not seen ($F(2,23) = 1.38$, $P = 0.305$).

In the OU group, three of seven bled animals appeared to shift activity patterns to diurnal activity during the second week after the bleeding process (Fig. 1), one appeared to shift to primarily nocturnal activity, and the remaining three did not appear exhibit a preference. In the LU group, four of six animals appeared to express diurnal activity during the second week post-bleeding period, while two animals appeared to express no preference for day/night. In the LRW group, one animal became active primarily during the day, while five animals appeared to express no preference.

Activity of the three bled groups decreased significantly after the bleeding process (Fig. 2). In the LRW and LU bled groups, there was a significant effect of time on mean daily distance moved ($F(3,12) = 3.82$, $P = 0.039$ and $F(3,15) = 3.51$, $P = 0.041$, respectively): bled animals decreased the distance they traveled during the second week after the bleeding process (Fig. 2; $P < 0.05$), while there was no effect of time on daily distance moved in the control groups (LRW: $F(3,9) = 0.52$, $P = 0.678$; LU: $F(3,15) = 0.514$, $P = 0.679$). In the OU bled group, time after bleeding also significantly affected mean daily activity ($F(3,18) = 4.02$, $P = 0.024$; Fig. 2, 3); percent of time spent active decreased ($P < 0.05$) during the second week after bleeding, while activity in the control group was not affected ($F(3,18) = 0.17$, $P = 0.92$). In the OU group, activity returned to pre-bleeding levels during the third week post-bleeding, while activity in the LRW and LU did not fully recover (Fig. 2).

In the OU group, six of seven bled animals appeared to exhibit normal to high activity levels for one to two days upon return to the water (percent of time active equaled or exceeded mean pre-bleeding levels; Fig. 3, top). This response was also seen in three of seven animals in the LU group, and one of seven animals in the LRW group. However, within the LU group, four of six animals exhibited a period of latency to initiate activity immediately after the bleeding process, with a “quiescent” period ranging 1.5 to 25.5 h (mean ± SE: 16.7 ± 5.5 h) prior to movement for four of the animals. Similarly, six of seven animals of the LRW group had a latency ranging from 2.8 to 21.6 h (8.6 ± 3.5 h) to initiate activity. In the OU group, the initial percent change in activity was significantly negatively correlated to the magnitude of the second-week activity decrease ($R^2 = 0.57$, $F(1,6) = 6.64$, $P < 0.05$; Fig. 3, bottom), with animals that exhibited highest initial increases in activity exhibiting larger activity decreases during week two post-bleeding.

Bled animals in the LRW and LU groups moved similar distances (LRW: 50.5 ± 10.7 m/day; LU: 62.3 ± 7.0; t(11) = 0.38, *P* = 0.715) prior to the bleeding process. During the second week after the bleeding process, LRW animals decreased activity by 66%, while LU animals decreased activity by 33%, with distance moved significantly less in the LRW than in the LU group (t(11) = 2.76, *P* = 0.04; LRW: 10.2 ± 2.9 m; LU: 42.8 ± 11.5 m). Similar to LU animals, OU animals decreased overall activity by 33% during the second week post-bleeding. Within each group, neither prosomal width nor percent of hemolymph extracted was correlated with percent reduction in activity during the second week post-bleeding for the three activity groups (prosomal width: OU: r(6) = 0.127, *P* = 0.811; LRW: r(6) = 0.361, *P* = 0.55; LU: r(6) = −0.275, *P* = 0.60; percent extracted: OU: r(6) = −0.308, *P* = 0.50; LRW: r(6) = 0.644, *P* = 0.24; LU: r(6) = –0.581, *P* = 0.23).

Linear and Angular Velocity—Linear and angular velocities were monitored in the LU group to assess changes in locomotor behaviors. While moving within the LU tank system, animals tended to exhibit three types of behaviors: traversals of the rectangular arenas, circles around the arena, or back and forth movements along one wall of the arena. Animals making both rapid traversals and circling movements exhibited high linear and angular velocity, while animals moving primarily along one side of their arenas exhibited lower velocities. During the 14 days before the bleeding process, both bled and control animals had similar linear (t(9) = 1.5, *P* = 0.167) and angular (t(12) = 1.02, *P* = 0.331) velocities. Bled animals decreased both linear (F(4,20) = 6.4, *P* = 0.002) and angular (F(4,20) = 2.99, *P* = 0.044) velocity during the first week after the bleeding process (Fig.4). While linear velocity returned to pre-bleeding levels during the third week post-bleeding, angular velocity remained suppressed (Fig. 4). In the control group, there was no effect of time on linear (F(4,24) = 0.93, *P* = 0.461) or angular (F(4,24) = 0.34, *P* = 0.849) velocity. Within the context of the LU system, the reductions in linear velocity suggest both slower locomotor rate during tank traversals and increased time spent moving along one side of the arena, while decreased angular velocity suggests slower rotational rates while circling arenas.

3.2 Alterations in Physiology

Hemocyanin—Hemocyanin loss was significantly affected by both bleeding (F(1,39) = 10.10, *P* = 0.003) and treatment condition (F(4,44) = 7.26, *P* = 0.001). Post-hoc analyses indicated that, six weeks post-bleeding, the percent of initial hemocyanin concentrations (pre-bleeding) remaining in the LRW, LU, and OU bled groups was significantly less than those of the corresponding control groups (*P* < 0.05). Further, hemocyanin percent losses in the LRW, LU, and LCT groups significantly exceeded those of the outdoor unrestrained group (*P* < 0.05; Fig. 5). The LRW bled and LU bled groups lost approximately 70% of the original concentration of hemocyanin, while the OU group lost 40%. On the day of the bleeding process, hemocyanin concentration did not differ significantly between bled and control animals of the four treatment groups (F(7,37) = 1.5, *P* = 0.2; data not shown).

When amount of hemocyanin lost as a result of the bleeding process was subtracted from initial hemocyanin levels, the hemocyanin reductions that occurred during the six weeks after the bleeding process did not differ between bled and control animals for the four groups combined (F(1,37) = 2.72, $P = 0.107$; data not shown), nor between bled and control animals within each treatment group (F(3,37) = 0.84, $P = 0.479$). Treatment conditions, however, affected total hemocyanin loss (F(3, 37) = 9.98, $P < 0.001$); specifically, subsequent losses in the OU group (bled and control) were significantly less than those laboratory groups.

In the LCT group, there was a significant effect of the bleeding process on hemocyanin concentration loss (F(1, 32) = 10.95, $P = 0.002$; data not shown), with a significant interaction of the bleeding treatment by time (F(3, 32) = 6.32, $P = 0.002$). Particularly, highest hemocyanin reductions in bled animals occurred immediately after the bleeding process (bled: 13.44 ± 2.8 mg*mL^{-1}week^{-1}, control: 2.31 ± 1.4 mg*mL^{-1} week $^{-1}$). This immediate loss was significantly greater than that in both bled and control animals before bleeding (bled: 4.48 ± 1.4 mg*mL^{-1} week $^{-1}$, control: 1.89 ± 2.1 mg*mL^{-1} week $^{-1}$) and during weeks 2–6 post bleeding (bled: 2.87 ± 0.7 mg*mL^{-1} week $^{-1}$, control: 2.17 ± 0.7 mg*mL^{-1} week $^{-1}$).

Mortality—There were five total mortalities (18%) among bled animals (Table 1), with 42% mortality in the LCT, 0% mortality in the OU group, and 14% mortality in the LRW and LU groups. Mortalities in the LCT and LU groups occurred on the third day after return to water, while the mortality in the LRW group occurred on day two. The mean percent of hemolymph extracted in the five animals that died (15.9 ± 1.8%) did not significantly differ from that extracted from the remaining animals (19.8 ± 1.2%; t(27) = −1.85; $P = 0.09$).

Discussion

In this study, we found that the biomedical bleeding process causes several sub-lethal behavioral and physiological changes. The most obvious behavioral effects were immediate (within one week) decreases in walking speed and latent (one week post) reductions in both overall activity and the expression of tidal rhythms. The greatest impact of bleeding on *Limulus* physiology was an immediate and sustained decline in hemocyanin concentrations.

Behavioral Effects

Activity Levels—The bleeding process caused, after a one-week delay, a period of reduced activity: during the second week after the bleeding process, animals decreased activity (distance moved and percent of time active) between 33% and 66%. Similarly, the stone crab (*Menippe mercenaria*, Davis *et al*., 1978) decreases activity for ten days after the fishery practice of declawing. Further, discarded (undersized) Norwegian lobsters (*Nephrops norvegicus*; Harris and Ulmestrand, 2004) exhibit diminished swimming performance for eight to fifteen days following harvest stress (trawl capture plus 1 h of emersion), activity reductions attributed to the energy costs of initial activity during capture, effects of aerial exposure, and recovery from trawl-related injuries. However, in *N. norvegicus*, the reduction in swimming ability is apparent immediately after harvest processes, a contrast to what we found in *L. polyphemus*.

While it is likely that the period of reduced activity in *L. polyphemus* is the result of the combination of blood loss, thermal stress, desiccation, and aerial exposure, the reason for the one week delay prior to a decline in activity is not clear. Animals varied in their initial behavioral responses to the bleeding process, with six of seven animals in the OU group, four of six animals in the LU group, and one of six animals in the LRW group exhibiting high activity levels immediately after re-immersion (Fig.1, LU bled, OU bled; Fig. 3) and then, after a 1 week delay, exhibiting a reduction in activity. The significant negative correlation between the immediate activity increase and the second week activity decrease in the OU group may suggest that the second week activity deficit is possibly a response to the initial damage from the bleeding process compounded by the depletion of energetic resources that may occur during initial high activity output. This initial activity may be a manifestation of an escape response (*L. polyphemus*, Rudloe and Hernnkind, 1976); alternatively, it may reflect disorientation incurred by the bleeding process (*L. polyphemus*; Kurz and James-Pirri, 2002) or foraging efforts to replace lost energy reserves (*Cancer pagurus*; Patterson *et al*., 2009). In contrast, the second-week activity decrease is likely to be due to an impact of the bleeding process that is more long-term; however, future work is needed to clarify the mechanisms responsible.

These results conflict with those of Rudloe (1983), who found no difference in overall activity between bled and control animals during the 28 days following hemolymph extraction. Several reasons may account for this discrepancy. Firstly, Rudloe (1983) examined activity in animals after a bleeding process that only included 3 h of aerial exposure, while our 52 h procedure more closely resembles the procedure typically used to extract blood for LAL. Secondly, in the Rudloe (1983) study, activity was assessed by the number of deflections of two rods suspended above a pool containing several animals, and thus individual activity was not examined; in contrast, our three separate activity monitoring systems enabled continuous collection of activity data from each individual animal, with the lowest resolution of one sample/minute. Finally, Rudloe (1983) compared activity between bled and control animals during a 2 h window each day over 28 days, while we used several weeks of continuous activity when making comparisons. Overall, our study both more closely replicated an actual biomedical bleeding process and allowed a closer monitoring of individual behaviors.

The degree of activity decline and recovery may have been differentially affected by treatment conditions in this study. Specifically, that complete recovery of activity levels only occurred in the OU group was most likely due to the fact that they had access to food, while the LU and LRW groups were not fed. Starvation both prolongs stress recovery periods (oyster, *Crassostrea gigas*, Li *et al*., 2009b) and decreases locomotion (*Cancer pagurus*, Ansell, 1973). Additionally, though the smaller animals of the LRW group exhibited the same trend in activity decline as did animals of both unrestrained groups, they appeared to have a larger activity loss (66%). However, among controls, LRW animals had the largest decreases in hemocyanin concentration, suggesting that conditions of the LRW system may have been more stressful than those of the LU and OU systems. As such, whether the smaller size of these animals or the experimental conditions affected the magnitude of the activity decline cannot be determined by this study, and it would be useful to compare activity of animals of varied sizes that are maintained in the same data collection system.

Expression of Tidal Rhythms—Decreased expression of tidal rhythms occurred contemporaneously with the decrease in activity levels. In the OU group, the transition from bimodal to unimodal behavioral patterns appeared to be due to a temporary disappearance of one of the two daily bouts of activity (four of five animals; Fig. 1, OU bled), with three animals appearing to be primarily active during the daytime high tide and one animal appearing to be active during the nighttime high tides. These transitions may serve as means of energy conservation, and may be directly related to the decreased overall activity during the second week after the bleeding process. Similarly, concomitant decreases in expression of tidal rhythms and overall activity occurs when *L. polyphemus* is exposed to decreased water temperature (4–11°C; Chabot and Watson, 2010). Additionally, in our laboratory animals that initially expressed primarily daily patterns of activity, the duration of the activity bout shortened during the second week after the bleeding process (Fig. 1, LU bled), and the loss of rhythmicity that occurred in three of the bled laboratory animals appeared to be a correlate of reduced overall activity.

Velocity—In the LU group, both the linear and angular velocity of the animals' movements significantly decreased during the first week after the bleeding process. Similarly, the great scallop, *Pecten maximus*, has significant reductions in swimming velocity during the first 24 h after dredge capture and aerial exposure (20 min; Jenkins and Brand, 2001). This transient effect is attributed to physical exhaustion after capture (Jenkins and Brand, 2001), while, in other species, immediate behavioral alterations appear to be caused by the physical trauma of harvest practices (Stoner, 2012a, b). However, the factors responsible for these immediate behavioral changes in *L. polyphemus* deserve further investigation.

Our linear velocity findings contrast with those of Kurz and James-Pirri (2002), who found no significant difference between movement rates of bled and non-bled female *L. polyphemus* returned to Nauset Estuary (Cape Cod, MA) after hemolymph extraction. This discrepancy could have been caused by the more intensive bleeding procedure we used: Kurz and James-Pirri (2002) performed a hemolymph extraction with a maximum of 30 min aerial exposure, while we used a high-stress bleeding treatment more closely analogous to the biomedical bleeding procedure (Hurton and Berkson, 2006). The heightened physiological impact of the high-stress treatment (higher mortality; Hurton and Berkson, 2006) is attributed to the synergistic effect of multiple stressors (hemolymph extraction, thermal stress, and aerial exposure); similarly, the presence of these compound stressors may evoke a heightened behavioral effect. However, Kurz and James-Pirri (2002) also observed that the bled females lacked a directional preference towards spawning beaches, contrasted to the directed movement patterns of controls towards these spawning beaches. Kurz and James-Pirri (2002) suggested this behavioral discrepancy may be caused by disorientation incurred by the bleeding process. Whether the velocity and activity changes we documented were laboratory manifestations of the movement patterns Kurz and James-Pirri observed in the wild remains to be determined and further work is also necessary in order to ascertain the connection between bleeding stress and relatively long-term alterations in behavior.

Physiological Effects

Hemocyanin Concentration Decrease—The bleeding process caused a prolonged period of hemocyanin loss: six weeks after the bleeding process, three of the bled groups exhibited significantly greater losses in hemocyanin concentration than the control animals in those groups (Fig. 5). Despite feedings, bled animals in the OU had only 60% of their original hemocyanin concentration six weeks after being bled. In contrast, OU controls lost just 10% of their original hemocyanin concentration. As the outdoor group experienced the most naturalistic conditions (freshly flowing bay water, sediment, and access to food), these results suggest that hemocyanin recovery in the wild may require a prolonged (>6 week) period. Similarly, James-Pirri *et al*. (2012) found significantly reduced total hemolymph protein in bled animals, compared to both wild caught and captive controls, 17 days after bleeding (James-Pirri *et al*., 2012), with hemolymph protein of bled animals approximately 20% less than that of controls. As hemocyanin constitutes over 90% of total hemolymph protein (Ding *et al*., 2005), our results support the suggestion that the bleeding process has a lingering impact on *L. polyphemus* hemolymph quality (James-Pirri *et al*., 2012), with no indication of recovery over six weeks even in animals that were fed.

The fact that the highest hemocyanin reductions occurred in the laboratory groups may be due to the both captivity stress and the lack of feeding. Not surprisingly, in the LCT group, hemolymph extraction caused significant hemocyanin loss during the first week after the process, indicating an immediate effect of the process of hemolymph quality. However, rates of decline between bled and control animals were similar during the following weeks, suggesting subsequent losses were most likely due to captivity stress (Coates *et al*., 2012); similarly, once hemocyanin loss due to hemolymph extraction was subtracted, net hemocyanin losses in all groups during the weeks following the extraction were equal, further suggesting that captivity stressors perpetuated these additional losses. Within the laboratory groups, the lack of feeding most likely exacerbated hemocyanin loss, as starvation can decrease total hemolymph protein through hemocyanin catabolism (*Cancer maenas*, Uglow, 1969) and slow protein synthesis (Li *et al*., 2009; Sokolova *et al*., 2012), an energetically demanding process (Hand and Hardewig, 1996).

Mortality Rates—The 18% total mortality across our four bled groups is lower than that reported by Leschen and Correia (30%; 2010) and by Hurton and Berkson (29%; 2006) for females that underwent either a 40% blood extraction (Hurton and Berkson, 2006) or a hemolymph extraction at a biomedical company (Associates of Cape Cod; Leschen and Correia, 2010) combined with 24–48 h aerial exposure. The low mortality in the LU and LRW groups, and the lack of mortality in the OU group, may be related to the percent of hemolymph we extracted (15–28%). Similarly, Hurton and Berkson (2006) found 0–6% mortality after a 48 h high stress process with 20–30% hemolymph extraction. However, the five deaths in our experiment appeared to be unrelated to percent of hemolymph extracted, with mean percent extracted for these five animals within the range of the overall mean extraction of 19.8 ± 1.2%. Additionally, the magnitude of the second week activity change did not appear to be related to the amount of hemolymph extracted, suggesting that, possibly, additional factors of the process (for example, air exposure or thermal stress) may be responsible for the observed behavioral and physiological effects.

The apparent variation in mortality among our four groups may be related to treatment conditions, notably the presence of feeding in the OU group and the weekly blood samples in the LCT groups. However, the disparate degrees of thermal stress in the bleeding processes may have also affected mortality: the laboratory groups experienced a maximum of 37°C while the outdoor unrestrained group experienced a maximum of 28°C. Thermal stress alone influences mortality and vigor in *Liocarcinus depurator* (Giomo *et al*., 2008) and *Nephrops norvegicus* (Lund *et al*., 2009), and, possibly, the higher temperature in our laboratory groups partially accounts for the increased mortality. The latency to initiate activity that occurred in 11 of the 14 animals of our laboratory groups may similarly be related to the degree of thermal stress these animals experienced, with both increased desiccation and accumulation of metabolic waste products potentially responsible for limiting activity until animals regained sufficient water volume. Both length of emersion (*Pandalus platyceros*, Stoner, 2012) and degree of thermal stress are positively correlated to degree of immediate behavioral and physiological impairments post-exposure in *Panulirus cygnus* (Paterson *et al*., 2005), *Nephrops norvegicus* (Ridgway *et al*., 2006), and *Homarus americanus* (Basti *et al*., 2010), and it would be useful to further investigate the importance of these variables on post-harvest behavior of *L. polyphemus*. Importantly, both the percent of hemolymph we extracted and the degree of stress to which we exposed the animals may be less than that of standard biomedical practices (ASMFC, 1998; Leschen and Correia, 2010), and the behavioral effects that we observed may underestimate the effects of a complete biomedical bleeding process with a full 30–40% blood extraction.

4.3 Ecological Implications

The changes we observed in activity levels, movement velocity, and expression of tidal rhythms may interfere with daily *L. polyphemus* activities, which would be particularly pronounced during the spawning season. Spawning necessitates several energetically costly trips to the intertidal zone (Leschen *et al*., 2006); larger females tend to make more excursions to the intertidal zone, often making multiple trips within the same week (Leschen *et al*., 2006). An activity deficit, such as that caused by biomedical bleeding, may either influence the number of those trips, or it may influence the timing of those trips. In the case of the latter, females may delay spawning activity while they are recuperating, and this could reduce their spawning output. In addition, modifications in the expression of tidal rhythms may alter the timing of excursions to mating beaches and cause a reduction in the probability they would find males with which to mate. As females are preferentially harvested (76%; Rutecki *et al*., 2003), these behavioral alterations during the spawning season may partially account for declining populations in heavily harvested regions (James-Pirri, 2012), specifically the declining proportions of females at spawning beaches (1 female: 8.5–14 males in Pleasant Bay), reduced egg abundances (James-Pirri, 2012), and the occurrence of single females attempting to spawn (James-Pirri, 2012).

The extended periods of low hemocyanin levels that we found may impact *L. polyphemus* fitness in the natural habitat. Hemocyanins have multiple physiological functions in invertebrates, including sclerotization and maintenance of cuticle integrity (Adachi *et al*., 2005; Terwilliger, 2007), wound repair, osmoregulation (Paul and Pirow, 1998), and involvement in the immune response (Coates *et al*., 2011). Reductions of this protein, combined with a reported four month deficit in amebocytes caused by the bleeding process (Novitsky, 1984), may result in a weakened organism, one that is both less able to contend with additional stresses and that exhibits increased susceptibility to infection. Further, the sustained declines in hemocyanin concentration that we found may partially account for the increased (10–11%) probability of mortality during the first two years post-bleeding in animals returned to the wild (Rudloe, 1983).

In summary, *L. polyphemus* females have decreased overall activity and expression of tidal rhythms during the second week after bleeding, decreased linear and angular movement velocities in the first week after the bleeding process, and long-term (>6 week) declines in hemocyanin concentrations. These results suggest that *L. polyphemus* experiences sub-lethal effects of the bleeding process, which, along with high mortality rates in females (Hurton and Berkson, 2006; Leschen and Correia, 2010), may partially account for the changing population characteristics in areas of heavy biomedical harvest. Whether these behavioral and physiological changes occur, or are possibly heightened, in the wild deserves further investigation in order to fully assess the implications of the harvest process for *L. polyphemus* spawning behaviors. Maintenance of populations of *L. polyphemus* is essential not only for the ecosystem as a whole, including subsistence of shorebird populations (Baker *et al*., 2004), but also for several commercial sectors. The use of horseshoe crabs in the production of LAL is of global importance, and a continued harvest is important in meeting the demands for LAL. However, to maintain the integrity of the stock needed to supply the industry, adaptive or flexible management strategies may need to be considered. In areas of population decline, harvest limits during the spawning season may help to minimize any potential population-level consequences incurred by individual behavioral and physiological changes.

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Abbreviations

LAL	(Limulus Amebocyte Lysate)

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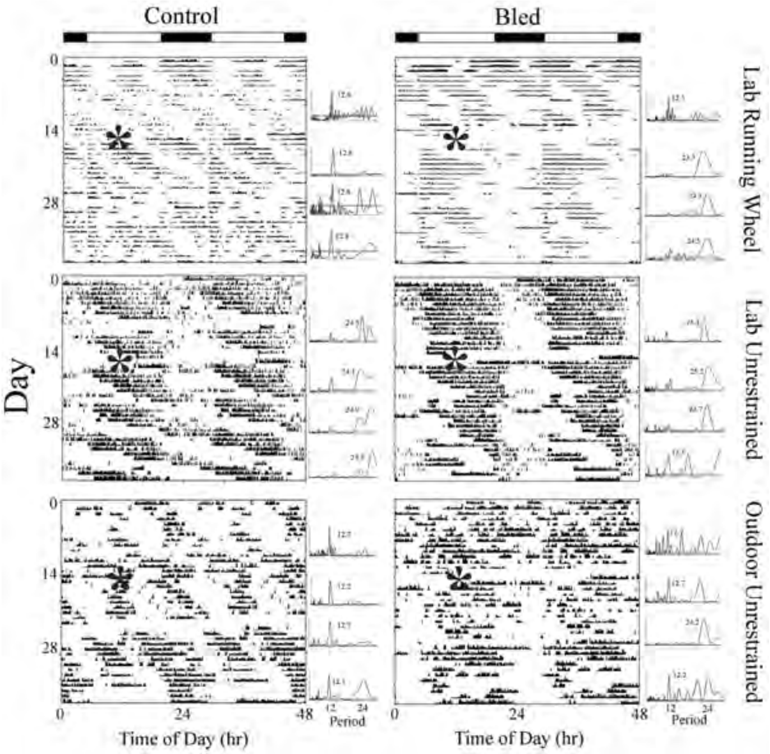

Figure 1. The effects of bleeding on locomotor activity and rhythms in representative *L. polyphemus*

Larger panels: Actograms are double-plotted, with size and position of black marks indicating the intensity and timing of activity. Asterisks indicate the start of the bleeding process; in the control actograms, the start is marked to facilitate comparison. The LD cycle (14:10) is indicated by light/dark bars at the top. Smaller panels: Lomb-Scargle periodograms to the right of each actogram indicate significant rhythms of activity during successive intervals; horizontal line above x-axis indicates $P = 0.001$. Number next to peak indicates most significant period of activity within circatidal (12–14 h) or circadian (22–16 h) range.

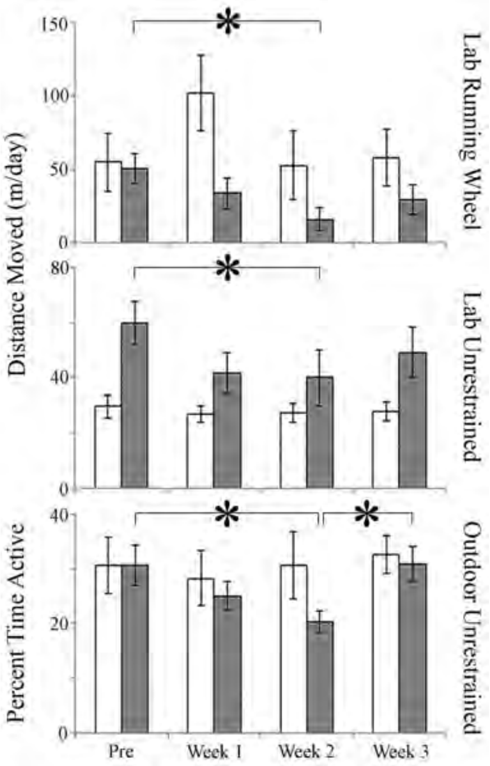

Figure 2. Effects of bleeding on activity (mean ± SEM) in the Laboratory Running Wheel, Laboratory Unrestrained, and Outdoor Unrestrained groups

White-Control; Grey-Bled. * - $P < 0.05$. Pre= 2 weeks before bleeding; Weeks 1–3= post-bleeding.

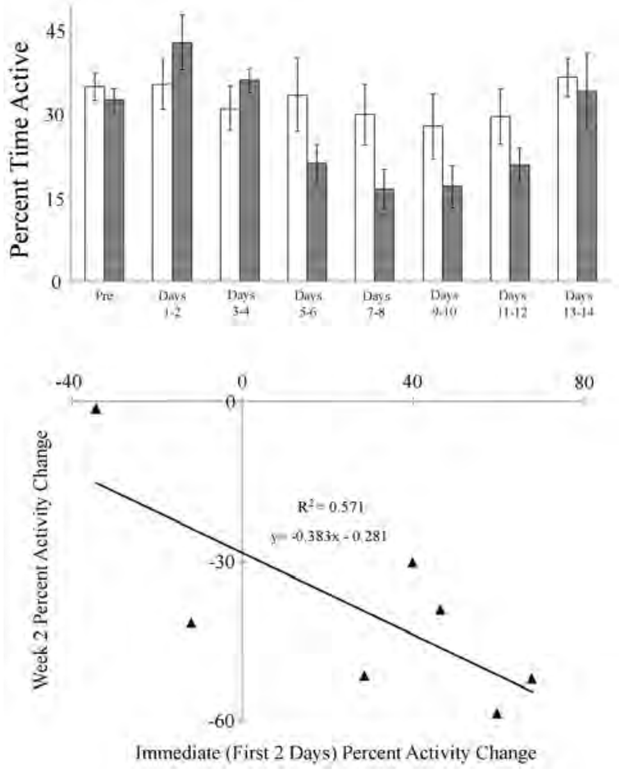

Figure 3. Effects of bleeding on activity (mean ± SEM) in the Outdoor Unrestrained group

Top: Mean activity; White-Control; Grey-Bled. Pre= 2 days before bleeding; Days 1–14 = post-bleeding. **Bottom**: Relationship between activity percent change during the first two days post-bleeding to activity percent change during the second week post bleeding.

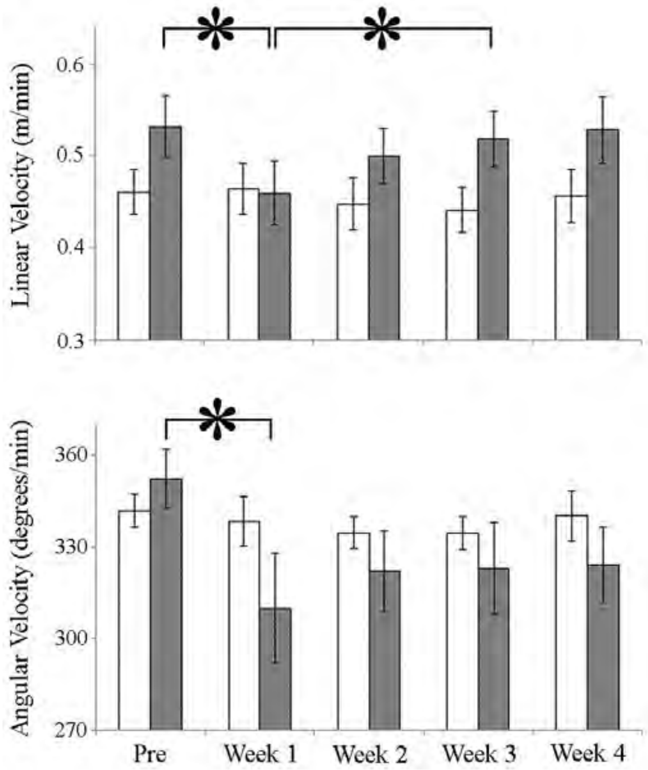

Figure 4. Effects of bleeding on linear and angular velocity in the Laboratory Unrestrained group

White bars: controls; grey bars: bled; *- $P < 0.05$. Pre= 2 weeks before bleeding; Weeks 1–4= post-bleeding.

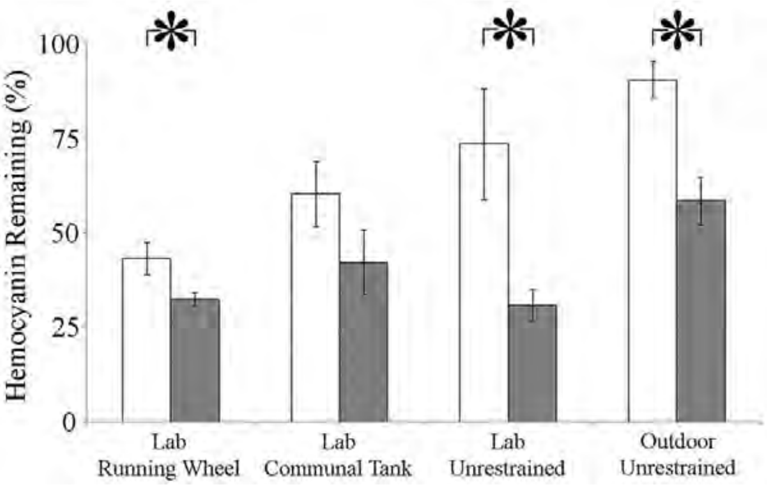

Figure 5. Percent of original hemocyanin concentration remaining six weeks post-bleeding
White bars: controls; grey bars: bled. *- $P < 0.05$.

Table 1

Size, amount of hemolymph extracted, and mortality in the four female bled groups.

Treatment	Prosomal Width (cm)	Extracted Hemolymph Volume (mL)	Estimated Percent Extraction	Total Mortality	Percent Mortality
Lab Running Wheel	17.5 ± 0.8	28.9 ± 4.7	15.5 ± 2.5	1	14
Lab Unrestrained	18.9 ± 0.3	47.6 ± 7.0	21.0 ± 3.0	1	14
Outdoor Unrestrained	19.1 ± 0.4	64.4 ± 2.8	28.1 ± 1.1	0	0
Lab Communal Tank	18.4 ± 0.4	29.0 ± 4.9	14.2 ± 2.4	3	42

Values are mean ± SEM; *n* = 7 per group. Estimated percent extraction determined by comparing actual amount extracted to expected hemolymph volume, calculated using prosomal width.

Table 2

Percent of *L. polyphemus* expressing tidal and daily rhythms (τ ± SEM) before and after the bleeding process.

Experimental Group	Rhythm Type	Pre	Week 1 Post	Week 2 Post	Week 3 Post
Lab RW Control	Tidal	86% (12.6 ± 0.3)	71% (12.1 ± 0.2)	57% (12.4 ± 0.2)	57% (12.3 ± 0.3)
	Daily	14% (23.8)	29% (23.0 ± 0.2)	43% (23.9 ± 0.7)	43% (24.6 ± 0.1)
Lab RW Bled	Tidal	83% (12.5 ± 0.1)	83% (12.5 ± 0.3)	33% (12.3 ± 0.2)	83% (12.5 ± 0.2)
	Daily	17% (24.3)	17% (23.5)	51% (24.3 ± 0.1)	17% (24.6)
Lab Unrestrained Control	Tidal	71% (12.6 ± 0.3)	57% (12.0 ± 0.2)	57% (12.3 ± 0.2)	71% (12.6 ± 0.3)
	Daily	28% (24.1 ± 0.4)	42% (23.3 ± 0.4)	43% (24.3 ± 0.3)	28% (25.0 ± 0.8)
Lab Unrestrained Bled	Tidal	83% (12.3 ± 0.1)	67% (12.7 ± 0.2)	33% (12.4 ± 1.0)	83% (12.5 ± 0.2)
	Daily	17% (23.2)	33% (24.5 ± 0.8)	51% (24.0 ± 0.2)	17% (23.8)
Outdoor Control	Tidal	86% (12.5 ± 0.1)	100% (12.2 ± 0.2)	86% (12.6 ± 0.2)	71% (12.2 ± 0.1)
	Daily	14% (26)	0%	14% (23.9)	29% (24.0 ± 0.5)
Outdoor Bled	Tidal	86% (12.7 ± 0.2)	86% (12.5 ± 0.1)	14% (11.2)	86% (12.3 ± 0.1)
	Daily	14% (23.7)	14% (23.1)	71% (24.4 ± 0.2)	14% (24.9)

The Impact of Biomedical Bleeding on Horseshoe Crab, Limulus polyphemus, Movement Patterns on Cape Cod, Massachusetts

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THE IMPACT OF BIOMEDICAL BLEEDING ON HORSESHOE CRAB, *LIMULUS POLYPHEMUS*, MOVEMENT PATTERNS ON CAPE COD, MASSACHUSETTS

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The purpose of this study was to determine if bleeding, for biomedical purposes, influenced the behavior of horseshoe crabs, *Limulus polyphemus*. In the summer of 2001, ten bled and ten control (unbled) female horseshoe crabs were tracked for 26 days using acoustic telemetry in a small estuary on Cape Cod, Massachusetts. All but three crabs, two bled and one control, were located during the study period. No mortality was observed in the control group, while 20% mortality was observed within the bled group. There was no significant difference in the average rate of movement or in the spatial distribution within the estuary between the two groups. However, a difference was detected in the movement patterns. Horseshoe crabs from the bled group had a random direction of movement compared to the directional movement pattern of the control group, suggesting that the bled crabs experienced more disorientation.

Keywords: Horseshoe crab; Behavior; Acoustic telemetry

INTRODUCTION

The American horseshoe crab, *Limulus polyphemus*, is exploited by both a commercial bait fishery and the biomedical industry (ASMFC, 1999). The biomedical industry uses blood from horseshoe crabs in the production of *Limulus* ameobocyte lysate or LAL, which is the standard test used to detect endotoxins pathogenic to humans in all injectable drugs and implantable medical devices (Novitsky, 1984). During the bleeding process up to 30% of the blood volume is removed from the horseshoe crab (Novitsky, 1984). All bled horseshoe crabs collected for LAL production must be released within 72 h of capture as mandated by the United States Food and Drug Administration (ASMFC, 1998). Previous studies have estimated that horseshoe

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crabs used for biomedical bleeding suffer an 8–15% mortality rate from the procedure (Rudloe, 1983; Walls and Berkson, 2000; Wenner and Thompson, 2000). However, the influence of bleeding on horseshoe crab behavior and long-term survival are unknown. This study evaluated the behavior patterns of bled and control (unbled) female horseshoe crabs in a natural setting using acoustic telemetry.

METHODS

Twenty female horseshoe crabs were collected from Pleasant Bay and Nauset Estuary within Cape Cod National Seashore, Cape Cod, Massachusetts in July of 2001. All horseshoe crabs used in this study were larger than 200 mm prosomal width (Table I) and free of epibionts, similar to individuals that are typically used by biomedical facilities for blood extraction. The twenty horseshoe crabs were treated in pairs, with one horseshoe crab from each of the ten pairs randomly chosen and bled. The individual that was to be bled was placed in a stand, similar in design to those utilized by the

TABLE I Summary of information for horseshoe crabs used in study. Number of days detected refers to the actual days where the individual's signal was located. Total number of data points refers to the number times a signal and corresponding GPS coordinates were recorded for the individual. The number of consecutive data points (within a 12 h period) for each individual is given in parentheses. Status indicates available current information on the location of the individual

Crab no.	*Treatment*	*Prosomal width* (mm)	*Blood volume removed* (mL)	*First day detected*	*Last day detected*	*No. of days detected*	*Total number and (consecutive) data points*	*Status as of spring 2002*
234	bled	232	120	7/7/01	7/31/01	4	11 (9)	unknown
245	bled	261	90	–	–	0	0 (0)	signal never detected
253	bled	215	76	7/4/01	7/19/01	5	11 (8)	dead (7/30/02)
267	bled	265	100	7/6/01	7/6/01	1	1 (0)	unknown
336	bled	221	82	7/4/01	7/31/01	7	10 (6)	dead (9/8/02)
344	bled	212	86	7/4/01	7/20/01	9	10 (4)	unknown
347	bled	217	104	7/6/01	7/10/01	2	3 (2)	unknown
353	bled	219	81	8/11/01	8/11/01	1	1 (0)	unknown
433	bled	221	85	7/9/01	7/25/01	4	6 (4)	unknown
457	bled	261	75	7/9/01	7/11/01	2	2 (0)	unknown
254	control	225	0	–	–	0	0 (0)	unknown
455	control	209	0	7/9/01	7/13/01	2	2 (0)	unknown
477	control	255	0	7/10/01	7/10/01	1	3 (3)	unknown
553	control	233	0	7/4/01	7/25/01	7	21 (19)	unknown
555	control	229	0	7/4/01	8/2/01	9	25 (20)	transmitter found spring '02
563	control	219	0	7/6/01	7/22/01	7	8 (2)	unknown
566	control	208	0	7/4/01	8/17/01	10	17 (9)	transmitter found spring '02
634	control	213	0	7/12/01	7/12/01	1	1 (0)	unknown
666	control	251	0	7/4/01	7/31/01	7	22 (21)	unknown
673	control	256	0	–	–	0	0 (0)	signal never detected

biomedical companies, to hold the horseshoe crab and expose the articular membrane. The articular membrane was swabbed with 70% isopropyl alcohol and a sterile, disposable, pyrogen-free, 14-gauge, 2-inch hypodermic needle was inserted into the articular membrane to drain blood from the cardiac sinus. The needle was removed once the blood flow from the cardiac sinus slowed to an intermittent drip. An average of 90 mL of blood (Table I) was removed from each of the ten female horseshoe crabs. Time out of the water was same for both treatment groups and did not exceed 30 min.

Sonic transmitters (Sonotronics, Tuscon AZ) 64 mm in length, 16 mm in diameter, and weighing 8 g, were attached to each of the twenty horseshoe crabs. The transmitter was fastened to the top of the prosoma using marine epoxy and a three-point monofilament harness. Each sonic transmitter emitted a unique signal, thus tracking multiple animals was possible. The sonic transmitters were detectable up to 1000 m and have a battery life of 14 months. A signal can only be detected when the transmitter is in the water, therefore if a horseshoe crab was out of the water the signal would not be detected. Alternatively, if an individual dies in the water, the signal would be detected but there would be no movement associated with that particular signal. All horseshoe crabs were released in pairs at the same location in Nauset Estuary from July 2 to 8, 2001 and were tracked using a hydrophone during July and August.

Nauset Estuary is a small system (600 hectares) within the boundaries of Cape Cod National Seashore. Horseshoe crabs within the estuary typically spawn from mid-May to early July, and a known spawning beach is located in the northern portion of the estuary (Fig. 2) (James-Pirri *et al.*, 2002). Due to its small size and shallow depths, locating signals from the transmitters was possible using a hydrophone from the platform of a kayak. A handheld geographic positioning system (GPS) unit was used to record the geographic coordinates of signals from the tagged horseshoe crabs. Once a signal was located, the individual was tracked for approximately 15 min (if possible) and the GPS coordinates and time of day were recorded every 3–5 min. Then the estuary would be searched for another signal and the process would continue. We also recorded the GPS coordinates of locations where no signals were detected to evaluate which areas of the estuary were frequented by horseshoe crabs. Often we would return to the location of previous signals one to five hours later to determine if the horseshoe crab was still in the area and to collect additional data. We refer to a signal from a transmitter and corresponding GPS coordinates and time as a datum point. Consecutive data points refer to data where an individual was tracked over time and information on its location was recorded at 3–5 min intervals.

Average rate of movement ($\mathrm{m\,min^{-1}}$) was calculated for horseshoe crabs where data points (a detected signal, corresponding GPS coordinates, and time) occurred within a 12 h period. The 12 h criterion was chosen since this was the time frame on any one day when the estuary could be searched for horseshoe crabs. If data points were included that spanned longer than 12 h, the rate of movement would be underestimated since crabs tended to return to the same areas on the each tidal cycle and thus distance traveled between data points would be small, whereas the time period between data points on different days would be great (1 or more days). To calculate the rate of movement, the time interval between consecutive data points within a 12 h period was determined and distance traveled was calculated from the GPS coordinates for those data points. Rate of movement for each individual (individuals were the replicates) was then calculated as the total distance traveled divided by the total time between consecutive data points within a 12 h period.

To determine differences between the rate of movement for bled and control groups, a One-way ANOVA was performed on the average rate of movement for each treatment group where consecutive data points on individuals were collected within a 12 h period. Prior to analyses, tests for normality and homogeneity of variances were conducted and the data were found not to violate the assumptions of ANOVA. Only 12 of the 17 horseshoe crabs that were located had consecutive data points that met the 12 h criteria, and thus only these individuals were included in the analyses of rate of movement (Table I).

GPS coordinates were entered into GIS software and superimposed with geographic data to display the spatial distribution of the horseshoe crabs within Nauset Estuary (Fig. 2a–c). To determine if the spatial distribution and movement of the two treatment groups displayed a nonrandom or random pattern, all data points were analyzed by Biotas® software from Ecological Software Solutions. Biotas® software provides a variety of analyses of spatial and temporal data including analyses of spatial dispersion, spatial autocorrelation, spatial randomness, and movement patterns. Additionally, GIS data can be used as an overlay to confine the analyses of spatial distribution and movement patterns to abiotic and biotic limits of the organism (e.g. home range, water bodies). Biotas® was used to evaluate the data with the Moran's I Test (Moran, 1950) and the Movements Analysis test within the boundaries of Nauset Estuary. The Moran's I Test uses a grid overlay on the pattern of geographic points within Nauset Estuary to analyze data in adjacent grids to determine if there is a spatial pattern such as spatial clustering. We used this test to evaluate if horseshoe crabs from the two treatment groups differed in their spatial autocorrelation (i.e. did the treatment groups have different spatial patterns) within Nauset Estuary. A high spatial autocorrelation would imply that the horseshoe crabs were not randomly distributed. The Movements Analysis takes a circular measure, a bearing, and converts it to a linear measure, an angle, and then uses the angle measure to test if changes in the animal's direction of movement (e.g. number of left and right turns) are significantly different than what would be expected from random chance. We used this test to determine if the pattern of movement, in terms of directional change, differed between the two treatment groups.

RESULTS

Seventeen (9 control, 8 bled) of the twenty horseshoe crabs were located after release. Three horseshoe crabs (one control and two bled) were not located during the study period. These three individuals could have moved out of Nauset Estuary, traveled to deeper water, or died with their bodies stranded out of the water where the transmitter signal would not be detected. Two of the horseshoe crabs from the bled treatment were found dead within Nauset Estuary 28 days and 68 days post bleeding. No mortality was observed among the control group. Horseshoe crabs from both groups frequented the same general area (northern portion) of the estuary (Fig. 2a,b). Locations where no signal was detected are shown in Fig. 2c.

There was no significant difference in rate of movement (One-way ANOVA, $p = 0.261$) between the bled and control horseshoe crabs (Fig. 1). However, due to the low sample size and associated variability, the power of this test was very low (power = 0.13, alpha = 0.05) indicating that the probability of failing to detect a

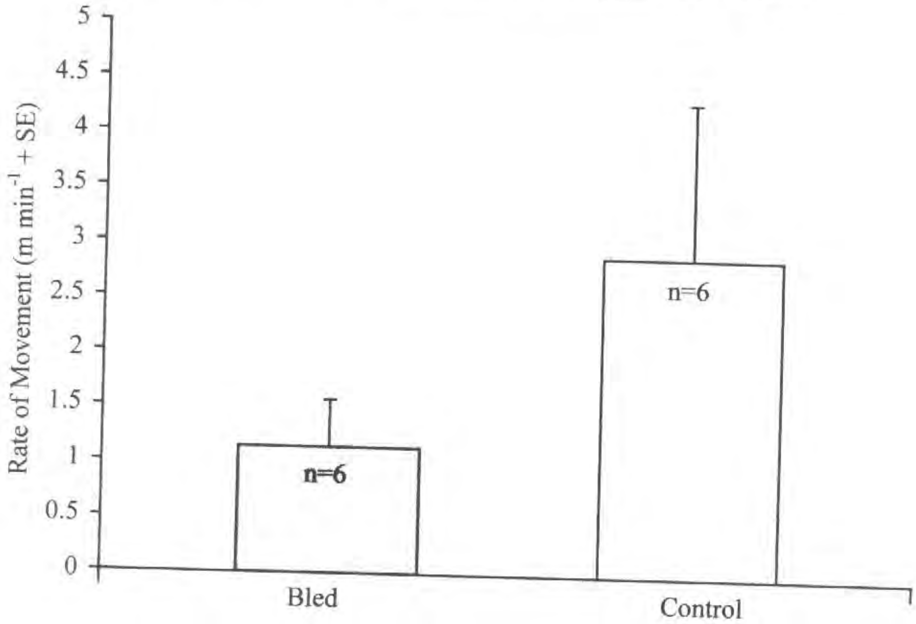

FIGURE 1 Rate of movement for bled and control horseshoe crabs.

difference between the treatments when a difference actually existed (i.e. Type II error) was 87%. To increase the power of the test to an acceptable level (i.e. power = 0.8, alpha = 0.05) we would have needed more than twice as many replicates within each treatment group.

The null hypothesis of no spatial autocorrelation was rejected by the Moran's I Test for both treatment groups (bled: *Z*-score = 4.517, $p > 0.05$; control: *Z*-score = 4.506, $p > 0.05$) indicating that both groups exhibited spatial autocorrelation (clustering), and therefore had a nonrandom distribution patterns within Nauset Estuary (Fig. 2a,b). The Movement Analyses indicated that the bled group exhibited a random direction of movement (*Z*-score = 0.43, $p < 0.05$), whereas the control group exhibited directional movement patterns (*Z*-score = 2.71, $p > 0.05$).

DISCUSSION

Our telemetry data show that horseshoe crabs from both treatment groups frequented similar areas of the estuary, particularly the northern portion that contains extensive tidal flats, while other areas were not extensively used by the horseshoe crabs. These shallow tidal flats are most likely an important foraging area since horseshoe crabs are predators on intertidal fauna (Botton, 1984a,b). It is interesting to note that the small beach used by horseshoe crabs for spawning in this area also has the highest horseshoe crab egg densities observed on beaches surveyed within Cape Cod (James-Pirri *et al.*, 2002). We have periodically searched for signals from the horseshoe crabs over the winter of 2001 through the summer of 2002, and have not yet located any of the animals from this study, although 2 transmitters that had fallen loose from the horseshoe crabs were recovered in the spring of 2002 within Nauset Estuary.

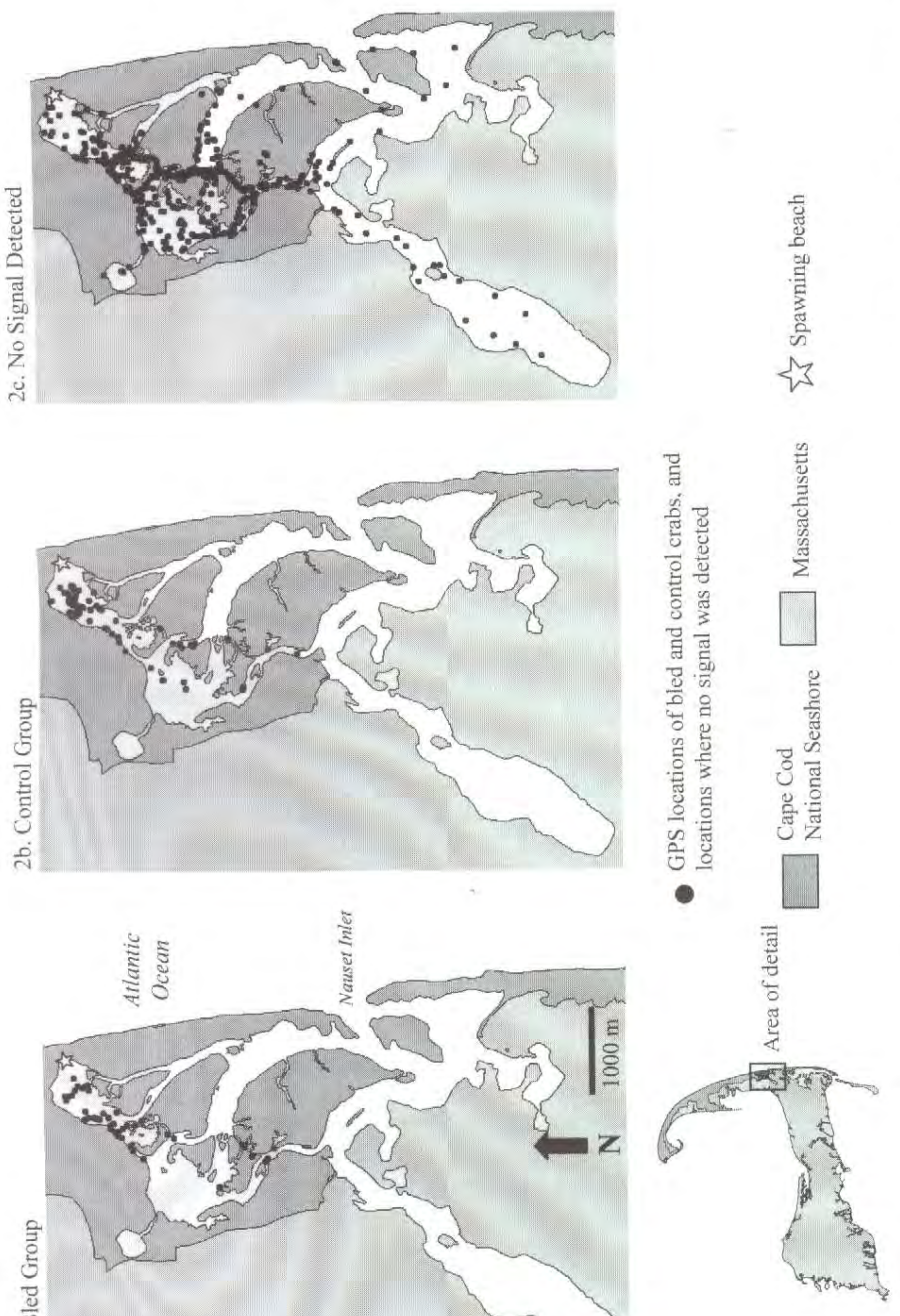

FIGURE 2 Map of Nauset Estuary showing the distribution of sonar signals for bled (2a) and control (2b) horseshoe crabs and locations where no signal was detected (2c).

The 20% mortality observed within the bled group is slightly higher than the 8–15% mortality estimated in previous studies (Rudloe, 1983; Walls and Berkson, 2000; Wenner and Thompson, 2000). However, the slightly higher mortality rate observed in this study may have been an artifact of low sample size.

We found no difference in the rate of movement between the bled and control groups, although there was a trend towards a lower rate of movement in the bled horseshoe crabs. Unfortunately, the statistical power of the test was low and probably of a Type II error was high. Both groups showed similar spatial distribution patterns, most likely related to foraging areas within the estuary. However, the bled horseshoe crabs did not exhibit the same type of movement as the control crabs. The bled group had a random movement pattern compared to the directional movement pattern of the control group, suggesting that the bled horseshoe crabs experienced more disorientation, presumably resulting from the bleeding procedure and the associated loss of blood volume. If this disorientation prevents horseshoe crabs from locating spawning beaches, bled horseshoe crabs may have a reduced reproductive output compared to those that are not bled. Unfortunately, due to the small number of horseshoe crabs where appropriate data were collected for statistical analyses, these results do not permit a conclusive statement about the influence of bleeding on horseshoe crab behavior and we believe that further investigation is warranted based on the observed disorientation and the trend of lower activity of the bled horseshoe crabs.

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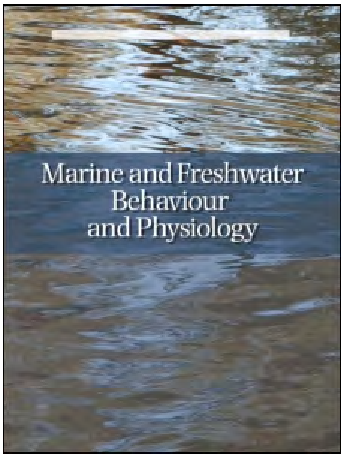

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Mortality in female horseshoe crabs (*Limulus polyphemus*) from biomedical bleeding and handling: implications for fisheries management

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Mortality in female horseshoe crabs (*Limulus polyphemus*) from biomedical bleeding and handling: implications for fisheries management

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Horseshoe crabs (*Limulus polyphemus*) are bled for biomedical purposes to produce Limulus Amebocyte Lysate (LAL), a valuable material used to detect endotoxins in medical devices and implants. Previous studies generally found mortality from the bleeding process of 5–15% with one study reporting 29% for females; the 15% value is now used for management purposes. However, most of these studies looked only at males, did not mimic handling practices typical of the biomedical facility in Massachusetts, or harvested females after the spawning season when much of the actual biomedical harvest occurs. Female mortality is of particular concern because they have greater importance than males for population dynamics, are preferentially harvested, and are more likely to be physiologically stressed than males because of energy outlay for egg production. We examined the mortality of unbled female horseshoe crabs versus that of crabs bled by the local biomedical company and either returned directly to water, or held overnight as is the current practice. Mortality of Control (unbled) crabs was low (3%) and differed significantly ($p < 0.001$) from that of either bled group (22.5% and 29.8% mortality, respectively); the two bled groups did not differ significantly from each other ($p = 0.31$). Mortality rates in bled treatment groups were double those used in current management of the biomedical fishery. The bait and biomedical fisheries are managed differently because biomedical is considered a low-mortality "catch and release" fishery. States and interstate management agencies may want to re-examine policies surrounding biomedical horseshoe crab harvest based on these results.

Keywords: horseshoe crab; *Limulus polyphemus*; biomedical bleeding; lysate; sex ratio; Pleasant Bay; harvest

Introduction

Horseshoe crabs (*Limulus polyphemus*) are a valuable and highly contested resource in Massachusetts, as elsewhere along the eastern seaboard of the United States. Historically, this species has been a ubiquitous feature along the coast because crabs come ashore to spawn in spring. Horseshoe crabs are much admired by the public and conservation groups because of the visible spectacle of their beach spawning events and the "prehistoric" nature of the animal: a fossilized xiphosurid displaying

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the typical horseshoe crab body plan has been aged at 445 million years (Rudkin et al. 2008). Horseshoe crabs also have important ecological value because of their eggs' role in fueling the northward migration of shorebirds (Botton et al. 1994; Karpanty et al. 2006; Atkinson et al. 2007) and the role they play as bioturbators in estuarine ecosystems (Botton et al. 2003).

In addition to the intrinsic "natural" value of the horseshoe crabs, they support several important commercial industries in Massachusetts. An extract of horseshoe crab blood cells, Limulus Amebocyte Lysate (LAL), is greatly valued as the raw material for a reagent critical to assuring safety of injectable pharmaceutical products and certain medical devices. LAL is used worldwide to detect endotoxins in virtually all medical products injected or implanted into humans, and currently has no substitute. Horseshoe crabs are essential to a multi-million dollar biomedical bleeding industry in Massachusetts. They are also the favored bait for the whelk (*Busycon* spp.) pot fishery, valued at approximately $3 million per year in Massachusetts (Massachusetts Division of Marine Fisheries – DMF – unpublished data). Finally, several bait dealers, draggers, and horseshoe crab fishermen rely on these animals as their primary source of income, and many more supplement their earnings with this species. For these reasons, maintaining horseshoe crab populations and the fishery at sustainable levels is important to both humans and the ecosystem. Recent concerns about overharvest of horseshoe crabs have highlighted the need for accurate assessments of the fishery's impact on populations in order to manage the fishery for sustainability.

Currently, biomedical and bait fisheries are treated separately under Massachusetts regulations. Fishermen hold permit endorsements to fish for one purpose or the other, but not both. Massachusetts does not currently manage biomedical crab fishery with quota, and their harvest is not counted against the annual quota of 165,000 bait crabs. In addition, fishermen with biomedical harvest permits have a daily limit of 1000 crabs, rather than the 400 crab per day limit imposed on fishermen with bait permits. Pleasant Bay, a large embayment on Cape Cod, Massachusetts (Figure 1), is closed to bait fishing, but biomedical harvest is allowed in the Bay except in areas which are part of the Cape Cod National Seashore. The Atlantic States Marine Fisheries Commission (ASMFC) has also treated coast-wide biomedical harvest differently from bait harvest. Addendum IV (ASMFC 2006) to the ASMFC Management Plan for Horseshoe Crabs (ASMFC 1998) maintains the provision that biomedical crabs are not subject to the same restrictions as those for bait use.

The two different sets of rules evolved because the biomedical fishery has been considered a "catch and release" fishery with low mortality. Crabs must be returned to the water body of origin after bleeding, and several previously published studies showed relatively low mortality resulting from bleeding (Rudloe 1983; Kurz and James-Pirri 2002; Walls and Berkson 2003; Hurton and Berkson 2006). Mortality in these studies fell mostly in the 5–15% range, which is the basis of Massachusetts policy, with Hurton and Berkson reporting 29% for females in the high-stress, high-bled group. ASMFC allows a maximum annual mortality of 57,500 crabs coast-wide from biomedical bleeding. The actual annual figure is calculated based on the assumption that 15% of the total biomedical harvest suffers mortality (ASMFC 2009). A mortality rate that is in fact higher than 15% could have implications for management policy in Massachusetts and with ASMFC and its member states.

Figure 1. Study area: Pleasant Bay, Cape Cod, Massachusetts.

We began to question the applicability of previous bleeding studies to Massachusetts due to the history of horseshoe crab fishing in Pleasant Bay and results of spawning surveys in this embayment and elsewhere on Cape Cod. Pleasant Bay has been consistently fished for biomedical purposes for nearly 30 years (Frank Germano, DMF, personal communication). For most of that time, bait fishery removals were small or nonexistent. Part of the Pleasant Bay coastline within the Cape Cod National Seashore was closed to all harvest in 2001. The bait fishery in the rest of the Bay rapidly expanded in 2005 and 2006 when a red tide forced the state to ban harvest of bivalves in most areas, and many shellfishermen turned to horseshoe crab harvest as an alternative income source. Bait harvest jumped from a few hundred crabs to 17,800 in 2005 and 40,700 in 2006 (DMF unpublished data) out of an estimated population of 500,000 (Carmichael et al. 2003). Concern by the biomedical company that their crab supply would be rapidly depleted prompted the DMF to place an emergency closure on bait harvest in Pleasant Bay, and to allow only the harvesting of biomedical crabs. The assumption behind this closure was that maintaining a biomedical-only harvest with low-mortality rate in this Bay would facilitate a sustainable fishery.

However, results of spawning surveys conducted in Pleasant Bay and other estuaries that are part of the National Seashore on Cape Cod prompted concern that

Table 1. Females as proportion of the count of horseshoe crabs on spawning beaches by region and year.

	Year					
Region	Historic (1950s)[a]	2000[b,d]	2001[b,d]	2002[b,d]	2008[c,d]	2009[c,d]
Buzzards Bay	N/A	N/A	N/A	N/A	0.41 (0.15–0.69)	0.44 (0.21–0.71)
Cape Cod Bay	0.29	0.24 (0–0.57)	0.26 (0–667)	N/A	0.38 (0–0.75)	0.28 (0–0.55)
Monomoy	N/A	N/A	0.33 (0.25–0.42)	0.38 (0.27–0.49)	0.31 (0.17–0.46)	0.45 (0.26–0.63)
Nauset Estuary	N/A	N/A	0.38 (0.12–0.75)	0.39 (0.17–0.61)	0.43 (0.23–0.64)	0.43 (0.31–0.54)
Pleasant Bay	0.29	0.18 (0.07–0.30)	0.10 (0.02–0.19)	0.17 (0.08–0.27)	0.09 (0.05–0.14)	0.12 (0.09–0.15)
Wellfleet Bay	N/A	0.22 (0–0.46)	0.29 (0–0.62)	N/A	0.32 (0.10–0.56)	0.30 (0.10–0.50)

Notes: Approximate 95% confidence limits are given in parentheses. N/A = data not available (surveys not conducted).

[a] From Shuster (1979). Raw data not available; so no confidence intervals are calculated.

[b] Data adapted from James-Pirri et al. (2005).

[c] DMF and James-Pirri (unpublished data).

[d] Counts of horseshoe crabs by sex were made on various spawning beaches within regions. The sampling utilized a cluster sampling design with multiple sampling of spawning beaches within regions over a spawning season. Approximately 95% confidence limits were constructed using the exact binomial formula using the average number of horseshoe crabs within samples for each region and year as an effective sample size.

biomedical harvest, possibly exacerbated by the 2-year spike in bait harvest, might be disproportionately affecting females. These surveys, modeled after those in Delaware Bay, were conducted in 2000, 2001, 2002, 2008, and 2009 (James-Pirri et al. 2005; James-Pirri unpublished data). During that time period, sex ratios have become increasingly male-dominated in Pleasant Bay, while remaining fairly constant in all other Cape estuaries surveyed (Table 1). Pleasant Bay is the only embayment where only biomedical harvest is allowed, and where this fishery has been in effect for 30 years. The uniqueness of this situation led us to ask whether the biomedical fishery might be behind the sex ratio trend particular to that bay.

This trend was of particular concern because females are much more important in population dynamics than males (Caswell 2000; Grady and Valiela 2007) under the assumption that enough males are available to fertilize females' eggs. We considered several possible explanations for why ratios may have become male-skewed. First, egg production in females of many species has been shown to be an energetically expensive process (Bryant and Hartnoll 1995; Nicol et al. 1995; Guillou and Lumingas 1999; Chaparro and Flores 2002; Lovrich et al. 2005; Jørgensen et al. 2006). Assuming that this is true in horseshoe crabs, this investment of energy prior to the spawning season could render females more physiologically stressed or depleted than males by the bleeding process. Over 50% of harvest and bleeding takes place during or shortly after spawning. Such stress could lead to either higher mortality rates than seen in males, or failure of some females to spawn after bleeding, either of which could explain their relative absence on spawning beaches.

Second, 66% (Rutecki et al. 2004; DMF unpublished data) of the reported biomedical harvest out of Pleasant Bay has been female since 2001, so any biomedical-associated mortality would disproportionately affect females. Third, Rutecki et al. (2004) found that harvested females were significantly larger on average than those in the general Pleasant Bay population; thus the harvest selects for larger animals. Since larger females are more fecund (Leschen et al. 2005), selective harvest, if associated with high mortality or spawning inhibition, could reduce the number of eggs being produced over time. Determining whether the prevailing assumption of 5–15% mortality applied to female horseshoe crabs became an important question, particularly in the case of Pleasant Bay.

In reviewing the literature, we found that the study designs and handling of crabs in previous studies on bleeding mortality were not comparable to the conditions experienced by crabs bled in the Massachusetts biomedical industry. This discrepancy brought into question the applicability of mortality estimates from these studies to the biomedical fishery in Pleasant Bay. Walls and Berkson (2003) used only males, whereas we were primarily interested in females. Kurz and James-Pirri (2002) focused primarily on movement patterns and had small sample sizes. Rudloe (1983) kept the crabs out of water only long enough to draw blood and did not account for the substantial handling that biomedically bled horseshoe crabs undergo during the harvesting/bleeding process. Handling of crabs in the Massachusetts biomedical industry has typically included time spent on an open boat deck during harvest, an hour drive in a non-air-conditioned truck to the bleeding facility, bleeding suspended upside down in racks until the blood clots naturally, and 24 h out of water stacked in 30 gal Rubbermaid containers at room temperature before being returned to the water after another 1 h truck ride and 15 min boat ride. In addition, crabs may have been further stressed by being bled more than once in a season, as no reliable system of marking was in place for bled crabs until 2009. In another study, Hurton and Berkson (2006) used crabs that had been caught in a dragger fishery in July and August, rather than hand-harvested during spawning season. Nevertheless, the latter study found that females that underwent the highest bleeding level and stress had a mortality of 29.4%. This figure, which resulted from conditions most closely resembling those practiced in Massachusetts, and which is nearly double the 15% currently used when setting quota management, led us to believe that a study focusing on females that underwent realistic handling would be prudent. Hurton and Berkson's finding of 29.4% mortality has not previously been used in management because that study did not focus on that group – rather the figure was "diluted" in the discussion by being averaged in with lower mortality rates of males and less-handled females.

Meanwhile, discussions with the biomedical industry about handling practices led them to agree to obtain a refrigerated truck in which crabs would be transported and stored overnight, fill the barrels only two-thirds full to lessen pressure on the bottom crabs, and keep barrels covered with wet burlap to minimize evaporation from the crabs' gills. To avoid re-bleeding the same crab within a season they also agreed to make a small notch on the edge of the opisthosoma, alternating sides each year. DMF also theorized that returning the crabs to the water the same day as bleeding would likely increase survival, but this practice would increase handling costs and time for both the company and the fisherman who returns them to the water. We decided to test (1) the difference in mortality between unbled control female crabs and female crabs handled and bled according to the new best management practices (BMPs) agreed upon by DMF and the Massachusetts biomedical industry and (2) the difference between mortality in those two groups versus an additional treatment wherein the crabs were subjected to new BMPs but were returned to the water the same day they were bled. We limited our study to females for the reasons described above, because Walls and Berkson (2003) had studied male mortality (exclusively), and because tank space was limited and we wanted room for an adequate sample size of females.

Methods

Crabs were divided into three treatment groups and held in six flow-through seawater tanks at the Marine Biological Laboratory (MBL) in Woods Hole. This facility is located approximately 1.25 h drive from Pleasant Bay, and 15 min from the biomedical company. Tanks were prepared by placing approximately 5 cm of sediment on the bottom so that crabs could burrow, and running seawater through for several days to clear out fines and detritus in the sediment. Lights are on during the day, but the facility is dark at night. The total number of crabs assigned to each tank approximated an equal number of crabs per area for all tanks. Tanks differed by volume and shared a common water flow. A similar number of crabs from each treatment group was assigned to each tank (±1 for sizes not evenly divisible by 3). The three treatment groups were as follows:

1. Group (Treatment) 1 – control (unbled)
2. Group (Treatment) 2 – bled and handled according to new BMP but crabs returned to water the same day.
3. Group (Treatment) 3 – bled and handled according to new BMP but returned to water the morning following bleeding.

Approximately 310 female horseshoe crabs were obtained from the biomedical fisherman in Pleasant Bay early in the morning of 2 June 2009. Crabs were randomly assigned to one of the 18 categories: three treatments and six tanks. Each crab was measured (prosomal width – PW in mm), labeled with two lobster bands (cut in half lengthwise to facilitate doubling over, two bands used for security in case one came off) on the telson. Bands on each crab were one of three colors depending on group (treatment) assignment. After measuring and labeling, crabs were placed in Rubbermaid barrels labeled with their group and tank number. Sorting was carried out inside an air-conditioned truck cooled to approximately 10°C at the dock. Barrels with crabs waiting to be sorted were covered with wet burlap, as were all barrels after sorting was finished.

After sorting was completed, the truck was driven to the biomedical company. Groups 2 and 3 were dropped off for bleeding, and Group 1 crabs were driven to the MBL and were placed immediately in their respective tanks (time out of water was 4 h). Groups 2 and 3 crabs were bled at the biomedical facility according to the standard procedures which included placing the crabs upside down in racks, puncturing the arthrodial membrane with a needle, and allowing blood to drain into a collecting vessel until bleeding stopped on its own. Group 2 crabs were then driven to the MBL, for a time out of water totaling 6 h. Group 3 crabs remained inside the facility (as they normally would) until the truck returned. They were then transferred into the refrigerated truck where they remained overnight with the thermostat set at 8.9°C. These crabs were driven to the MBL at 6 am the next morning, for a time out of water totaling 25 h, as is the current practice. After being dropped at the MBL, Groups 2 and 3 barrels were left in the parking lot for 15 min and shaken occasionally, to simulate the 15 min boat ride they would normally have in Pleasant Bay before being returned to the water. They were then distributed to their assigned tanks.

Twelve crabs were rejected during sorting due to injury or other damage. Another 17 crabs (5.7% – 11 from Treatment 2 and six from Treatment 3) were sorted but later classified as not bleedable by the biomedical company because they did not meet qualifications for bleeding – injured, too small, damaged arthrodial membrane, etc. – were excluded from the study without replacement. A total of 99 crabs received Treatment 1 (Control), 89 received Treatment 2, and 93 received Treatment 3. Thus, crabs deemed not bleedable by the company were included in the Control group but not the treatment groups.

The tanks were checked daily for mortalities and dead crabs were removed. PW, band color, and date of death were recorded. MBL staff caring for the crabs was blind to treatments and did not know the details of the study or band color code until the study's conclusion. Temperature (T) and dissolved oxygen (DO) were recorded for each tank daily. Since crabs would presumably be foraging if returned to the wild, they were fed three times per week for the 17-day duration of the study. This time period was primarily a function of tank and funding availability.

Mortality rate was modeled as a function of treatment and tank with PW as a covariate using a generalized linear model (GLM) logistic regression with binomial errors using R software (R Development Core Team 2009). Several candidate models, ranging from a full model with interactions between treatment, tank and PW to just marginal treatment effects were evaluated using Analysis of Deviance. Marginal effects of treatment were examined using a model with treatment only. Difference in T and DO among the tanks was calculated for tank and day effects using two-way ANOVA.

Results

The first mortality was recorded on the third day of the study, and 84.3% of mortalities in the bled groups had occurred by day 6. Highest mortalities occurred on days 4 (13.7%), 5 (49%), and 6 (19.6%). The general pattern of die-off was consistent in the two bled groups. The Controls died on days 6, 7, and 17 (the one on day 17 had a previously undetected injury that may have caused its death).

Analysis of Deviance indicated that treatment and tank were significant factors in explaining mortality, but tank-interaction and PW as a covariate (Figure 2) were not significant (Table 2). Predicted mortality rates with approximately 95% confidence intervals by treatment and tank are provided in Table 3. Mortality rate for the Control was significantly different from Treatment 2 ($p < 0.001$) and Treatment 3 ($p < 0.001$). Treatments 2 and 3 were not significantly different from each other ($p = 0.31$).

Tank effects were relatively small compared with treatment effects. The odds ratio of dying in tanks 2–6 compared to Control in tank 1 ranged from 0.4 to 3.7. The odds ratio of dying from Treatments 2 and 3 compared to the Control was 10.3 and 14.6, respectively. The odds ratio for Treatment 2 compared to Controls in tank

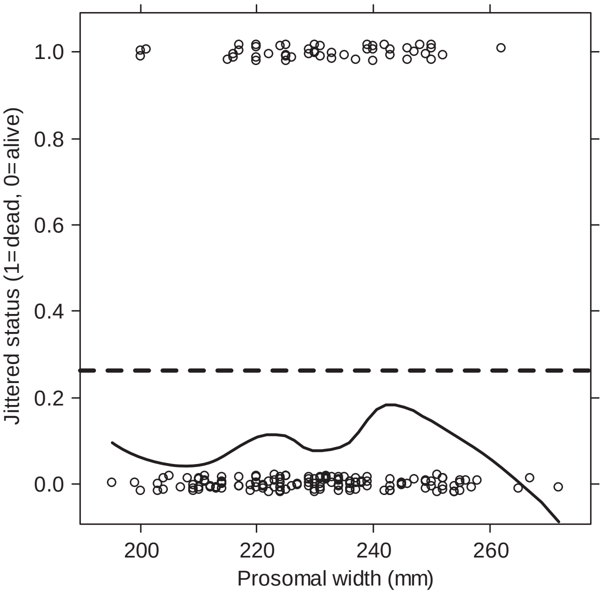

Figure 2. Jittered status (1 = dead, 0 = alive) by PW for Treatments 2 and 3 pooled. Black solid line is Loess fit, representing mortality at PW. Dashed line is mean mortality rate (0.26) for the two groups combined over all PWs.

Table 2. Analysis of Deviance for select GLM models.

Candidate model	Df	Residual deviance	Df	Deviance	Probability	Akaike information criteria
Treatment*tank*PW	245	200.884				272.88
Treatment*tank + PW	262	212.670	−17	−11.786	0.81	250.67
Treatment + tank + PW	272	223.833	−10	−11.163	0.35	241.83
Treatment + tank	273	223.919	−1	−0.086	0.77	239.92
Treatment	278	236.168	−5	−12.250	0.03	242.17

Notes: Model formula based on Wilkinson–Rogers notation.
*Indicates model includes estimating interaction term between factors or separate intercepts and slopes for covariates.

1 ranged from 4.4 to 38.2, while the odds ratio for Treatment 3 compared to Controls in tank 1 ranged from 6.3 to 54.4 (Table 3).

In the model including only marginal treatment effects, the mortality rate was 0.031 in Control, 0.225 in Treatment 2, and 0.298 in Treatment 3 (Table 4). Treatments 2 ($P < 0.001$) and 3 ($P < 0.001$) were significantly different from the Control. Treatments 2 and 3 were not significantly different ($P = 0.26$). Treatment 2 had 9 times and Treatment 3 had 13 times the odds of dying as in the Control group (Treatment 1).

Differences in densities between tanks were negligible (Table 5), and marginal tank predicted mortality was not related to tank densities. DO ranged from 5.91 to

Table 3. Predicted mortality rates by treatment and tank with approximately 95% confidence interval.

Treatment	Tank	Total crabs	Predicted mortality rate	Lower 95%	Higher 95%	Odds ratio
1 (Control)	1	16	0.017	0.004	0.069	
	2	21	0.028	0.008	0.096	1.6
	3	16	0.061	0.018	0.190	3.7
	4	13	0.008	0.001	0.046	0.4
	5	16	0.043	0.012	0.143	2.6
	6	16	0.024	0.006	0.090	1.4
2	1	15	0.152	0.063	0.321	10.3
	2	19	0.225	0.116	0.391	16.7
	3	13	0.400	0.228	0.601	38.2
	4	14	0.072	0.017	0.255	4.4
	5	14	0.314	0.167	0.511	26.2
	6	14	0.203	0.094	0.384	14.6
3	1	17	0.203	0.091	0.392	14.6
	2	21	0.293	0.163	0.467	23.7
	3	14	0.487	0.300	0.678	54.4
	4	9	0.099	0.024	0.329	6.3
	5	15	0.395	0.226	0.592	37.3
	6	18	0.265	0.135	0.455	20.7

Note: Odds ratio compares odds of dying in any cell with odds of dying as a control in tank 1 (0.02 : 1).

Table 4. Predicted mortality rate with approximately 95% confidence limit from a model using only treatments.

Treatment	Predicted mortality rate	Lower 95%	Higher 95%	Odds ratio compared with odds of control
1 (Control)	0.031	0.01	0.09	
2	0.225	0.15	0.32	9.2
3	0.298	0.21	0.40	13.4

Note: Odds ratio compares odds of dying in a treatment to odds in controls (0.032 : 1).

Table 5. Crabs m^{-2} in each tank after unbleedables removed from sample.

Tank	Crabs m^{-2}	DO (ppm) Mean (range)	T (C) Mean (range)
1	7.2	9.2 (8.4–9.7)	15.4 (14.8–16.1)
2	6.7	8.9 (7.2–9.6)	15.7 (14.8–16.3)
3	6.6	9.4 (8.4–9.9)	15.4 (14.8–16.0)
4	6.1	8.6 (5.9–9.9)	15.6 (14.9–16.4)
5	6.9	9.1 (8.0–9.8)	15.4 (14.8–15.9)
6	7.2	8.6 (7.6–9.1)	15.6(14.8–16.4)

Note: DO and T mean and range in each tank.

9.91 ppm with an overall mean of 8.95, well above the minimum of 5.0 ppm recommended for aquaculture systems (Timmons et al. 2001). Temperature ranged from 14.8 to 16.4°C with an overall mean of 15.5. A two-way ANOVA of DO indicated that tank ($p < 0.001$) and day effects ($p < 0.001$) were significant for DO. Similarly, ANOVA of T indicated significant tank ($p < 0.001$) and day effects ($p < 0.001$). Unlike other tanks, tank 4 showed a declining trend in DO over the first 8 days. Low values of 5.91 and 6.80 ppm were recorded on days 8 and 9 in tank 4, at which point it was discovered that flow into that tank was low; this was corrected, and DO values increased. A correlation between mean tank DO and marginal predicted tank mortality was 0.73 with a 95% $\text{CI} = -0.20 - 0.97$ and a ($p = 0.10$). Although not significant at 5% significance, the relatively high correlation and low probability suggests that variation in DO may have contributed to tank effects. No relationship was observed between marginal predicted tank mortality and either temperature or crab densities. Tank 4 contributed towards low mortality and had the lowest mean DO while tank 3 had the highest mean DO and highest marginal tank mortality.

Discussion

Our study showed higher female mortality from biomedical bleeding than previously reported, except the group of females with the highest level of bleeding and handling in Hurton and Berkson (2006), where mortality was virtually identical to that of our Group 3 (29.4% and 29.8%, respectively). These mortality rates are considerably higher than those currently used for management in both Massachusetts and by ASMFC (15%). It is worth noting that our study did not include males; as such, we recommend management to use the male mortality based on previous studies such as Walls and Berkson (2003).

In this experiment, 12 crabs were rejected by DMF because of injury or other defects at the dock. Another 17 crabs were rejected by the biomedical company from Treatments 2 and 3 as unfit for bleeding. Our estimate of mortality rate does not include the harvest-induced mortality component of rejected crabs. Since crabs deemed not bleedable were included in the Control group but not in the treatment groups, the odds ratios for the treatments compared to the Control may have been underestimated if non-bleedable crabs have higher mortality rates in captivity. This potential bias would have the impact of underestimating the difference in mortality between the control and the two treatments. The biomedical company's typical percentage of crabs that do not meet criteria for bleeding due to injury or death for MA biomedical crabs is 5.0%; it was 5.7% in this study.

Although we observed a significant tank effect, the only obvious pattern that emerged is that tank 4 exhibited particularly low mortality (Table 3). Although DO and T varied among tanks, the lowest DO was recorded in tank 4, where mortality rate was the lowest. No obvious positional, lighting, or other differences among the tanks were discernable that could explain the difference in mortality. The source of variation in mortality among tanks remains unexplained and may simply reflect type I error.

Until now, the absence of annual quota for the biomedical horseshoe crab fishery in Massachusetts and the ASMFC annual biomedical quota have been based on an assumption of no more than 15% mortality. Our mortality rate of 30% for the treatment mimicking current practices may, if anything, underestimate actual bleeding mortality for several reasons. First, crabs in our study were handled with more care than has been the practice of the biomedical company over the past 30 years – air-conditioned truck and storage, less-full barrels, barrels covered with wet burlap, and notching to prevent re-bleeding. Mortality under the old practices may have been even higher. Second, harvest-associated mortality of rejected crabs was not followed or included in the data. We note that crabs can be rejected due to injury as well as damage to the arthrodial membrane.

Our study did not address the question of whether there are sublethal effects on spawning behavior. This question deserves more study since sex ratios are becoming highly skewed toward males, yet the biomedical harvest, which occurs away from the spawning beaches, is still highly skewed toward females. Although some bled (notched) crabs have been sighted on spawning beaches, this apparent inconsistency in sex ratios on and off the spawning beaches may indicate that some bled females still present in the population may not be appearing on beaches to spawn.

The biomedical industry contributes not only to local economies where they are sited, but also provides essential products necessary for human health and safety. Curtailing their operations by restricting harvest may not be desirable in the short term. However, having marked decline in populations could permanently reduce biomedical harvest and undermine the industry. Results of this study show that previously reported mortality rates of bled horseshoe crabs may not accurately reflect deaths of crabs harvested and handled in the Massachusetts biomedical industry.

Conclusion

Amidst growing concern about horseshoe crab populations in Massachusetts and sex ratios in Pleasant Bay, managing the fishery for sustainability is increasingly important. Management must therefore be based on realistic estimates of total mortality that reflect handling and bleeding procedures to which local biomedical crabs are subjected. This study provides estimates of female mortality, which are disproportionately more important than that for males, both because of their importance to population dynamics and because they are preferentially harvested.

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Effects of the Biomedical Bleeding Process on the Behavior of the American Horseshoe Crab, *Limulus polyphemus*, in Its Natural Habitat

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Abstract. Horseshoe crabs are harvested by the biomedical industry in order to create *Limulus* amebocyte lysate to test medical devices and pharmaceutical drugs for endotoxins. Most previous studies on the impacts of the biomedical bleeding process on horseshoe crabs have focused on mortality rates and sublethal impacts in the laboratory. In this study, we investigated the effects of the bleeding process on the behavior of horseshoe crabs after they had been released back into their natural environment. A total of 28 horseshoe crabs (14 control and 14 bled) were fitted with acoustic transmitters and released into the Great Bay Estuary, New Hampshire, during the spring of 2016. The acoustic tags transmitted information about the activity and depth of each animal, and these data were logged by an array of passive acoustic receivers. These data were collected from May to December 2016 and from March to October 2017. Bled animals approached mating beaches less than control animals during the first week after release, with the greatest differences between bled and control females. Bled animals also remained significantly deeper during the spawning season than control animals. However, overall, bled and control animals expressed similar biological rhythms and seasonal migrations. Thus, it appears as if the most obvious impacts of the bleeding process take place during the first one to two weeks after crabs are bled.

Introduction

The American horseshoe crab, *Limulus polyphemus*, is an ecologically and economically important species found in bays and estuaries along the Atlantic coast of North America, including the Great Bay Estuary (GBE), New Hampshire. Horseshoe crabs play an important ecological role as bioturbators, as a result of foraging for food (Krauter and Fegley, 1994; Lee, 2010); and their eggs are a vital food source for 425,000 to 1 million migratory shorebirds (Walls *et al.*, 2002; Botton *et al.*, 2010). They are also harvested for use as bait for the eel and whelk fisheries (ASMFC, 1998, 2012) and for their blood, which is used to create *Limulus* amebocyte lysate (LAL) (Novitsky, 2009). LAL is used in the biomedical industry to test medical devices, vaccines, and pharmaceutical drugs for pathogenic gram-negative bacteria (Novitsky, 2009; Chen and Mozier, 2013).

Currently, while quotas and regulations have been placed on the bait fishery (ASMFC, 2012), the biomedical fishery remains fairly unrestricted; and harvest rates continue to increase in certain areas (ASMFC, 2013), which could have deleterious effects on populations of this valuable marine species. A quota system and several complete closures of coastal waters have been implemented for harvesting horseshoe crabs for the eel and whelk fisheries (ASMFC, 1998, 2012), and this led to a significant decrease in the commercial harvest levels of horseshoe crabs, from around 2 million crabs in 2000 to 600,000–700,000 crabs in 2014 (ASMFC, 2013). In contrast, the number harvested for the biomedical industry continues to increase, with levels climbing from 340,000 crabs in 2004 to 610,000 crabs in 2012 (ASMFC, 2013).

With the growing demand for LAL as the global population expands, medical advancements improve, and medical needs increase, it is critical to understand the consequences

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Abbreviations: BMP, best manufacturing process; GBE, Great Bay Estuary; IO, interocular; JEL, Jackson Estuarine Laboratory; LAL, *Limulus* amebocyte lysate.

of the biomedical bleeding industry on horseshoe crabs' fitness and population dynamics. The capture process for this industry includes multiple stressors, such as air exposure (time on docks, boats, and trucks) and warm temperatures (on boat decks during the summer or in poorly temperature-controlled facilities and transport vehicles). In addition, the blood extraction process itself can compromise the health of the horseshoe crab, because ~30% of the estimated blood volume of individual horseshoe crabs is extracted (James-Pirri *et al.*, 2012). Female horseshoe crabs are preferentially chosen for this process because of their larger size and subsequently greater blood volume (Rutecki *et al.*, 2004; James-Pirri *et al.*, 2012); this has led to skewed sex ratios in some areas (Leschen and Correia, 2010). Each of the four major biomedical companies that bleed horseshoe crabs has slightly different collection, handling, and bleeding processes; and the extent to which they follow best manufacturing practices (BMPs) likely varies, depending on region. These BMPs include keeping them moist, avoiding bleeding injured animals or those that can be identified as having been bled before, and returning them to their point of capture within 24 hours.

Mortality rates associated with the bleeding process range from 5% to 30% (Rudloe, 1983; Thompson, 1998; Walls and Berkson, 2000, 2003; Kurz and James-Pirri, 2002; Hurton and Berkson, 2006; Leschen and Correia, 2010; Anderson *et al.*, 2013), with a differential mortality rate between sexes (15% mortality in males and up to 29% in females; Leschen and Correia, 2010; James-Pirri, 2012). Sublethal impacts include delayed blood volume recovery, reduced blood protein levels, and behavioral deficits. Specifically, Novitsky (2009) found that in laboratory holding tanks it takes three to seven days for a bled horseshoe crab to regain its total blood volume and up to four months for amebocytes to return to baseline levels. Captive bled animals exhibit significantly lower blood protein values, signifying that biomedical bleeding may have prolonged impacts on horseshoe crab physiology; and bled crabs released back into their natural environment displayed a more random pattern of movements than control animals (Kurz and James-Pirri, 2002; James-Pirri *et al.*, 2012). Finally, Anderson *et al.* (2013) found changes in the bled horseshoe crab's activity levels, expression of circatidal rhythms, linear and angular movement velocities, and hemocyanin levels.

Although the harvest process used by the biomedical fishery is considered low impact and classified as "minimally harmful to horseshoe crabs" (ASMFC, 2012), the aforementioned detrimental effects could alter population dynamics and could lead to long-term declines (Krisfalusi-Gannon *et al.*, 2018). For example, because females are preferentially bled and because there is a higher mortality rate in females, this could lead to an overall decline in female fecundity and altered sex ratios (Le Moullac and Haffner, 2000; James-Pirri *et al.*, 2005; Leschen *et al.*, 2006; Leschen and Correia, 2010). The sublethal effects of biomedical bleeding on activity levels, expression of tidal rhythms, and movement velocities (Anderson *et al.*, 2013) may disrupt activities such as foraging and spawning and may reduce crabs' ability to find mates and appropriate spawning beaches, thus leading to declines in reproductive output (Powers and Barlow, 1985; Barlow *et al.*, 1986, 2001; Herzog *et al.*, 1996; Barlow, 2001). Finally, extended periods of reduced hemocyanin levels may cause additional respiratory stress and increased susceptibility to infection because hemocyanin plays a major role in immune function and wound repair (Adachi *et al.*, 2005; Coates *et al.*, 2011).

With the exception of a study by Rudloe (1983) on the mortality rates of bled animals and a study by James-Pirri *et al.* (2012) on the impacts of bleeding on horseshoe crab orientation, all relevant studies regarding biomedical bleeding effects on horseshoe crabs have been carried out in the laboratory. Therefore, the major goal of this project was to determine the behavioral and physiological effects that the bleeding process has on horseshoe crabs that are released back into their natural environment. The animals in this study were collected from, and released back into, the GBE. This population of horseshoe crabs has not been previously harvested for biomedical bleeding (ASMFC, 2012), and a great deal is already known about the behavior of horseshoe crabs in this estuary. For example, Schaller *et al.* (2010) and Watson *et al.* (2016) found that horseshoe crabs remained in GBE year-round but that they changed depths and locations in the estuary as temperatures changed throughout the year. In the spring (March–April), when water temperatures exceeded 10–11 °C, animals traveled to shallower areas and moved to spawning beaches at high tides. After spending the summer and early fall scouring the mudflats for food, they moved down the estuary into deeper waters in the late fall to overwinter. If bled horseshoe crabs express these same patterns of behavior, then we can conclude that the bleeding process does not impact them when they are released back into their natural habitat.

In this study, before being released into GBE, designated animals underwent the bleeding process; then all animals, both bled and controls, were fitted with acoustic transmitters to monitor their movements, the times when they were active, and their preferred depths. These data were collected for about two years and were used to discern whether the bleeding process had an impact on spawning activity, the expression of daily and tidal rhythms, overall activity and distances traveled, and seasonal migrations.

Materials and Methods

Animal collection and tagging

A total of 28 (14 male, 14 female) healthy adult American horseshoe crabs, *Limulus polyphemus* (Linnaeus, 1758), were hand collected during high tide from a spawning beach on Adams Point, Durham, New Hampshire, in May 2016 (Fig. 1). All captured crabs were brought back to the University of New Hampshire's Jackson Estuarine Laboratory (JEL) and held in flow-through estuarine-water tanks until they under-

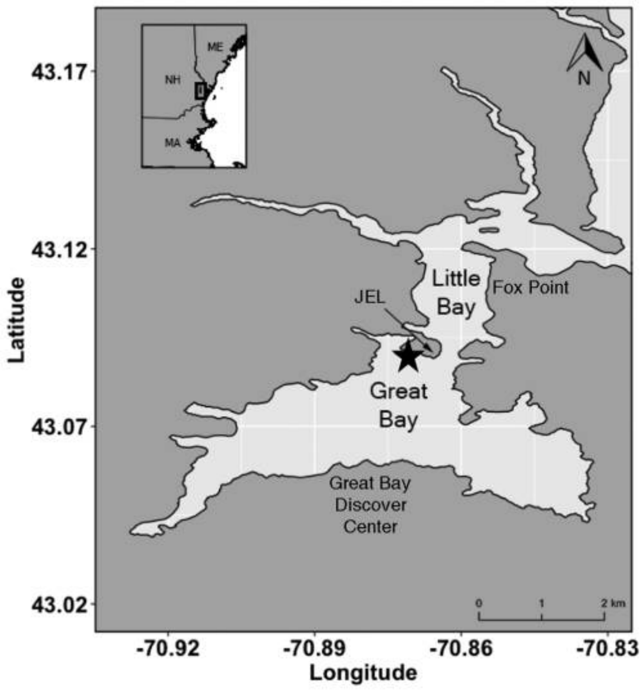

Figure 1. Map of the study location in the Great Bay Estuary, New Hampshire. The star represents the collection and release site of horseshoe crabs at Adams Point, Durham, New Hampshire. The arrow indicates the location of the Jackson Estuarine Laboratory (JEL)

went their designated treatment. Half of the animals (7 males: inter-ocular [IO] width = 8.21 ± 0.61 cm [SD]; 7 females: IO width = 11.56 ± 0.52 cm) were used as controls, while the remaining half (7 males: IO width = 8.31 ± 0.47 cm; 7 females: IO width = 11.42 ± 0.63 cm) were bled according to the industry standard procedures typically followed by the biomedical bleeding facilities, as outlined below (see *Bleeding procedure*). However, it should be noted that these procedures might vary from facility to facility and from state to state.

After treatments (bleeding or not), all horseshoe crabs were fitted with VEMCO V13AP ultrasonic transmitters (69 kHz, 147-dB low-power output, 13-mm diameter, 48-mm length, 6.5 g in water, estimated battery life of ~530 days; VEMCO, Bedford, Nova Scotia, Canada). The V13AP transmitters were programmed to transmit acceleration and depth data at random intervals about every three minutes. They were also programmed to turn off in December 2016 and then turn back on in March 2017. A transmitter was attached to the dorsal carapace of each individual, using the following method. First, it was superglued into a piece of plastic tubing that had two cable ties attached to it. The cable ties were then affixed to the carapace by using small screws. Finally, duct tape was superglued over the entire harness to ensure that the fixture would not become caught on underwater obstructions. In addition, male claspers were secured in the closed position with cyanoacrylate glue to eliminate their ability to attach to females. This ensured that data from males represented their activities and not those of a female to which they were attached. After the transmitters were attached, the animals were released into the GBE at the same spawning beach where they had been previously collected.

Bleeding procedure

Pre-bleeding treatment. The bleeding process, replicating industry standard procedures, took a total of three days (May 13–15, 2016). Half of the 28 horseshoe crabs ($n = 14$, 7 males, 7 females) were randomly selected to undergo the bleeding procedure. The animals in this treatment group were evenly distributed between two 50-gallon plastic barrels. HOBO temperature loggers (Onset Computer Corporation, Bourne, MA) were placed in each of the barrels to record temperature. The control animals (7 males, 7 females) remained in the flow-through tanks at JEL (14.1 ± 1.4 °C, mean ± SD) until transmitters were attached, and then they were released at the same location where they had been collected, which was also the same location where the bled animals were released.

The 50-gallon barrels with the treatment animals were placed outside of JEL in direct sunlight for 4 h, or next to a space heater in the JEL greenhouse (depending on the temperature and ambient sunlight during the selected day), to replicate the duration of time spent on the deck of a boat or a dock prior to transport to biomedical facilities. The average temperature that the animals experienced during this time was 32.6 ± 2.7 °C. After the first 4 h, the barrels were placed in the back of a car and were driven for an additional 4 h, to simulate time spent in a truck traveling to a bleeding facility (23.2 ± 1.7 °C). After these 4 h, the barrels were placed indoors at JEL for 16 h, to simulate time spent overnight at a bleeding facility (20.7 ± 0.6 °C). Finally, after 16 h, hemolymph was extracted as described below.

Hemolymph extraction. Hemolymph was extracted following the procedure of Armstrong and Conrad (2008), with modifications from Anderson *et al.* (2013). The arthrodial membrane between the prosoma and the opisthosoma of each horseshoe crab was exposed, and the hinge joint was sterilized with 70% ethanol. An 18-gauge syringe needle was inserted into the membrane, and hemolymph was collected in pre-chilled 50-mL conical tubes until 30% of total hemolymph volume had been reached or until the blood flow stopped. Total hemolymph volume for each individual was calculated using the following equation from Hurton *et al.* (2005):

$$H = 25.7\ e^{0.1928(IO)}\ [H = \text{hemolymph volume (mL)};\ IO = \text{interocular width (cm)}].$$

Post-bleeding treatment. To minimize the amount of handling the treated horseshoe crabs experienced, immediately after they were bled transmitters were attached to them, and they were returned to their respective barrels. For the control animals, transmitters were attached at the same time, but they were returned to the flow-through tanks at JEL. The bled animals remained in their barrels overnight to replicate a second night at a bleeding facility (20.5 ± 1.1 °C). Then the barrels were placed back into a car for 4 h (21.9 ± 0.9 °C) to simulate transportation back to the dock, where they would be loaded on vessels and returned to their capture location. Finally, all 28 horseshoe crabs were returned to their collection site at Adams Point and released into the estuary.

Acoustic telemetry/tracking

An array of VR2W acoustic monitoring receivers ($n = 11$; 69 kHz; VEMCO) was set up throughout the estuary, ranging from Fox Point to the Great Bay Discovery Center (Fig. 1). The receivers were deployed ~0.5 km apart and were attached to a mooring line suspended ~5–10 m from the bottom, or placed in an empty lobster trap, depending on the depth of the listening station (some areas were only ~2 m deep at low tide). Based on previous range tests, each receiver was capable of detecting a horseshoe crab with a transmitter attached when it was within 200–500 m of the receiver. However, this range varied as a result of currents, turbidity, topography, weather events, and high winds. The transmitters were programmed to take depth (m) and triaxial (x, y, z) acceleration (m s^{-2}) readings at 5 Hz within a 25-s period. Then, every 70–140 s the acceleration data (root mean square of the three axes [m $s^{-2} = (x^2 + y^2 + z^2)^{1/2}$ averaged over T, time]) obtained during the most recent 25-s period (T), or the depth at that time, were transmitted (depth and acceleration transmissions would alternate). Based on specifications for these transmitters, the proportion of time for which acceleration was measured was only 12% of every 2–5 min. Each time a transmission was received, the receiver would record the date and time and either the acceleration or the depth of the animal.

Receivers were downloaded in VUE software 2.3.0 (VEMCO) every one to two weeks in the spring and summer and about once every month in the fall, and they were removed from the water in the winter after the date the transmitters were programmed to shut off. After each download, if the receiver had multiple detections of different animals, it would be kept in the same location. If the receiver did not contain a viable number of detections, we would move it to a more suitable location. HOBO temperature data loggers were attached to several of the receiver mooring lines to record water temperature every five minutes for the duration of the project. The temperature data loggers were placed near the release site, at JEL, and in the middle of Little Bay. Near the middle of Great Bay, temperature data were collected by a buoy that was deployed by the NOAA National Estuarine Research Reserve System (NERRS). This buoy was located on the outskirts of the deep channel, with the temperature logger ~1 m below the surface of the water.

A VR100 acoustic receiver and a VH165 omni-directional hydrophone (VEMCO) were also used to manually track horseshoe crabs. The hydrophone was plugged into the VR100 receiver and was slowly towed behind a research vessel to locate tagged horseshoe crabs. If a horseshoe crab was within range, the geographical position (GPS coordinates), depth, and/or acceleration were logged in the VR100. These data were downloaded after each trip in VUE software and were used to help determine the location of animals and the best positions for deploying receiver stations.

Data analyses. A previously determined threshold value of 0.1 m s^{-2} (Watson *et al.*, 2016) was used to classify an animal as either active or inactive based on accelerometer tag output. Data were lumped into 10-minute bins; and if an animal exceeded the threshold value during any of the minutes in that 10-minute period, the animal was considered to have been active during that 10-minute period. These values were entered into the program ActogramJ to create actograms that could be used to determine the types of rhythms expressed by individual horseshoe crabs (Schmid *et al.*, 2011). Periodograms, using the Lomb-Scargle method, were used to determine when animals expressed significant circatidal (~12.4-h) or circadian (~24-h) rhythms (peaks exceeding $\alpha = 0.001$; tidal: 10–14-h range; daily: 22–26-h range; arrhythmic: no significant peaks).

For most analyses, we used data only from animals that were detected for more than seven days in a row in a given month. Three-way ANOVAs were used to test for effects of treatment group (bled and control), years (2016 and 2017), and sex (males and females) on days at large and also on ranges of movements in the estuary. Days at large were calculated as the first day in a season an animal was detected until the last day an animal was detected. Ranges were measured as the distance from the animal's farthest up-estuary position to its farthest down-estuary position. Two-way ANOVAs were used to test for effects of treatment groups and sex on mating and also on depth changes. A MANOVA was used to look at the impacts of sex, treatment groups, and months on percentage of time active and depth in different animals. In all cases, the time active, movements, and rhythms of control animals were compared to the experimental animals. Tukey's honest significant difference *post hoc* analyses (with a level of significant difference set at $P < 0.05$) were used to examine differences between means of treatment groups, monthly depths, rhythms expressed at different depths, and sexes. Correlational and single linear regression analyses were used to determine relationships between temperature and years, as well as between activity and depth across months. Unpaired Student's t tests were used to compare depth or activity between treatment groups.

To determine whether horseshoe crabs were approaching spawning beaches, changes in animals' depths and tide heights were examined together, along with their activity. Water depth and tide height data were obtained from the Squamscott River Monitoring Station (data provided by the NOAA Tide Predictions website, https://tidesandcurrents.noaa.gov/noaatidepredictions.html?id=8422687) and the Great Bay

Buoy (data provided through the Northeastern Regional Association of Coastal Ocean Observing Systems, Portsmouth, NH, and University of New Hampshire Center for Coastal Ocean Observation and Analysis, Durham, NH). Horseshoe crabs were considered "spawning" if they showed high activity levels (>0.1 m s^{-2}; Watson *et al.*, 2016) around the times of high tides, commensurate with a movement to a shallower location, during the peak of the spawning period in 2016. Only data from the first two weeks after release were used for these analyses, for two reasons: (1) this time period represented the peak of the spawning season in 2016 in the GBE, and (2) most of the animals were still within the VR2W array and near the spawning beaches where we collected them, which enabled us to obtain the maximum possible amount of data from the most animals before they dispersed. For each animal we determined how many high tides occurred while we were collecting continuous data from them. Then, to determine the spawning percentage, we divided the number of apparent spawning events (identified as described above) by the number of available high tides they experienced.

Results

Days at large and days detected

A total of 28 horseshoe crabs were successfully tracked in the GBE, from May 15 to December 6, 2016 (205 days); and data were also obtained from 23 of these between April 14 and October 4, 2017 (191 days). In 2016, animals were at large for an average of 158 ± 59 days (max = 205, min = 7) and were detected for 84.3 ± 50 days (max = 180, min = 8). In 2017, the 23 animals were at large for 91 ± 56 days (max = 172, min = 1) and were detected for 32.4 ± 41 days (max = 171, min = 0).

Potential mating events

Based on data from New Hampshire *Limulus* spawning surveys, which are conducted each year, including during this study (Cheng *et al.*, 2016), the mating season for 2016 started on May 9, when temperatures reached 11.2 °C; it peaked around May 21–26; and it ended around June 10. The mating season for 2017 started on May 16, when temperatures reached 11.3 °C; it peaked around June 10–16; and it ended around June 20. We used these data to focus our analyses of potential impacts of the bleeding process on mating behavior on the peak spawning period that occurred right after animals were released.

The criteria outlined in Materials and Methods were used to determine whether horseshoe crabs approached spawning beaches to possibly mate (Fig. 2). Out of all the times animals were detected during each high tide within the first week after they were released, females appeared to mate less than males, bled animals less than controls, and bled females much less than control females (Fig. 3; Table A1). More precisely, control females appeared to spawn 4.8 ± 2.5 times during the first week after they were released, while bled females spawned only 2.0 ± 0.7 times. Out of the animals that were detected for at least two weeks, there was no difference in apparent mating events between the first and second weeks post-release (Student's paired t test, $P = 0.16$; data not shown). These analyses were not performed in 2017 because there were not enough animals present near the spawning areas that were being monitored with VR2W receivers, and they did not appear to approach beaches to mate for a long enough time period to provide sufficient data for a rigorous analysis.

Biological rhythms

2016. Horseshoe crabs from which we obtained sufficient data for biological rhythm analyses (*i.e.*, at least seven days of continuous data) exhibited both tidal and daily rhythms. In June, all animals expressed tidal rhythms ($n = 12$; $\tau = 12.4$ h; Table 1; Fig. 4). This corresponds to the mating season in 2016 in GBE. In the following months they exhibited a combination of arrhythmic, daily, and tidal rhythms (Table 1; Figs. A1, A2). While there were more control animals detected, the majority of both treatment groups expressed clear daily or tidal rhythms from June to October 2016. When we added up all of the months during which a given animal expressed a clear rhythm, control animals were rhythmic during 30 of 32 months, while bled animals expressed clear biological rhythms in 15 of 16 months (Table 1). All animals that expressed daily rhythms ($\tau = 24$ h; Fig. A1) were more active during the day than at night, except for Animal 75, which was more active during the night in July and September. There was no significant difference between the depths where animals resided in months that they displayed daily rhythms (4.4 ± 1.4 m) *versus* tidal rhythms (3.3 ± 1.3 m; unpaired Student's t test, $P = 0.5$). This was also true for the relationship between depths and rhythms for animals that switched from one kind of rhythm to another (Fig. A2). There was also no clear relationship between sexes and the expression of different rhythms (Table 1). Finally, four animals switched from a tidal rhythm to a daily rhythm in July, directly after the mating season (Table 1; Fig. A2).

2017. In 2017, these same animals continued to express the same variety of rhythms (Table 1). Since different animals were detected at different times in 2017, there was a lower sample size and a greater distribution of rhythms, so we have chosen to discuss the two years separately. In April, before the start of the mating season, horseshoe crabs exhibited tidal rhythms or were arrhythmic. Interestingly, all of the bled animals ($n = 4$) were arrhythmic. After April, there was no clear difference between the types of rhythms exhibited by both groups. In June, only 1 animal expressed a tidal rhythm in 2017, while 13 did so in 2016; but there were also fewer animals detected overall. As in 2016, all animals that displayed daily rhythms were more active during the day than at night. In contrast to 2016, there was a significant relationship between the depths

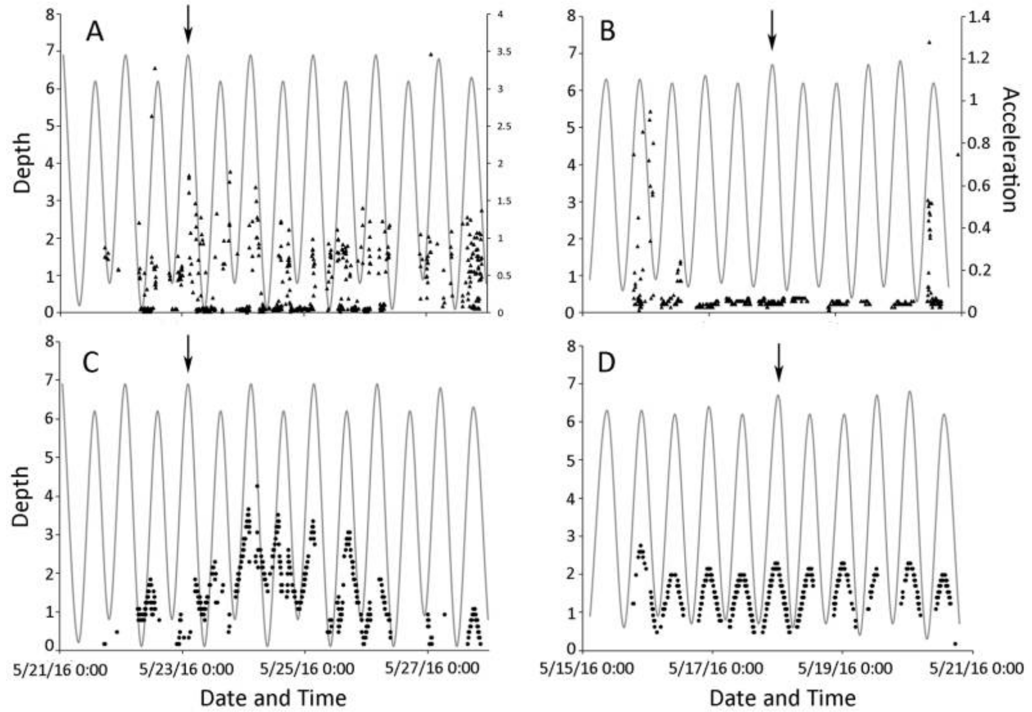

Figure 2. Potential mating events recorded from one control female (A, C) and one bled female (B, D) horseshoe crab. The gray lines in all graphs indicate the natural tides (in feet, so they can be distinguished from the depth of animals, which is shown in meters). The top graphs show the accelerometer output (m s^{-2}) of the tags on a control female (A) and a bled female (B). The filled circles in the bottom two graphs show the depth (in meters) of both females. Note that the bled female did not move much, so her depth followed the tides very closely. In contrast, the control female was very active, especially during high tides, and sometimes she moved into shallow water during a high tide (at times indicated by the arrows in A and C). We considered these to be potential mating events.

occupied by animals and the types of rhythms they expressed. Animals that were arrhythmic resided in deeper water (9.8 ± 4.5 m) than animals that expressed tidal rhythms (4.7 ± 2.4 m) or daily rhythms (4.6 ± 1.1 m; ANOVA, $F_{2,21} = 8.22$, $P = 0.002$).

Ranges of animal movements

All of the horseshoe crabs appeared to remain within the GBE for the duration of the study. The farthest an animal moved from the release site toward the coast was 3.2 km, and the farthest an animal traveled up-estuary was 3.4 km. In 2016, the mean annual range of movement (distance from the animal's farthest up-estuary position to its farthest down-estuary position) was 3.3 ± 1.7 km, and in 2017, it was 3.2 ± 1.3 km. There was no difference in range for years, between treatments, or between sexes (Table A2).

In 2017, 5 out of the 23 detected animals returned to their release site (Fig. A3). Four of these animals returned during the mating season in May–June. All four were males, three were controls, and one was a bled animal. The fifth animal was a control female that returned to the release site in late July. Because this is generally two to three weeks after horseshoe crabs stop spawning in the GBE (Schaller *et al.*, 2010; Cheng *et al.*, 2016; Watson *et al.*, 2016), it probably was not spawning at that time.

Overall seasonal changes in behavior

The water temperature in the GBE ranged from 9.5 to 25.4 °C during the time when we were tracking animals, and there was no difference in the mean water temperature between the 2 years of the study (2016: 18.9 ± 3.6 °C; 2017: 18.12 ± 3.2 °C; $P < 0.0001$). We also ran a regression comparing the daily temperatures in each year, and they were highly correlated ($r = 0.85$, $P < 0.0001$).

The majority (21 of 23) of the animals that were detected in 2017 were first detected near the location where they were last detected in 2016. The 2 animals that were not detected at their exact location from the previous year were still in Little Bay but ±2.3 km up- or down-estuary from their last location. Therefore, although transmitters were turned off during the winter season, all of the animals appeared to have remained in the same location throughout the winter months.

Seasonal changes in depth and activity. Only animals that were detected and active for at least seven days in a given month were used for depth and activity analyses (2016: $n = 21$; 2017: $n = 11$). In 2016, there was a significant correlation

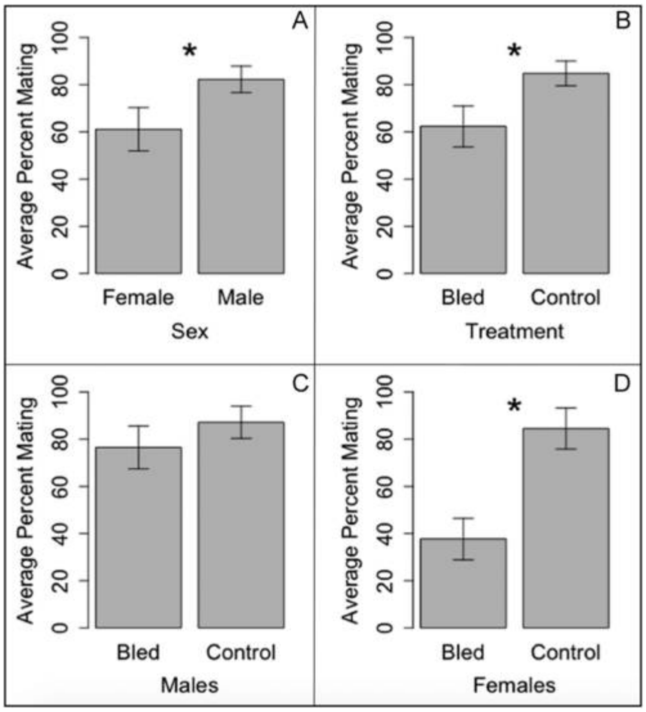

Figure 3. Average percent (±SEM) of times that horseshoe crabs appeared to be mating as a function of the number of high tides they experienced during the first week after they were released. (A) Females ($n = 10$) appeared to mate less than males ($n = 13$). (B) Bled animals ($n = 12$) appeared to mate less than control animals ($n = 11$). (C) There was no difference between control males ($n = 6$) and bled males ($n = 7$). (D) Bled females ($n = 5$) appeared to mate less than control females ($n = 5$). Asterisks indicate statistically significant differences ($P < 0.05$).

between activity and depth ($r^2 = -0.35$, $P = 0.01$), with animals being more active when they were in shallower water (Fig. A4). In addition, animals tended to be more active from May to August when water temperatures were warmer (≥~18 °C). There was no difference between bled and control animals in terms of the percentage of time when they were active (unpaired Student's *t* test, $P = 0.4$). As water temperatures started to decrease in the fall, animals moved deeper and exhibited a lower amount of activity, and bled animals spent significantly more time in deeper water in 2016 than control animals (Fig. 5; Table A3). Finally, 15 of the 21 animals that migrated from Great Bay to Little Bay (toward the coast) moved in late July to early August when temperatures peaked at 22 °C. The following year, 14 of 15 of those animals moved back into Great Bay when temperatures reached 11.2 °C in May.

In 2017, only four animals (three males and one female) were detected continuously, and were active, for at least seven days in a month. Based on data from only these four animals, there was a significant difference between month and depth (MANOVA, $F_{6,19} = 3.32, P = 0.021$) but no significant difference between activity and month (MANOVA, $F_6 = 1.410$, $P = 0.262$).

Summary of seasonal movements. Most of the animals in this study followed the same seasonal trends of activity and depth preferences that were reported in two previous studies in the GBE (Fig. 6; Schaller *et al.*, 2010; Watson *et al.*, 2016). Animals overwintered in deep water and remained there until water temperatures started to exceed ~11 °C in the spring. At this time, they moved up into the estuary, where it tends to be shallower, and they had a higher level of activity during warmer months of the year. Then in the fall they returned to deeper water as temperatures began to drop. It should also be noted that when water temperatures exceeded 20 °C, 8 of the animals in 2017 moved out of Great Bay to Little Bay, where the water is a bit cooler, which is exactly what these same animals did at the same time in 2016 (late July to early August).

Between 2016 and 2017, there were no major differences in the seasonal migration trends, or annual ranges, of bled *versus* control animals (Fig. 6). However, there were some distinct changes in the seasonal movements of some of the bled animals from one year to the next. One noticeable trend was that in May and June of 2017, the bled animals did not approach shallower areas in Great Bay, preferring to remain in deeper channels. Out of all of the animals that were detected in 2017 (regardless of whether they were also detected at the same time in 2016), bled animals remained in deeper waters than controls (unpaired Student's *t* test, $P < 0.001$).

Discussion

While some of the impacts of the biomedical bleeding process have been studied in the laboratory and in the field, this seems to be one of the first comprehensive studies designed to determine the behavioral impacts that the bleeding process has on the horseshoe crabs that are released back into their natural environment. The two most obvious impacts we found were that (1) bled females appeared to approach beaches to mate less frequently than control females, and (2) bled animals remained in deeper water than control animals during certain times of the year. However, there was no difference in the annual ranges of bled *versus* control animals or in their overall distribution in the GBE across months or years.

Mating

Since it is well known that horseshoe crabs mate at high tide at spawning beaches during the spring and early summer (Rudloe, 1980; Cohen and Brockmann, 1983; Barlow *et al.*, 1986; Cheng *et al.*, 2016), it was possible to use a combination of depth, tide, and accelerometer data to determine possible mating events of animals fitted with appropriate acoustic transmitters. We found that control animals probably mated, or at least moved inshore toward mating beaches during the high tides, more often than bled animals during the first tracking season, especially when comparing bled *versus* control females. These data suggest that the bleeding process might have an immediate impact on the reproductive output of fe-

Table 1

Rhythms expressed by individual horseshoe crabs for each month in 2016 and 2017

			2016					2017					
Animal no.	Treatment	Sex	June	July	August	September	October	April	May	June	July	August	September
69	B	F	T	T									
70	B	F	T	T		T		A	A	A		A	
71	B	F	T	A									
39	B	M	T										
73	B	M			T				T				
74	B	M			T			A	T			D	
75	B	M		T/D	D	D		A					D
85	B	M			D			A	A				D
87	B	M	T	T									
37	C	F	T	T/D	T	T	A						
38	C	F	T	T									
76	C	F	T	D	D								
77	C	F		T	T				D			D	
80	C	F	T	T/D	T	T			D		D		
37	C	F	T	T/D	T	T	A	T	D	T	D		
38	C	F	T	T	D								
68	C	M	T	T	T		D						
36	C	M	T	T	T		D						

A, arrhythmic; B, bled; C, control; D, daily rhythm; T, tidal rhythm; T/D, both tidal and daily rhythms. Empty cells indicate that data were not sufficient to identify a type of rhythm.

male horseshoe crabs for that season. These data are also consistent with the decreases in activity in bled animals that were documented by Anderson *et al.* (2013) in previous laboratory experiments. Since female horseshoe crabs are preferentially selected for in the biomedical bleeding process because of their size (James-Pirri *et al.*, 2012), and their mortalities are consistently greater than males (Leschen and Correia, 2010; James-Pirri, 2012; Krisfalusi-Gannon *et al.*, 2018), both the lethal and sublethal impacts of the bleeding process can alter the effective sex ratio and reproductive output of the population. For example, in several areas where horseshoe crabs are harvested, such as Pleasant Bay, Massachusetts, there has already been a 20% decrease in female appearances at spawning beaches (Carmichael *et al.*, 2003; Malkoski, 2010); and egg abundances have also significantly decreased at spawning sites (James-Pirri, 2012). Although Massachusetts regulations do not permit the harvesting of horseshoe crabs during the 5 days including and surrounding the new and full moons during the mating season, this does not always coincide with the peak of the spawning season; and many horseshoe crabs mate during other high tides during the ~1.5-month-long spawning season (Smith *et al.*, 2010; Cheng *et al.*, 2016). Therefore, it might be prudent to revisit harvest regulations and consider not harvesting horseshoe crabs at all during the majority of the spawning period.

Males are more frequently detected at mating beaches than females (Loveland and Botton, 1992). Furthermore, females usually only spawn several times within one week and do not return until the next year, whereas males return to these beaches more often (Rudloe, 1980; Brockmann and Penn, 1992; Leschen *et al.*, 2006). This trend was also evident in this study, further supporting the hypothesis that the skewed sex ratios observed near most mating locations are due to behavioral differences between males and females and are not indicative of the actual sex ratios of the local populations.

In this study, we restricted our analyses of mating behavior impacts to just 2 weeks during the peak of the mating season, allowing for ~14–15 potential mating events per week. Control animals appeared to mate, on average, three to four times a week, whereas bled animals mated one to two times per week. For control animals, this meant that they were likely to be mating during 22%–29% of the potential high tides, while bled animals appeared to be mating during only 7%–14% of them. Although these numbers are consistent with the results reported by Watson and Chabot (2010) and Brousseau *et al.* (2004), they are slightly lower. This difference could be due to two main factors. First, because our animals were released midway into the spawning season and because we examined only two weeks of data, some of our subjects might have already mated, or attempted to mate, several times before they were captured. Second, environmental factors could have affected spawning. During our spawning surveys, in both years, we noted that there was (1) heavy rain, (2) increased detritus on beaches on days immediately following large storms, and (3) overcast conditions. All of these factors appear to be correlated with fewer mating animals. A similar pattern was observed during spawn-

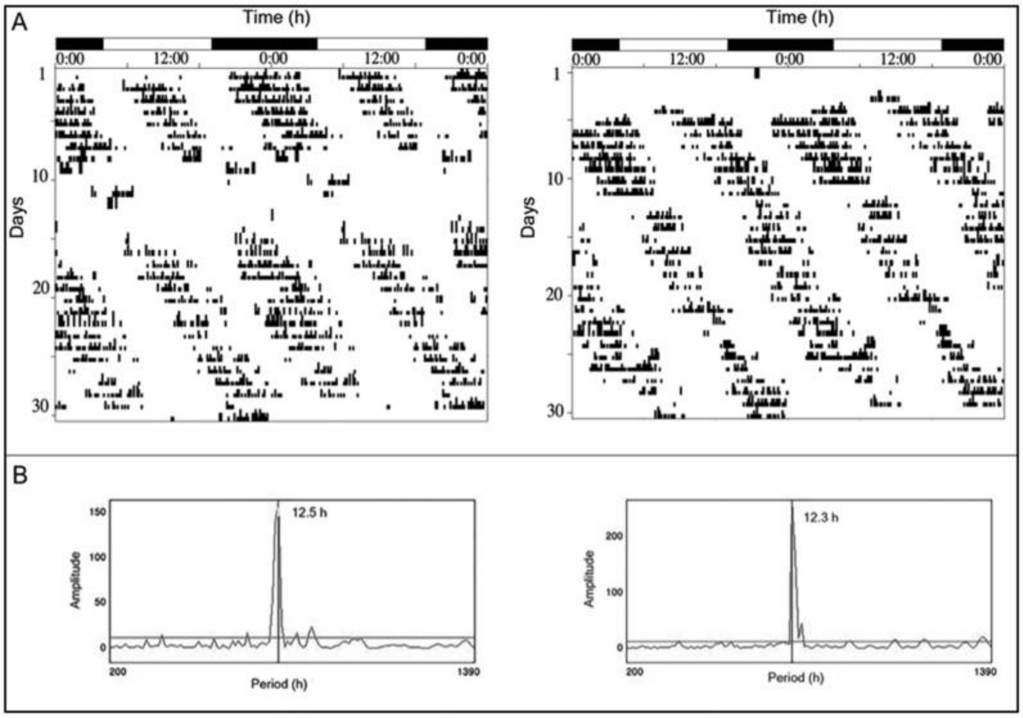

Figure 4. Representative tidal rhythms expressed by two horseshoe crabs in June. (A) Actograms showing the activity of a control female (left) and a bled female (right). The lack of activity by the control female around days 11–14 may represent a span of time when the animal was not within the detection range of a receiver. Each small black tick mark represents the amount of activity per 10 min. The *x*-axis is double plotted to make rhythms appear more evident, showing a span of 48 h for each line. The white and black horizontal bars at the top in (A) represent the light and dark periods of the day, respectively. The *y*-axis represents the 30 days in June 2016. (B) Periodograms displaying significant periods of activity ($P < 0.05$) for each animal for the amount of time shown in the actograms. The control female on the left and the bled female on the right had similarly strong tidal rhythms ($\tau = 12.5$ h *vs.* $\tau = 12.3$ h).

ing surveys in the GBE in 2012 and 2013, when periods of heavy rain were correlated with periods of decreased spawning activity (Cheng *et al.*, 2016).

Behavioral rhythms

Horseshoe crabs possess endogenous clocks (Chabot *et al.*, 2007) that allow them to anticipate changes in environmental factors, specifically changes in tides, so they can synchronize their spawning and foraging activities to particular phases of the tides (Cohen and Brockmann, 1983; Barlow *et al.*, 1986; Watson and Chabot, 2010). Any disruptions to the rhythms controlled by these clocks could have negative implications for reproduction and survival. In 2016, all animals that expressed activity in June had tidal rhythms. This is likely because this was the spawning season, when they approach mating beaches at high tide (Rudloe, 1980; Shuster and Botton, 1985; Barlow *et al.*, 1986). During the following summer, animals expressed both tidal and daily rhythms or became arrhythmic; and then in October no animals expressed tidal rhythms. This same seasonal transition was reported in previous studies, and it is likely due to the fact that as winter approaches, crabs move into deeper waters and prepare for overwintering (Chabot and Watson, 2010; Watson *et al.*, 2016).

In 2016, animals that had daily rhythms were more active during the day than at night. It has been previously argued that horseshoe crabs spawn more and increase their activity at night (Cavanaugh, 1975; Rudloe, 1980, 1981; Barlow, 1983; Barlow *et al.*, 1986; Finn *et al.*, 1990; Swan *et al.*, 1991, 1993; Smith *et al.*, 2010). In the GBE, this hypothesis has not been supported by previous telemetry studies or spawning surveys; in contrast, horseshoe crabs have actually been shown to be significantly more active during the day (Watson *et al.*, 2009; Watson and Chabot, 2010). The data from this study are consistent with this pattern of activity.

In 2017, there appeared to be some impact of the bleeding process on the expression of biological rhythms, as indicated by the fact that more bled animals were arrhythmic (four of nine animals) in 2017 than control animals (zero of three animals). For example, Animal 70, a bled female, remained arrhythmic throughout all of the months that it was active, including during the spawning season. However, the animals that were arrhythmic were also deeper and mostly manifested these behaviors in April, before the start of the spawning sea-

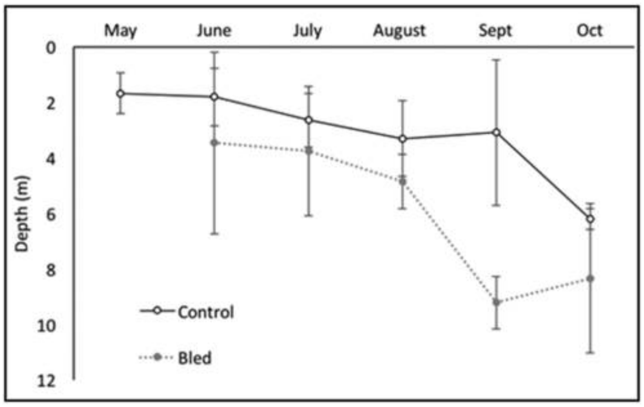

Figure 5. Mean monthly depth (±SEM) of horseshoe crabs from May to October 2016. Bled horseshoe crabs tended to remain deeper than control horseshoe crabs. Data are not shown for bled horseshoe crabs in May because there were insufficient data from the bled animals. Sample sizes for activity and depth for each month were as follows (Control, Bled): May (4, 0); June (6, 3); July (6, 4); August (5, 4); September (2, 2); October (2, 3); November (2, 1).

son, which is not too unusual. They usually do not begin to express clear rhythms until temperatures increase in late spring (10–11 °C; Schaller *et al.*, 2010). Nevertheless, two other bled animals were also arrhythmic in May and June during the spawning season, and this could be a result of having been bled, especially considering that all of the horseshoe crabs expressed tidal rhythms in June in 2016 but only two of five bled animals in May and June of 2017. However, it should also be noted that only one of three control animals expressed a tidal rhythm in 2017, so other factors could have played a role. Therefore, it is possible that the bleeding process could have delayed and extended impacts on the expression of rhythms and depth preferences, and thus on both foraging and mating behaviors. However, we have no explanation for how the bleeding process could lead to behavioral impacts that manifest themselves about a year after the procedure. Clearly, more studies are necessary to confirm that bled animals tend to reside in deeper water and approach mating beaches less often than control animals many months after the procedure.

Movements and migrations

The 28 animals that were tracked in 2016 appeared to remain within the GBE, along with the 23 that were detected in 2017. In fact, 21 of the 23 animals detected in 2017 were first detected at the same location where they were last detected in 2016. This is consistent with previous findings in the GBE (Schaller *et al.*, 2010), as well as in other embayments, such as Pleasant Bay, Massachusetts (James-Pirri, 2010). Horseshoe crabs are thought to be philopatric to the embayments where they spawn, and in New England specifically, horseshoe crab populations appear to be more localized and do not seem to migrate offshore (Baptist *et al.*, 1957; Botton and Ropes, 1987; James-Pirri *et al.*, 2005; Moore and Perrin, 2007). Therefore, if horseshoe crabs are harvested from a particular area, it is unlikely that they would be replenished from adults or larvae from a different area. In addition, if they are removed from one area, bled, and returned to a different area, they might not have the ability to adjust their migration and spawning behaviors to the new region.

There were no clear differences in the annual range of movements or seasonal migrations between control and bled animals. These annual ranges were similar to those found in Cape Cod, Massachusetts, and Maine embayments (Kurz and James-Pirri, 2002; James-Pirri *et al.*, 2005) and followed the same patterns that have been described in other New England estuaries and in the GBE. In the spring, when the water temperature reached 10–11 °C (Schaller *et al.*, 2010), the horseshoe crabs moved up-estuary to shallow areas near mating beaches. In 2017, this was clearly shown in our data because the transmitters turned on before the mating season. There was a clear trend of animals moving from deeper waters where they stayed in April to shallower waters in May, when the temperature threshold was reached. Then, after mating, when the estuary was the warmest in July and August, animals moved down-estuary; and they eventually overwintered at the farthest down-estuary portion of their annual range. As previously mentioned, in 2017, when our transmitters re-activated, 21 of the 23 animals were in the same location where they were last detected in December 2016, when the transmitters were de-activated. These seasonal movements are thought to be driven by temperature preferences, with animals seeking warmer water during the spawning season, then moving toward the coast when the water temperature rises above ~20 °C, and finally seeking deep water in the fall, which tends to be warmer in the winter (Schaller *et al.*, 2010). These thermal preferences could be an important factor driving mating behaviors because temperature plays a large role in egg development (French, 1979). Moreover, recent studies have demonstrated that horseshoe crabs can detect changes in water temperature and that, when given a choice, they prefer slightly warmer water (Cheng, 2015).

Although there was no significant difference in the annual range of movements between bled and control animals, or in the overall seasonal migration trends, there were some distinct changes in the seasonal movements of some animals from one year to the next. Most of the control animals followed similar patterns, but there were several bled animals that had very different migration routes between 2016 and 2017. One noticeable trend was that in May and June, the bled animals did not approach shallower areas in Great Bay but remained in the deeper channels. This trend is consistent with data showing that bled animals remained deeper throughout the year in 2016 and that bled females mated less than control females. In other words, some of the immediate impacts of bleeding that we observed in 2016 continued in 2017. There are two potential reasons why these bled animals remained in deeper water. First, in the laboratory, disrupted orientation and a random di-

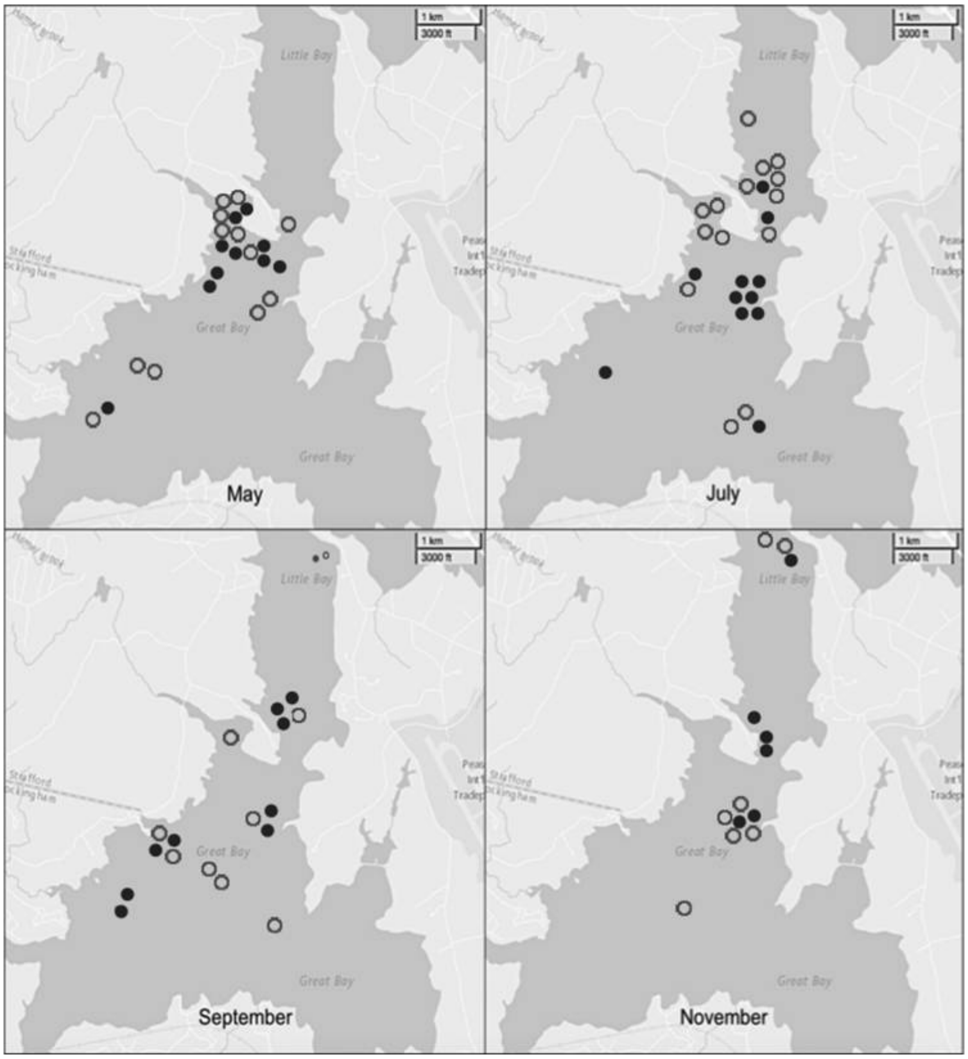

Figure 6. Maps of the location of horseshoe crabs, by season, in 2016. Filled dots represent bled animals, and open dots represent control animals. It should be noted that these locations are based on detections by acoustic monitoring receivers and, thus, do not necessarily represent the exact locations of each animal.

rection of movements have been observed in bled horseshoe crabs (Anderson *et al.*, 2013). Moreover, in one of the previous field studies comparing the impact of biomedical bleeding on movement patterns in Cape Cod, it was found that the bled group tended to have more random movements than control animals (Kurz and James-Pirri, 2002). This disorientation could prevent horseshoe crabs from locating spawning beaches and could also explain why the bled animals remained deeper and farther away from spawning beaches. Second, bled animals may not have been as motivated to spawn, and, therefore, they did not move toward spawning beaches as often as controls. This lack of motivation could be due to the fact that bleeding influenced their energy utilization and shifted it from reproduction to stress remediation. For example, Hu *et al.* (2011) showed that starvation of two Asian horseshoe crab species led to a decrease in their respiration rates and ammonia excretion rates, which was correlated with a decrease in scope for growth.

Interestingly, 5 out of the 23 animals in 2017 returned to their original release site where they had been captured while spawning. Four were control animals, and one had been bled. In a telemetry study completed in Delaware Bay, 77% of animals did not return to the same beach to spawn (Smith *et al.*, 2010), so we did not necessarily expect all of the animals to show up at their original spawning location in 2017. Therefore, while the 4 : 1 ratio of controls to bled animals is interesting, it is possible that this relates not to an inability of bled animals to find the same spawning beaches from year to year but to the fact that most animals do not return to the same spawning beach each year.

Overall impacts

Overall, there were some immediate impacts of the bleeding procedure on mating behaviors and the expression of biological rhythms, and there also appeared to be a tendency for bled animals to reside in deeper water during both the first and second years of the study. If bled animals, especially females, have alterations in their biological rhythms and mating behaviors, it is likely to further alter the sex ratio on spawning beaches, reduce reproductive output, lower population levels, and decrease the fitness and survival of this keystone species. Further studies should investigate the immediate effects of the bleeding process on horseshoe crabs during the mating season to obtain a more precise and accurate assessment of their behavior in the vicinity of mating beaches. Also, the orientation of bled animals in their natural habitat should be examined because one laboratory study and one field study indicate that some bled horseshoe crabs do not orient in the same manner as normal adult horseshoe crabs. Taken together with the results of many previous investigations, it appears as if the biomedical bleeding process might have some behavioral impacts that could impact the sustainability of harvested *Limulus* populations; and, thus, there is a need for further investigations into possible improvements that might reduce these effects.

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Appendix

Table A1

Two-way ANOVA results from likely mating events for horseshoe crabs tracked in 2016 comparing different sexes (males and females) and treatments (bled and controls)

	Likely mating events			
Source	df	MS	*F*	*P*
Sex	1	2568.405	6.019	**0.024**
Treatment	1	3800.770	8.906	**0.008**
Sex × treatment	1	2449.484	5.740	**0.027**
Error	19	426.746		
Total	23			

Significant differences are shown in bold ($P < 0.05$). MS, mean square.

Table A2

Three-way ANOVA results comparing the ranges of horseshoe crabs tracked in 2016 and 2017 comparing different years (2016 and 2017), sexes (males and females), and treatments (bled and controls)

	Range			
Source	df	MS	*F*	*P*
Sex	1	1.555	0.613	0.438
Treatment	1	0.475	0.187	0.667
Year	1	0.010	0.004	0.950
Sex × treatment	1	2.842	1.120	0.296
Sex × year	1	0.153	0.060	0.807
Treatment × sex	1	0.017	0.007	0.936
Year × sex × treatment	1	4.583	1.807	0.186
Error	40	2.537		
Total	48			

MS, mean square.

Table A3

MANOVA results from depth and activity data for horseshoe crabs tracked in 2016 comparing different sexes (males and females), treatments (bled and controls), and months (May–November)

		Depth and activity			
Source		df	MS	*F*	*P*
Month	Depth	6	36.683	3.947	**0.008**
	Activity	6	309.357	0.895	0.515
Sex	Depth	1	30.426	3.274	0.084
	Activity	1	0.047	0.000	0.991
Treatment	Depth	1	63.789	6.863	**0.016**
	Activity	1	184.634	0.534	0.472
Month × sex	Depth	5	2.676	0.288	0.915
	Activity	5	38.196	0.111	0.989
Month × treatment	Depth	6	7.608	0.819	0.567
	Activity	6	86.332	0.250	0.954
Sex × treatment	Depth	1	1.157	0.124	0.728
	Activity	1	70.64	0.204	0.656
Month × sex × treatment	Depth	1	0.141	0.015	0.901
	Activity	1	13.061	0.038	0.848
Error	Depth	22	9.295		
	Activity	22	345.489		
Total	Depth	44			
	Activity	44			

Significant differences are shown in bold. ($P < 0.05$). MS, mean square.

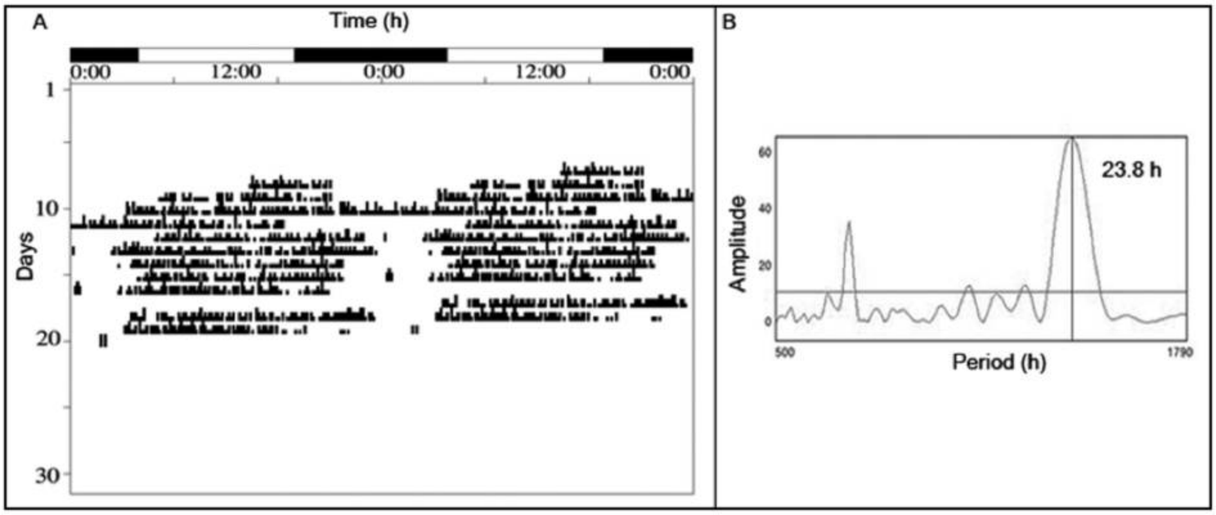

Figure A1. Representative example of the expression of a daily rhythm in a horseshoe crab. (A) Actogram of a control female that expressed a daily rhythm and was more active during the day. (B) Periodogram showing that this animal had a significant rhythm ($P < 0.05$) with $\tau = 23.8$ h. Each small black tick mark represents the amount of activity per 10 min. The *x*-axis is double plotted to make rhythms appear more evident, showing a span of 48 h for each line. The white and black horizontal bars at the top in (A) represent the light and dark periods of the day, respectively. The *y*-axis represents the 30 days in July 2016. The horizontal line above the *x*-axis in (B) indicates $P = 0.001$.

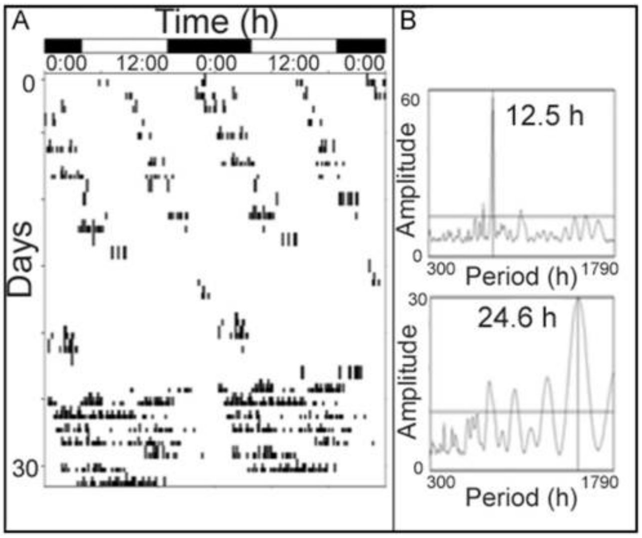

Figure A2. An example of a horseshoe crab that switched from a tidal rhythm to a daily rhythm. (A) This animal expressed a tidal rhythm during the first 25 days and then a daily rhythm during the last 8 days. (B) Periodograms showing that this animal had a significant rhythm ($P < 0.05$) of 12.5 h during the first 25 d and 24.6 h during the last 8 d. Each small black tick mark represents the amount of activity per 10 min. The *x*-axis is double plotted to make rhythms appear more evident, showing a span of 48 h for each line. The white and black horizontal bars at the top in (A) represent the light and dark periods of the day, respectively. The horizontal line above the *x*-axis in (B) indicates $P = 0.001$.

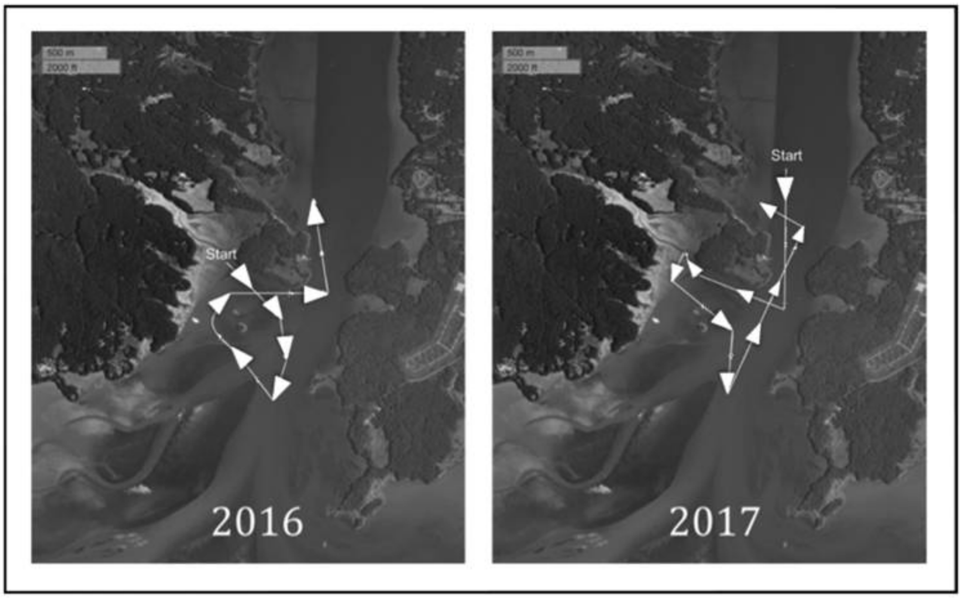

Figure A3. An example of a control horseshoe crab that returned to the mating beach where it was initially captured and released (indicated by “Start” on the left in 2016). Arrowheads indicate the direction of the path traveled by the individual in each year.

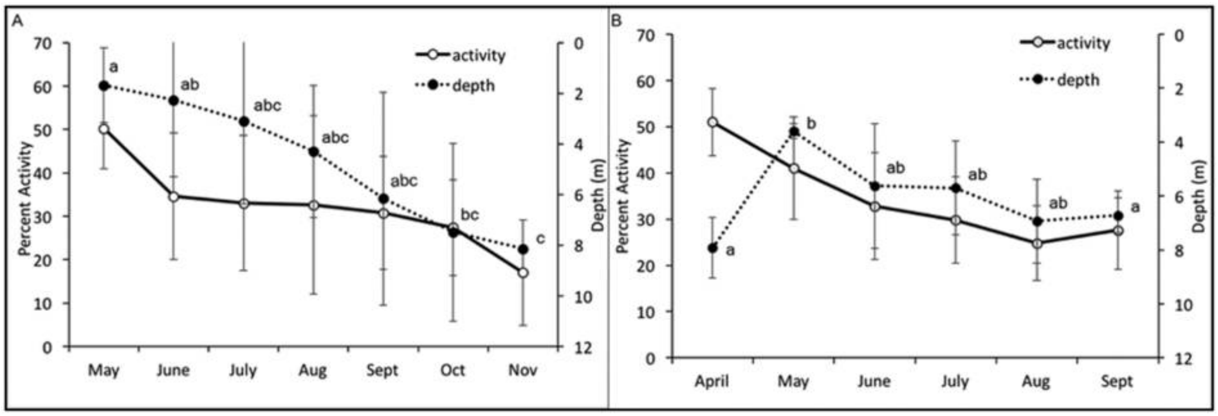

Figure A4. Mean (±SEM) monthly horseshoe crab (both bled and control) activity and depth for 2016 (A) and 2017 (B). Activity was calculated as the amount of time per day that animals were moving, based on accelerometer data. Data for these analyses were based on individuals that were detected for at least one week for each month. Sample sizes for activity and depth for each month were (2016, 2017) as follows: April (0, 5); May (4, 9); June (10, 2); July (10, 3); August (11, 4); September (4, 3); October (5, 0); November (3, 0). Different lowercase letters associated with the means represent significant differences for monthly depths (ANOVA, Tukey's honest significant difference *post hoc*, $P < 0.05$).

EXHIBIT C

Reference: *Biol. Bull.* **240:** 000–000. (June 2021)

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Assessing the Viability of American Horseshoe Crab (*Limulus polyphemus*) Embryos in Salt Marsh and Sandy Beach Habitats

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Abstract. For animal embryos that develop externally, the physio-chemical environment can substantially affect offspring viability. In the case of the American horseshoe crab (*Limulus polyphemus*), sediment conditions along estuarine shorelines influence development rates and embryonic viability. Sandy beach habitats are considered to have optimal conditions for horseshoe crab embryonic development; however, spawning is often observed outside of these optimal habitats, in areas such as salt marshes, where reduced oxygen availability is thought to decrease the viability of eggs laid in these sediments. We excavated horseshoe crab eggs, embryos, and trilobites laid naturally in marsh and beach sediments in South Carolina to compare their development and viability between habitats. We found all developmental stages in both marsh and beach habitats. For two of three sampling areas, trilobites were more likely to be found at beaches than at marshes. Multivariate analyses demonstrate that the prevalence of early and middle developmental stages was similar between habitats but that beaches had a greater proportion of late-stage trilobites than marshes. The lower likelihood of finding trilobites at some marshes may reflect differences in spawning phenology between habitats or reduced rates of embryonic development in marshes compared to beaches, leading to potentially different developmental timelines rather than a true reduction in viability. Nevertheless, the substantial proportions of eggs laid in salt marshes that survive to the trilobite stage indicate that spawning in this habitat could represent a previously underappreciated source of recruitment for horseshoe crab populations that may need to be incorporated into population assessments.

Introduction

The nature of the external environment into which eggs are released can significantly impact subsequent extraparental embryonic development. For example, environmental conditions such as temperature, oxygen availability, moisture content, and salinity can influence development time and offspring viability (*e.g.*, Alderdice and Forrester, 1971; Dos Santos and Nascimento, 1985; Przeslawski, 2004; Noiva *et al.*, 2014). Spawning is generally expected to occur in areas where the environmental conditions are optimal for embryonic development (Viertel, 1999; Haramura, 2008; Snickars *et al.*, 2010); however, animals sometimes spawn in conditions that are sub-optimal (Lewis *et al.*, 2002; Reglero *et al.*, 2018). This can occur for several reasons, including high spawning densities in optimal environments (Falcy, 2015; Huntsman *et al.*, 2017), the threat of predation (Korb and Linsenmair, 1999), food availability (Ventura *et al.*, 1997), and anthropogenic changes to environmental conditions (Jackson and Moser, 2012; Nelson *et al.*, 2016; Laurel and Rogers, 2020). If spawning commonly occurs in environments sub-optimal to embryonic development, and if developmental success therein is significantly reduced, recruitment into the population may be adversely affected. Thus, it is important to understand the effects of environmental conditions on embryonic development and viability to assess the impacts of spawning activity in sub-optimal habitats on overall population health.

The American horseshoe crab, *Limulus polyphemus* (Linnaeus, 1758), lives in coastal and offshore environments along the Atlantic and Gulf coasts of the United States and the Yucatan Peninsula of Mexico. Along portions of its range,

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Abbreviations: db-RDA, distance-based redundancy analysis; $\text{Location}_{\text{StDev}}$, standard deviation of sample location; NMDS, non-metric multidimensional scale; SCDNR, South Carolina Department of Natural Resources.

L. polyphemus plays important roles in ecological interactions, supports important commercial fisheries, and/or serves critical functions in the biomedical industry. Its ecological importance is highlighted through its spawning behavior, which leads to substantial bioturbation along estuarine shorelines (Jackson *et al.*, 2020), and through its production and deposition of eggs, which supports shorebird migrations (Smith *et al.*, 2017). Highly nutritious horseshoe crab eggs and embryos, for instance, provide a critical food resource to the federally threatened *rufa* red knot, *Calidris canutus rufa* (A. Wilson, 1813), as it stops to feed in areas, such as Delaware Bay (Karpanty *et al.*, 2006) and South Carolina (Takahashi, 2016), during its annual migration from southern South America to the Arctic (Haramis *et al.*, 2007; USFWS, 2014; Smith *et al.*, 2017). Juvenile and adult horseshoe crabs are also prey for alligators *Alligator mississippiensis* (Daudin, 1802) (Reid and Bonde, 1990), as well as endangered Kemp's ridley (*Lepidochelys kempii* Garman, 1880) and loggerhead (*Caretta caretta* (Linnaeus, 1758)) sea turtles (Seney and Musick, 2005; Witherington and Witherington, 2015). Horseshoe crabs are also economically important because they are harvested as bait for eel and whelk fisheries in some US states (Rutecki *et al.*, 2004). Additionally, a product made from an extract of horseshoe crab hemolymph, called *Limulus* amebocyte lysate (LAL), is used extensively in the biomedical industry to test for contamination by bacterial endotoxins (Krisfalusi-Gannon *et al.*, 2018). Effective management strategies necessary to conserve this critically important species should include a comprehensive understanding of reproductive ecology, including habitat use by spawning adults and variability in embryonic development (ASMFC, 2019).

Horseshoe crabs generally spawn in the spring and early summer, although the exact timing depends on location (Thompson, 1998; Brockmann and Smith, 2009; Sasson and Brockmann, 2016; Smith *et al.*, 2017). Warming temperatures provide a cue for adult horseshoe crabs to move from deeper waters to the intertidal zone, where spawning often occurs (Shuster, 1982; Moore and Perrin, 2007; Watson *et al.*, 2009; Schaller *et al.*, 2010; Cheng *et al.*, 2015; Smith *et al.*, 2017). During spawning, females excavate the sediment and deposit between 14,500 (Leschen *et al.*, 2006) and 88,000 (Shuster, 1982) eggs, typically at depths of 5 to 20 cm (Shuster, 1982; Cohen and Brockmann, 1983; Loveland and Botton, 1992). Eggs are fertilized by males as females extrude and deposit them into sediments (Cohen and Brockmann, 1983; Penn and Brockmann, 1994). Sandy beaches are generally considered the optimal spawning habitat for horseshoe crabs because the sediment is porous and well oxygenated, allowing for the circulation of gases and water necessary for embryonic development (Shuster, 1982; Botton *et al.*, 1988; Penn and Brockmann, 1994; Loveland *et al.*, 1996; Vasquez *et al.*, 2015a).

While considered to spawn primarily on sandy beaches, horseshoe crabs have also been observed spawning in a variety of other habitats, including those with peat and mud sediments, such as salt marshes (Sekiguchi *et al.*, 1977; Botton, 1995; Beekey and Mattei, 2008). These habitats are typically thought to provide sub-optimal conditions for horseshoe crab development (*e.g.*, Botton *et al.*, 1988) and, thus, have received relatively little attention in terms of studies of embryonic development and viability. While adult horseshoe crabs are known to exhibit broad tolerances for environmental factors (*e.g.*, Fraenkel, 1960; Roberston, 1970; Ehlinger and Tankersley, 2003), field studies on sandy beaches and laboratory studies suggest that optimal developmental conditions for embryos are narrower (Laughlin, 1983; Penn and Brockmann, 1994; Vasquez *et al.*, 2015a, b). Along many portions of its geographic range, the estuarine shoreline is dominated by salt marsh comprised of dense mud and smooth cordgrass, *Spartina alterniflora* Loisel. The South Carolina Department of Natural Resources (SCDNR) has received reports from its staff, members of the public, and suppliers of horseshoe crabs to the biomedical industry of extensive horseshoe crab spawning in the salt marshes of South Carolina, suggesting that this habitat potentially supports viable development of horseshoe crabs. Since South Carolina and Georgia have the largest expanses of estuarine vegetated wetlands on the east coast of the United States (Tiner, 2013), salt marsh habitats in this region could represent a significant, but previously underappreciated, source of horseshoe crab embryos that may contribute substantially to the adult population. The incorporation of salt marsh into habitat models for horseshoe crabs could, therefore, enhance the development of effective management strategies for this species.

Horseshoe crab embryos develop in sediments through four embryonic molts before hatching into the juvenile stage (Shuster, 1982; Botton *et al.*, 1988; Loveland *et al.*, 1996). Morphological characteristics associated with these molt stages, as well as patterns of intra-molt development, have been used as the basis for categorizing horseshoe crab embryos into a series of developmental stages (Sekiguchi *et al.*, 1988; Shuster and Sekiguchi, 2003; Botton *et al.*, 2010). Following fertilization, it takes about two to four weeks for horseshoe crab eggs to reach the post-embryonic trilobite stage (Botton *et al.*, 1992); but variation in abiotic conditions, such as temperature, salinity, moisture, and oxygen availability, has significant effects on developmental rates (Botton *et al.*, 1992; Penn and Brockmann, 1994; Jackson *et al.*, 2008; Vasquez *et al.*, 2015b). The low oxygen availability generally characterizing salt marsh sediments (Bradley and Morris, 1990) may impede horseshoe crab embryonic growth and development (Botton *et al.*, 1988; Vasquez *et al.*, 2015a). Hypoxic conditions (<2% oxygen saturation) have been shown to prevent the completion of the first embryonic molt (Funch *et al.*, 2016), suggesting that development to the trilobite stage is unlikely to occur in the absence of sufficient dissolved oxygen. Additionally, salt marsh sediment temperatures may be different from those of sandy beach habitats, with potentially important effects on rates of embryonic development (Vasquez *et al.*, 2015b). While anecdotal evidence suggests that the use of salt marsh habitat by horseshoe crabs for spawning may be extensive, it is unclear whether developmental timelines and viability of eggs laid in salt marshes are similar to those of eggs laid in sandy beaches. Laboratory studies indicate that the conditions found in salt marsh habitats, including high levels of hydrogen sulfide, are likely to be detrimental to horseshoe crab embryonic development (Vasquez *et al.*, 2015a, b); but *in situ* measurements of the developmental progress of eggs spawned in salt marshes are rare.

In this study we compared the developmental progression and viability of horseshoe crab eggs collected from salt marsh and sandy beach habitats located across central and southern South Carolina. A finding of earlier-stage embryos and/or a lower proportion of viable embryos in marsh habitat than in sandy beach habitat may indicate that marsh habitat is suboptimal for early development. Quantifying the developmental differences between eggs laid in these habitat types will help to inform our understanding of their relative importance to the reproductive success of this ecologically, commercially, and biomedically important benthic invertebrate species.

Materials and Methods

Sample collection and developmental staging

In June 2019, we conducted sampling to collect early-developmental-stage horseshoe crabs (*Limulus polyphemus* (Linnaeus, 1758)) (*i.e.*, eggs, embryos, and trilobites) in salt marsh and sandy beach habitats at three discrete island locations in South Carolina, as follows: Edisto Island (32°29′38″ N, 80°20′46″ W), Harbor Island (32°24′43″ N, 80°26′6″ W), and Turtle Island (32°4′8″ N, 80°53′28″ W) (Fig. 1). Horseshoe crab spawning in South Carolina generally occurs from March to June, peaking in late May, so we sampled in June to ensure that we would collect early developmental stages. We chose sites within each of these three locations on the basis of previous reports of documented horseshoe crab spawning activity provided to the SCDNR *via* multiple reporting

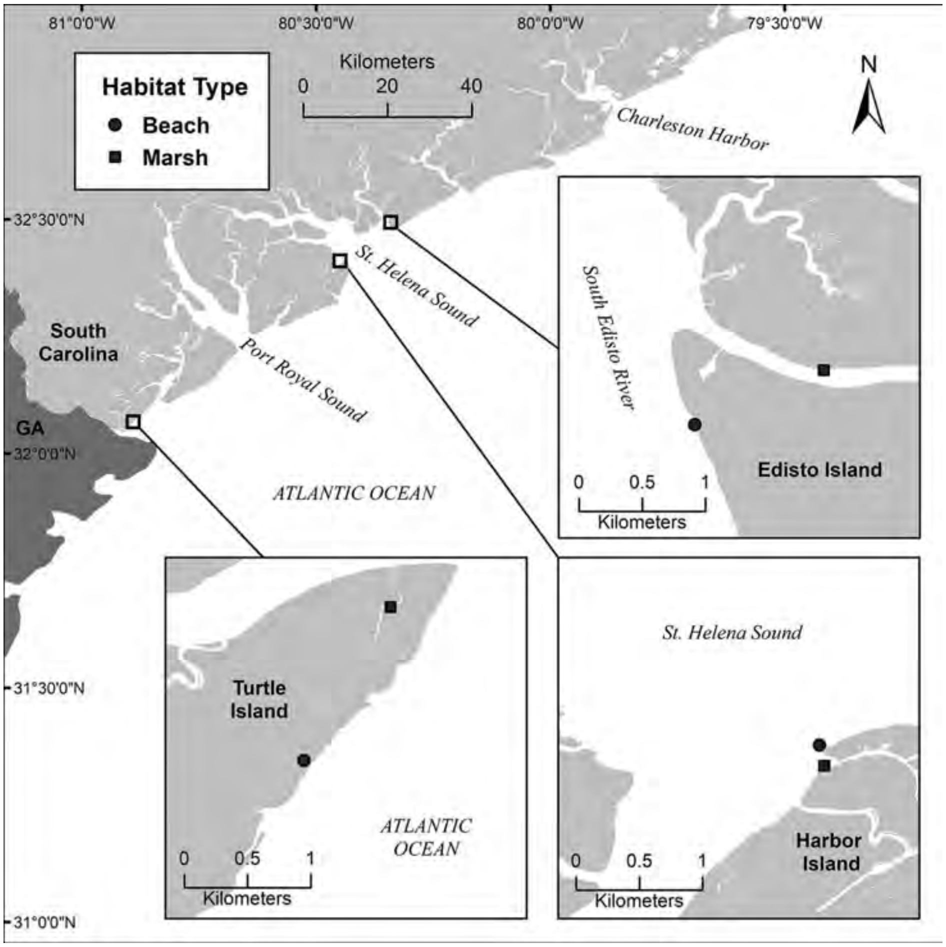

Figure 1. Map of coastal South Carolina showing the three discrete horseshoe crab (*Limulus polyphemus*) sampling locations (Edisto Island, Harbor Island, and Turtle Island). Inset maps for each location show the proximity of marsh and beach sites for each location.

channels (*e.g.*, public reporting outlets, personal observations, and collaborations with suppliers of horseshoe crabs to the biomedical industry). We excavated early-developmental-stage horseshoe crabs from replicate sites (Edisto Island: n = 11 marsh, 10 beach; Harbor Island: n = 10 marsh, 11 beach; Turtle Island: n = 7 marsh, 11 beach) on the same day for each location (Turtle Island: June 13; Harbor Island: June 17; Edisto Island: June 19).

We conducted sampling near low tide because semi-diurnal tides characteristic of the region inundate both marsh and beach locations twice daily. At beach sites, we visually assessed areas for evidence of horseshoe crab eggs by identifying depressions in the sediment where females may have burrowed during spawning and by observing shorebird behaviors unique to feeding on horseshoe crab eggs (Fraser *et al.*, 2010). At marsh sites, we chose excavation sites along the creek-marsh edges (Fig. 2A). This creek-marsh edge is often referred to as the creek levee and shows biogeochemical properties, such as redox potential and hydrogen sulfide concentrations, that are often distinct from interior ponded marsh areas (Seliskar *et al.*, 2002; Koretsky *et al.*, 2005). To search for early developmental stage horseshoe crabs, we used hand trowels to dig into the sediments along creek levees at marsh sites (Fig. 2A, B) and near the high tide line at beach sites (Fig. 2C). Once we successfully located early developmental stages, we excavated an approximately 5 cm × 5 cm × 20 cm (length × width × depth) area and preserved all excavated horseshoe crabs from each individual site in 95% ethanol as a discrete sample.

From each sample, we randomly subsampled at least 50 eggs, embryos, or trilobites and assigned them to 1 of 7 morphologically based developmental stages under at least 20–60× stereoscopic magnification, using a Nikon SMZ1500 microscope (Table 1; Fig. 3; Botton *et al.*, 2010). Because of the difficulties associated with distinguishing early developmental stages (Shuster and Sekiguchi, 2003; Botton *et al.*, 2010), we binned embryos into stages that broadly correspond to the synopsis of development described by Sekiguchi *et al.* (1988) and Botton *et al.* (2010), as follows: stage A = stages 0–14, stage B = stages 15–17, stage C = stage 18, stage D = stage 19, stage E = stages 20-1 and 20-2, stage F = stage 21, and stage G = stage 21. Specific descriptions of these stages are provided in Table 1 and shown in Figure 3. We measured the sizes of a subset of 20 randomly selected embryos at each stage under magnification as described above and using a Nikon D300 camera. We then used ImageJ (ver. 1.53a; Schneider *et al.*, 2012) to measure the width of each embryo at its widest point, drawing the measuring line through the center of the embryo. We measured the size of trilobites (stages F and G) by drawing the measuring line across the widest part of the opisthosoma (Sasson *et al.*, 2012).

Additionally, some samples contained eggs or embryos that were fully or partially discolored either brown or black. For each sample, we noted whether brown or black discoloration was present in the subsample of eggs used for staging (Fig. 4). A pilot study conducted in 2020 (DAS, unpubl. data) to rear eggs collected from the field suggests that eggs with discoloration are less viable than eggs without discoloration (12 of 60 discolored eggs developed to the trilobite stage compared to 155 of 183 non-discolored eggs).

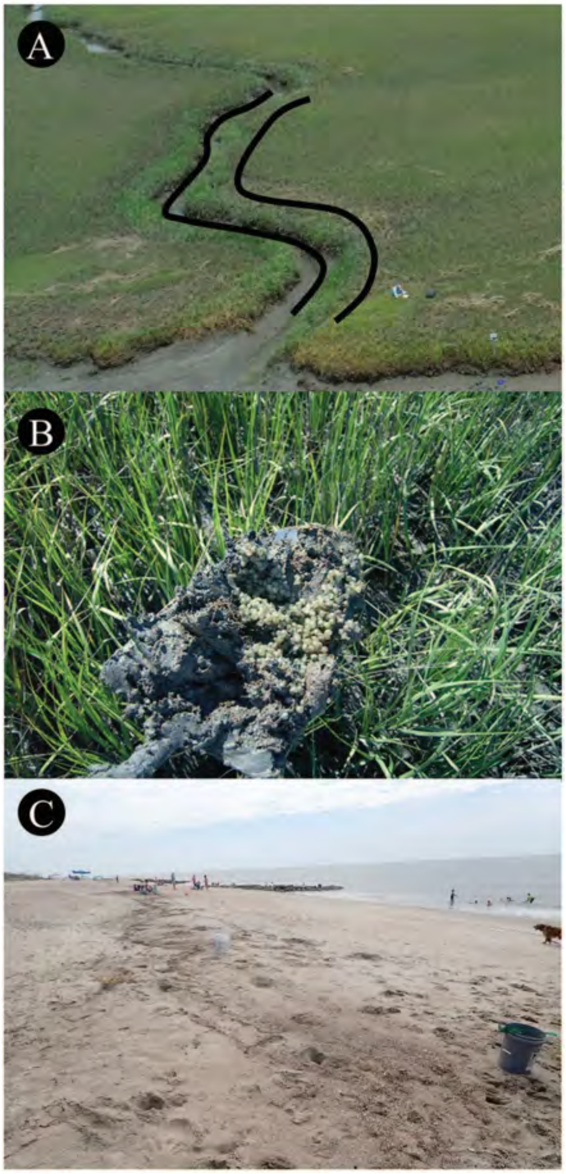

Figure 2. Photographs of representative habitats used by spawning horseshoe crabs (*Limulus polyphemus*). (A) Salt marsh habitat, with an example of the creek levee area sampled during this project shown between the two overlaid black lines. Note that the smooth cordgrass, *Spartina alterniflora*, in this creek levee area is more brightly colored than the surrounding marsh platform (photo captured on Edisto Island, South Carolina, on May 18, 2020). (B) Early developmental stage horseshoe crabs excavated from salt marsh sediments (photo captured on Harbor Island, South Carolina, on June 17, 2020). (C) Beach habitat sampled near the high tide line (photo captured on Edisto Beach, South Carolina, on June 30, 2020).

Table 1

*Description of early-developmental-stage horseshoe crabs (*Limulus polyphemus*)*

Stage	Description
A	Eggs are unfertilized or at an early stage of fertilization and have a large volume of yolk. They are typically greenish-blue, greenish-gray, or pink. The egg surface is smooth and may have a dent in the surface.
B	Limb bud tissue is starting to develop but is not yet defined. Other tissues are beginning to appear in the egg, but they are not yet discernible. This stage occurs prior to the first embryonic molt.
C	Early limb bud development is clear. Legs are short and close to the body. This stage follows the first embryonic molt.
D	Limb buds are elongated, starting to unfold from the body, and are becoming more defined. This stage follows the second embryonic molt.
E	The embryo can easily be seen with the unaided eye. Segmentation along the dorsal surface is evident. Book gills are forming. Legs are fully segmented, and the eyes are visible. The embryo is still within the egg membrane (*i.e.*, unhatched). This stage follows the third embryonic molt.
F	This is the trilobite stage (first instar). This stage has usually hatched from the egg membrane. The telson is attached to the fan-shaped opisthosoma edge. This stage follows the fourth embryonic molt.
G	This is the advanced trilobite stage. The telson is separated from the fan-shaped opisthosoma edge and is beginning to grow past the opisthosoma.

Statistical analysis

To determine differences in the probabilities of finding specific developmental stages across habitats, we used individual egg, embryo, and trilobite data ($n = 3427$) to develop generalized linear mixed models using the lme4 package in R (Bates *et al.*, 2015), with binomial distributions (presence or absence) and logit link function (Zuur *et al.*, 2009) for each developmental stage or category. We used sample number ($n = 60$) nested within sampling location (*i.e.*, island, $n = 3$) as a random effect and assessed significance using chi-squared likelihood ratio tests (LRTs; Bates *et al.*, 2015). Each developmental stage was categorized as present or absent for each egg, embryo, and trilobite; and separate models were developed to test for habitat differences of each of the seven developmental stages, incorporating a Bonferroni correction for multiple comparisons. We also developed similar models to test specifically for differences in early developmental stages (stages A and B in Fig. 3), trilobites (stages F and G in Fig. 3), and discolored eggs across locations for marsh and beach habitats. We performed *post hoc* analyses from Tukey-corrected pairwise comparisons of estimated marginal means, using the emmeans package (Lenth, 2020).

In addition to the univariate approaches used above, we also explored developmental differences between locations and between habitat types by using multivariate approaches. We

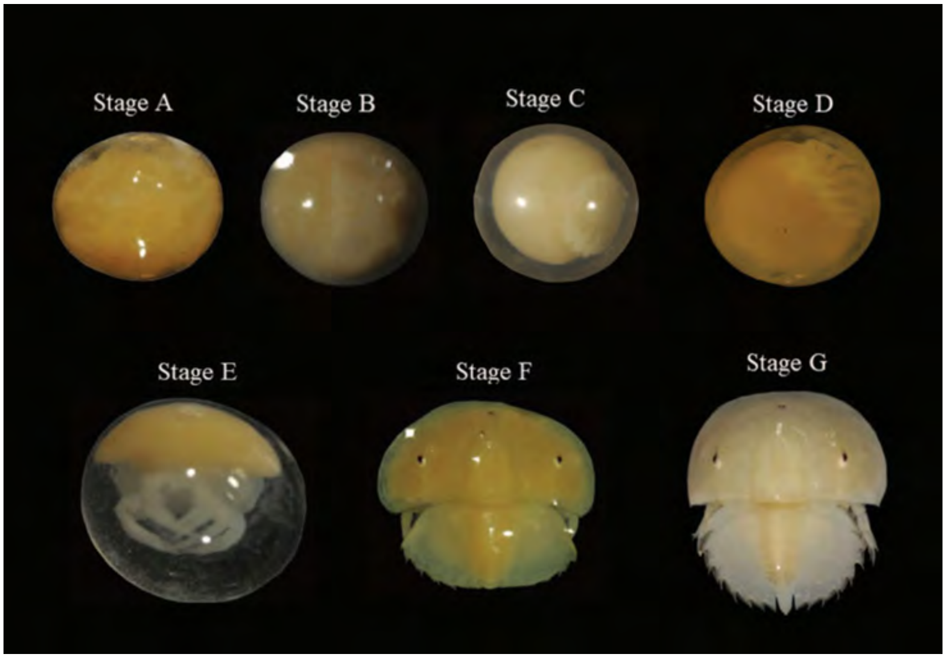

Figure 3. Developmental stages of horseshoe crabs (*Limulus polyphemus*) as photographed under at least 20–60× magnification from ethanol-preserved specimens. Sizes for each stage are based on a subset of samples as follows (mean ± SE): stage A (1.98 ± 0.13 mm), stage B (2.02 ± 0.12 mm), stage C (2.09 ± 0.11 mm), stage D (3.48 ± 0.14 mm), stage E (3.70 ± 0.18 mm), stage F (2.83 ± 0.20 mm), and stage G (2.99 ± 0.13 mm).

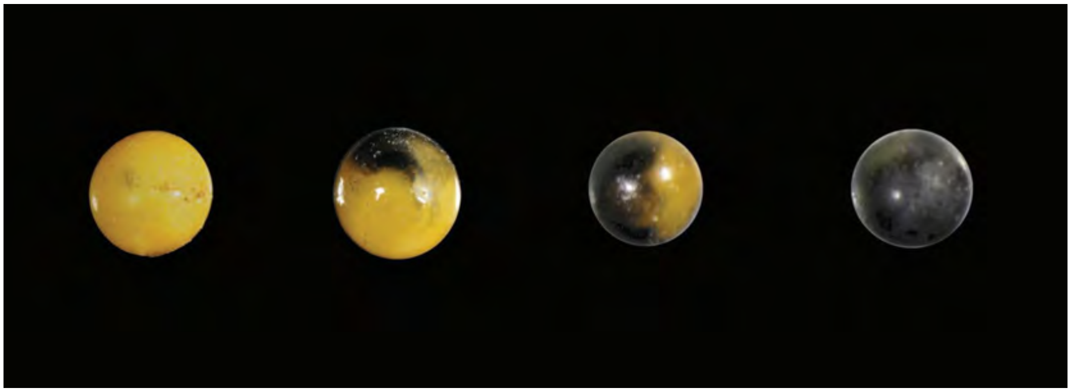

Figure 4. Unfertilized horseshoe crab (*Limulus polyphemus*) eggs and/or eggs at an early stage of fertilization, ranging from no discoloration (left-hand image), to partially discolored eggs (middle two images), to fully discolored eggs (right-hand image). Eggs averaged 1.98 mm in diameter.

used samples from all collection sites to compare the relative abundance of developmental stages by using both unconstrained (non-metric multidimensional scale [NMDS]) and constrained (distance-based redundancy analysis [db-RDA]) approaches (Legendre and Anderson, 1999). The constrained approach used the dissimilarity matrix of egg composition as the dependent variable, habitat as the independent (constraining) variable, and location as a condition for permuting data. This approach allowed for a more comprehensive analysis of variability in developmental stages between habitat types and locations that complements the univariate approaches. We conducted multivariate analyses using the vegan package (Oksanen *et al.*, 2018) in the R statistical computing platform (R Core Team, 2018).

Results

We observed all developmental stages at both marsh and beach sites (Fig. 5). Stage E was more likely to be found at marsh sites than beach sites, while stage G was more likely to be found at beach sites (Fig. 5). Test results for fixed effects (χ^2 and *P*-value), random effects (standard deviation of sample location; hereafter, $\text{Location}_{\text{StDev}}$), and sample number nested within sample location (hereafter, $\text{Num:Location}_{\text{StDev}}$) of each comparison are as follows: stage A ($\chi^2 = 2.542$, $P = 0.111$, $\text{Location}_{\text{StDev}} = 1.23$, $\text{Num:Location}_{\text{StDev}} = 2.07$), stage B ($\chi^2 = 3.997$, $P = 0.046$, $\text{Location}_{\text{StDev}} < 0.001$, $\text{Num:Location}_{\text{StDev}} = 2.39$), stage C ($\chi^2 = 4.453$, $P = 0.035$, $\text{Location}_{\text{StDev}} < 0.001$, $\text{Num:Location}_{\text{StDev}} = 2.18$), stage D ($\chi^2 = 6.566$, $P = 0.010$, $\text{Location}_{\text{StDev}} = 0.472$, $\text{Num:Location}_{\text{StDev}} = 1.96$), stage E ($\chi^2 = 7.638$, $P = 0.006$, $\text{Location}_{\text{StDev}} = 1.03$, $\text{Num:Location}_{\text{StDev}} = 2.11$), stage F ($\chi^2 = 0.103$, $P = 0.749$, $\text{Location}_{\text{StDev}} = 0.75$, $\text{Num:Location}_{\text{StDev}} = 1.94$), and stage G ($\chi^2 = 9.378$, $P = 0.002$, $\text{Location}_{\text{StDev}} < 0.001$, $\text{Num:Location}_{\text{StDev}} = 4.25$).

Models showed that the probability of finding early-stage eggs and embryos (stages A and B combined) did not differ between habitat types ($\chi^2 = 1.151$, $P = 0.283$) but did differ by location ($\chi^2 = 11.803$, $P = 0.003$); however, no significant habitat × location interaction was found ($\chi^2 = 0.668$, $P = 0.716$, $\text{Location}_{\text{StDev}} < 0.001$, $\text{Num:Location}_{\text{StDev}} = 2.42$; Fig. 6A). *Post hoc* analyses showed that clutches from Turtle Island were more likely to have early developmental stages than Edisto Island (*z*-ratio = 2.364, $P = 0.048$) and Harbor Island (*z*-ratio = 3.340, $P = 0.002$), but there was no difference between Harbor Island and Edisto Island (*z*-ratio = 1.018, $P = 0.565$).

For the probability of trilobite occurrence (stages F and G combined), clutches from beach sites were more likely to contain trilobites than clutches from marsh sites ($\chi^2 = 7.323$, $P = 0.007$; Fig. 6B). There was no significant effect of location ($\chi^2 = 5.548$, $P = 0.062$) but a significant interaction between habitat and location ($\chi^2 = 13.594$, $P = 0.001$, $\text{Location}_{\text{StDev}} < 0.001$, $\text{Num:Location}_{\text{StDev}} = 2.51$). *Post hoc* analyses showed significantly greater likelihood of finding trilobites at beach habitats compared to marsh habitats at Turtle Island (*z*-ratio =

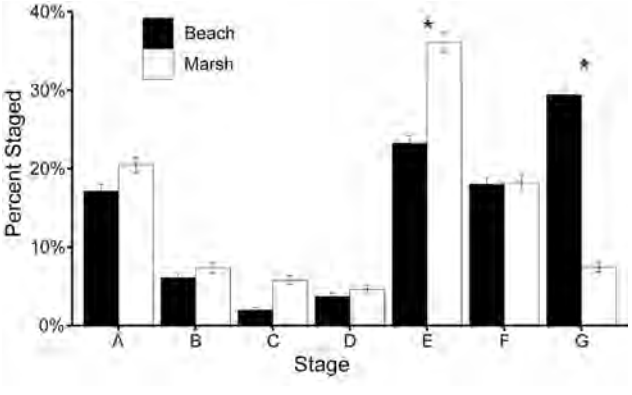

Figure 5. Mean percent (±SE) of horseshoe crab (*Limulus polyphemus*) embryos that were categorized into seven discrete developmental stages across all samples. Bonferroni-corrected significance ($\alpha = 0.007$) of generalized linear mixed models (GLMMs) testing differences between marsh habitats ($n = 1624$) and beach habitats ($n = 1803$) for each stage is indicated by an asterisk.

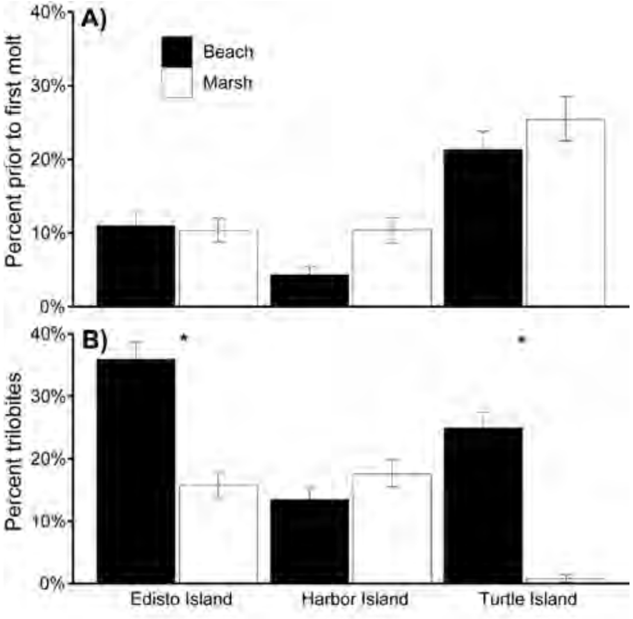

Figure 6. Comparison of early and late developmental stages across sampling locations. (A) Mean percent (±SE) of horseshoe crab (*Limulus polyphemus*) eggs and embryos staged as being prior to their first embryonic molt (*i.e.*, stages A and B). (B) Mean percent (±SE) of horseshoe crab embryos that developed to trilobites (stages F and G) in marsh and beach sediments collected from the three sampling locations. Significant differences ($\alpha = 0.017$) of Tukey-corrected pairwise comparisons of individual egg occurrence between habitats at each location are indicated by an asterisk.

3.391, $P < 0.001$) and Edisto Island (z-ratio = 2.216, $P = 0.027$) but showed no difference at Harbor Island (z-ratio = 0.866, $P = 0.386$).

We found significant relationships between the occurrence of discolored eggs across habitats ($\chi^2 = 5.757$, $P = 0.016$), with no significant effect of either location ($\chi^2 = 1.453$, $P = 0.484$) or the interaction between habitat type and location ($\chi^2 = 5.4699$, $P = 0.065$). *Post hoc* analyses showed that clutches from marsh sites had greater probability of containing discolored eggs or embryos than beach habitats at Turtle Island (z-ratio = 3.163, $P = 0.002$), but there was no significant difference in the probability of finding discolored eggs or embryos at Harbor Island (z-ratio = 0.706, $P = 0.480$) or Edisto Island (z-ratio = 0.907, $P = 0.364$).

The NMDS analyses showed large amounts of overlap in the composition of developmental stages between marsh and beach sites. This is demonstrated by the lack of separation between the polygons drawn around the marsh and beach samples (Fig. 7A). When constraining analyses to distinguish potential differences based on habitat type in the db-RDA, however, we found significant differences between habitats ($F = 3.95$, $P = 0.005$; Fig. 7B). Despite these differences, habitat explained only 6.6% of the variability in this analysis, indicating that most of the variability in developmental stages was not associated with differences in habitats. While not mutually exclusive to the percent of variability explained by habitat type, far greater variability (16%) was explained by the grouping factor of location in the model. Nonetheless, differences in the multidimensional scores of stages E and G, as represented by their placement at opposite ends of both x- and y-axes (Fig. 7B), highlight that differences between habitats are largely due to variation in the relative abundance of these two developmental stages.

Discussion

In this study we have documented the presence of horseshoe crab eggs, embryos, and trilobites in both salt marsh and sandy beach habitats. While previous studies have acknowledged some level of spawning by horseshoe crabs in salt marsh habitats (Smith *et al.*, 2017), these habitats are generally considered sub-optimal (Botton *et al.*, 1988), and previous evaluations of embryo viability in non-sandy habitats are rare. Previous studies have documented the broad physiological tolerances of horseshoe crab eggs and embryos (Kingsley, 1892; Robertson, 1970; Ehlinger and Tankersley, 2003), but environmental conditions clearly influence rates of embryonic development (Penn and Brockmann, 1994; Vasquez *et al.*, 2015b). While sub-optimal environmental conditions may lead to longer developmental time for horseshoe crab eggs laid in salt marsh sediments, our results showing viable horseshoe crab development to the trilobite stage in salt marsh sediments indicate that these habitats may be an important source of recruitment for this species.

Some of the differences in embryonic development between habitat types that we observed could point to stages at which horseshoe crab embryos are particularly vulnerable to environmental stressors. Both univariate and multivariate analyses revealed important differences in embryonic development between habitat types and revealed lower proportions of late-stage trilobites at salt marsh sites compared to sandy beach sites. For marsh sites, we observed high relative abundances of pre-trilobite embryos (*i.e.*, stage E) but low relative abundances of late-stage trilobites (*i.e.*, stage G). This contrasts with high relative abundances of stages E and G at beach sites. Large differences in trilobite abundance at two of the three locations (Edisto Island and Turtle Island), which were not observed at the third location (Harbor Island), may be driving this relationship.

Multiple hypotheses could explain these differences, including the direct effects of abiotic conditions on embryonic development. Salt marsh sediments often have low levels of dissolved oxygen (Bradley and Morris, 1990; Timmerman and Chapman, 2004), and while horseshoe crab embryos are tolerant of low oxygen availability prior to their first embryonic molt, later developmental stages require greater oxygen availability (Funch *et al.*, 2016). This ontogenetic increase in oxygen demand, coupled with the generally reduced availability of oxygen in salt marsh sediments (Wenzhöfer and Glud,

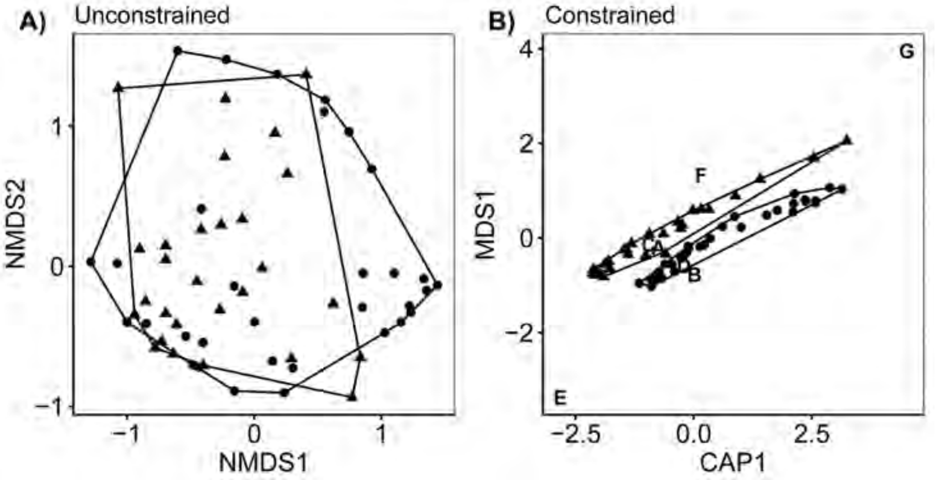

Figure 7. Multivariate analyses showing unconstrained (A) and constrained (B) ordination plots of horseshoe crab (*Limulus polyphemus*) developmental stages. Each point represents an individual sample (and its location within multivariate space) from either a marsh site (triangles) or a beach site (circles). Large overlap is observed between points from each habitat in the unconstrained plot (A; 2D-stress = 0.13), indicating overall similar composition of egg stages between the two habitat types. The ordination plot constraining variability to that explained by the habitat variable, shown in (B), reveals some differences in compositions of developmental stages (indicated by A–G) between the two habitat types, driven largely by significantly greater proportions of stage E embryos and lower proportions of stage G trilobites found in marsh sites compared to beach sites. CAP, constrained analysis of principal coordinates; MDS, multidimensional scaling; NMDS, non-metric multidimensional scale.

2004; Taillefert *et al.*, 2007), may explain the similarity in the patterns of occurrence of stage A eggs as well as the reduced number of late-stage trilobites in marsh sites compared to beach sites that we observed in this study. Furthermore, while temperatures outside of developmental optima can slow embryonic development in horseshoe crabs (Vasquez *et al.*, 2015b), we do not know whether sediment temperature differed between habitat types in this study; thus, its potential effect on differences in embryonic development rates between habitat types remains unclear. Most likely, multiple environmental factors (*e.g.*, temperature and dissolved oxygen) contribute to differences in developmental rates between the two habitat types (Jackson *et al.*, 2008; Vasquez *et al.*, 2015a, b).

For eggs laid synchronously, slower development in the salt marsh, due to temperature, oxygen availability, or other environmental conditions, may lead to lower proportions of advanced trilobites being present at marsh sites in June, when this study took place. The environmental conditions in salt marshes, however, may not ultimately represent a developmental barrier to later embryonic stages. Because of the semidiurnal tidal regime, tidal inundation of creek levees occurs twice daily in this region. If spawning occurs during spring tides of the new moon and the full moon, inundation of eggs laid at the high tide line of the beach occurs less frequently than eggs laid in creek levees. These differences in inundation regime potentially influence rates of development and trilobite emergence from the sediments. Because sea level rise is already leading to increasing inundation times of salt marshes in the region (Crotty *et al.*, 2020), understanding the role that inundation time plays in viability outcomes of horseshoe crab embryos in these environments will be critical. The snapshot approach used in this study may not accurately reflect the total survival of horseshoe crab embryos to trilobite or juvenile stages. Additional research is required to determine how differences in abiotic conditions between salt marshes and sandy beaches might affect the progression of developmental timelines for horseshoe crab embryos.

Differences in the timing of spawning activity at individual sites could also have influenced the composition of egg stages presented here. While spawning activity can be synchronous around the timing of the full moon and the new moon in some areas of the horseshoe crab's geographic range (Barlow *et al.*, 1986), many studies have shown that other local environmental factors can more strongly influence spawning (Rudloe, 1985; Cheng *et al.*, 2016; Sasson *et al.*, 2020). Anecdotal information from suppliers of horseshoe crabs to the biomedical industry indicates that in the early spring, horseshoe crabs may spawn on sandy beaches two to three days before spawning in marsh habitats; but variation in spawning locations appears to be driven primarily by local weather conditions and does not systematically differ between habitats as the season progresses. Anecdotal reports also suggest that sporadic, late-season spawning may predominantly occur in salt marsh habitats. Differences in the timing of spawning at our study sites, as a result of either local environmental conditions or inherent behavioral differences, could lead to the observed differences in the proportions of developmental stages between habitat types. Broad-scale surveys combined with *in situ* developmental assays may be necessary to determine the degree to which the timing of spawning influenced our findings.

We also found that marsh sites are more likely to contain discolored eggs. Egg discoloration may be due to the generation of necrotic tissues in response to anoxic conditions, the leaching of dissolved organic carbon (DOC) compounds as the eggs come into contact with the surrounding sediment and DOC-rich porewater, or other factors. If discolored eggs are less viable, which seems probable based on our preliminary rearing experiments (DAS, unpubl. data), a lower proportion of eggs laid at marsh sites would survive to the trilobite stage, ultimately reducing overall reproductive contributions from these habitats. For eggs without discoloration, our preliminary experimental data show that development to the trilobite stage is similar for marsh and beach sites (DAS, unpubl. data). Given the potential importance of egg discoloration as an indicator of viability, mechanisms associated with egg discoloration and how it affects embryonic development need further attention.

Although widespread in the southeastern United States, salt marshes are dynamic and heterogeneous environments (Mitsch and Gosselink, 2015); and not all areas may be equally suitable for horseshoe crab embryonic development. Highly reduced conditions characteristic of interior marsh platforms, for instance, can lead to high levels of hydrogen sulfide (Gallagher *et al.*, 1988; Ornes and Kaplan, 1989), a compound shown to be detrimental to horseshoe crab embryonic development (Vasquez *et al.*, 2015b). Collections of early-developmental-stage horseshoe crabs from marsh habitats in this study were generally made from levees bordering tidal creeks (Tiner, 2013). In creek levees, the topography is slightly elevated (Koretsky *et al.*, 2002), there are greater concentrations of macrofaunal burrows (Valiela *et al.*, 1978; Koretsky *et al.*, 2002; Seliskar *et al.*, 2002), and the redox potential tends to be less reduced, leading to lower concentrations of hydrogen sulfide compared to marsh platforms (Koretsky *et al.*, 2005). The less reduced conditions and lower levels of hydrogen sulfide in these creek levees may be more conducive to the development of horseshoe crab embryos than other areas of the salt marsh. The full extent of horseshoe crab spawning in the salt marsh, however, is unclear, because we primarily sampled creek levees. A more thorough characterization of the environmental and biogeochemical properties of the salt marsh sediments where horseshoe crabs spawn, especially accounting for diel variability, is required for relating horseshoe crab development and viability to specific factors.

While environmental factors, such as oxygen availability, are critically important to the viability of horseshoe crab eggs, other factors contribute to the overall importance of spawning habitats for recruitment to later life stages. The South Carolina coast is comprised of far more marsh habitat than beach habitat (Tiner, 2013), for instance, and the broad availability of marsh habitat may support substantial adult spawning and embryo development. Spawning adults also prefer habitats that offer some protection from high-energy waves, which can flip adults or exhume buried eggs (Penn and Brockmann, 1994; Jackson *et al.*, 2005; Smith *et al.*, 2002). The marsh habitats that we sampled were generally in areas with lower wave energy than many of the front-facing beaches in South Carolina. Given differences in habitat availability, physical factors, and environmental conditions, a landscape-scale perspective may be important for understanding the relative importance of different habitat types to horseshoe crab recruitment dynamics. Multivariate analyses showed that habitat explained a relatively low amount of variability in the composition of egg stages, indicating that factors other than coarse habitat characteristics may be contributing to some of the differences observed in this study. Increasing the sample size and adding relevant model factor terms to the analysis that we could not include (*e.g.*, days since spawning) may help to reduce the amount of unexplained variation in this model.

While spawning in sub-optimal habitat may at first appear to be detrimental to the fitness of individuals doing so, there are several potential reasons for this behavior. Bet-hedging is a strategy that trades mean fitness for reduced variation in fitness as a response to temporally and/or spatially heterogenous environments (*e.g.*, Seger and Brockmann, 1987; Childs *et al.*, 2010). Horseshoe crabs nesting in both the marsh and the beach may be an example of diversified bet-hedging, that is, not putting all their eggs in one basket (Seger and Brockmann, 1987; Oloffson *et al.*, 2009), which may increase lifetime fitness at the expense of fitness in any given year by mitigating the consequences of catastrophic occurrences in one habitat due to unforeseen events (*e.g.*, erosion, predation, *etc.*; Lee and Doughty, 2003). There may also be a genetic component to habitat selection if genotypes differ in their preferred habitat or their likelihood of switching between spawning habitats. Genetic and mark-recapture studies of spawning adults may be able to resolve these questions.

Results from this study highlight the need to account for reproductive activity across a range of physical and environmental conditions for any given species rather than focusing on those associated with habitats considered to be optimal or preferred. Reproduction in habitats deemed sub-optimal is not only common (Lewis *et al.*, 2002; Falcy, 2015; Reglero *et al.*, 2018), but also can constitute a substantial component to reproduction and, therefore, contribute significantly to the source-sink dynamics of a population (Huntsman and Petty, 2015). An understanding of species tolerances to a range of environmental conditions, including temperature, salinity, and other physio-chemical parameters, will be required to effectively predict how land use and environmental changes may affect reproduction and recruitment of a species in the future (Nelson *et al.*, 2016; Laurel and Rogers, 2020).

Management of horseshoe crabs along the Atlantic coast of the United States occurs at the state level as well as in coastwide collaboration through the Atlantic States Marine Fisheries Commission (ASMFC). Data from horseshoe crab beach spawning surveys along the Atlantic coast of the United States are often incorporated into stock assessments and adaptive resource management (ARM) frameworks for both horseshoe crabs and the federally threatened *rufa* red knot, *Calidris canutus rufa* (ASMFC, 2019). Migratory birds such as *C. canutus rufa* that rely on horseshoe crab eggs for food are more likely to feed on eggs laid at beach sites than on those laid in salt marshes (Burger *et al.*, 1997). While recent population assessments of horseshoe crabs in the southeastern United States indicate that horseshoe crab populations are healthy (ASMFC, 2019), if a significant proportion of horseshoe crab spawning is occurring in salt marsh habitats, these population censuses may not be representative of the availability of horseshoe crab eggs to migratory shorebirds feeding at the beach. Our results suggest that future assessments of horseshoe crab populations that incorporate indices based on spawning abundance should potentially include surveys of habitats generally thought to be sub-optimal. In addition, an improved understanding of how horseshoe crab population censuses relate to egg availability for migratory shorebirds would increase the effectiveness of adaptive management strategies. Surveys of habitat considered sub-optimal may become increasingly important for accurate population assessments as the reduction of available beach spawning habitat due to sea level rise and other anthropogenic effects continues.

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Data Accessibility Statement

All data used in statistical analyses presented in this paper have been deposited in the Figshare repository with the following Digital Object Identifier: 10.6084/m9.figshare.12980780 and at the following web link: https://figshare.com/articles/dataset/Kendrick_et_al_Staging_and_Staining_Data/12980780.

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EXHIBIT D

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Short communication

Physiological impacts of time in holding ponds, biomedical bleeding, and recovery on the Atlantic horseshoe crab, *Limulus polyphemus*

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ABSTRACT

Atlantic horseshoe crabs, *Limulus polyphemus* (HSC), are commercially harvested along the eastern U.S. coast and bled for hemolymph used in pharmaceutical safety testing. In South Carolina, some HSCs are held in outdoor ponds before transport to facilities where they are bled and then released to the wild. This study examined whether the time HSCs are held before bleeding, bleeding itself, or the duration of the recovery after bleeding affects HSC mortality and physiological condition. Female HSCs were collected from Coffin Point Beach, South Carolina (April 22–24, 2016), held in ponds for 2, 4, 6, or 8 weeks, then bled or held as controls. Body weights, hemocyanin concentrations, and hemocyte densities were measured prior to treatment (bled/control) and at 2, 6, and 12 days of recovery. Hemocyanin concentrations declined significantly in HSCs held in ponds for 8 weeks prior to bleeding and were excluded from further analyses. Compared to some studies, HSC mortalities were low (11%). Impacts of time in holding ponds, bleeding, and recovery from bleeding on physiological measures were assessed using 3-way fixed-effects ANOVA. While duration of recovery had main effects on physiological measures, significant interactions were also present. There was an interaction of treatment and recovery duration, with control crabs having higher hemocyte densities than bled animals at days 2 and 6 of recovery. There were two significant two-way interactions influencing hemocyanin concentration: pond time and recovery, and treatment and recovery. Our study suggests both main and synergistic effects are important when assessing the physiology and mortality of HSCs harvested for biomedical purposes.

The Atlantic horseshoe crab (HSC), *Limulus polyphemus*, is harvested and bled to produce *Limulus* Amebocyte Lysate (LAL), a cellular extract derived from the hemolymph used to screen pharmaceutical drugs and medical instruments for contamination by bacterial endotoxins. A 67% increase in biomedical harvest (2004–2017) and lethal harvest of HSCs for bait likely contributed to the recent declines documented for HSCs in parts of the United States (Eyler et al., 2018). The Atlantic States Marine Fisheries Commission (ASMFC) assumes a 15% mortality rate for biomedical bleeding in HSCs (Eyler et al., 2018), but estimates range from 8% (Walls and Berkson, 2003) to 30% or higher (Hurton and Berkson, 2006). Sublethal effects also have been associated with biomedical bleeding, and some evidence supports the idea that holding conditions between initial harvest and hemolymph extraction can be detrimental alone and in combination with bleeding (James-Pirri et al., 2012; Anderson et al., 2013; Smith et al., 2017).

The steps involved in the biomedical harvest of HSCs to produce LAL vary. Animals collected by licensed harvesters may be placed temporarily in ponds or immediately transported to extraction facilities. After bleeding, HSCs are often returned to the ocean. The South Carolina (SC) fishery is distinct in that HSCs can be held in ponds from 1 day to multiple weeks between mid-April and mid-June before biomedical bleeding. In SC, ponds used to hold HSCs must be aerated, mortalities must be reported, and all HSCs are required to be released by the end of the harvest season, per state-issued permits. It is well

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Abbreviations: ASMFC, Atlantic States Marine Fisheries Commission; HSC, horseshoe crab; LAL, *Limulus* Amebocyte Lysate; SC, South Carolina

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known, however, that changes in water quality (temperature, dissolved oxygen, salinity) can be harmful to marine organisms (reviewed by Burnett and Stickle, 2001; Fotedar and Evans, 2011) and HSCs in particular (Crabtree and Page, 1974; Hurton and Berkson, 2006; Coates et al., 2012).

Here, we examined whether the length of time HSCs are kept in outdoor holding ponds prior to bleeding, biomedical bleeding, and recovery after bleeding can separately or synergistically affect HSC physiological condition and mortality. The physiological condition of individual animals was monitored by measuring body weight, hemocyte density, and hemocyanin concentration in the hemolymph. These hemolymph variables have been used to monitor the physiological condition of many marine species, including HSCs (Coates et al., 2012; James-Pirri et al., 2012; Anderson et al., 2013; Kwan et al., 2014).

Between April 22 and 24, 2016, 101 female HSCs were hand-collected from Coffin Point Beach, SC (32.433802, −80.473438) and transported to the Waddell Mariculture Center in Bluffton, SC in open air trucks covered by tarpaulins. HSCs were placed randomly in three outdoor, aerated, open-bottom ponds (each approximately 1000 m^2) filled with unfiltered, natural seawater to approximately 1-m depth. These ponds simulated the environmental conditions of commercial HSC holding ponds. All HSCs were introduced into ponds at the same time and were divided between the three ponds (~34 HSCs per 1000 m^2). HSCs were not fed, and pond water was not changed, but continually aerated. Data loggers measured salinity, temperature, dissolved oxygen and pH in each pond every 15 min over the duration of the experiment (Table S1). Ponds were checked daily for mortality.

Every two weeks over an eight-week period, eight HSCs were selected randomly from each of the three experimental ponds ($n = 24$) and processed as described below. At each time point, the first animals to be processed were the biomedically bled treatment group. Four animals were removed from each of the three ponds and processed as a set; individual HSCs in each set were weighed and prosoma width was measured. Next, 1 mL of hemolymph was sampled from the large cardiac sinus for measurements of hemocyte density and hemocyanin concentration. To do this, the surface of the arthrodial membrane was disinfected with 10% betadine, a sterile 21-gauge needle was inserted through the arthrodial membrane, and 1 mL of hemolymph was drawn with a syringe. Following the 1 mL hemolymph sampling, HSCs in the biomedically bled treatment group were left exposed to air for 2 h to mimic the time out of water during transport to the bleeding facility. Afterwards, HSCs were biomedically bled simulating the commercial bleeding process used in SC. This was achieved by placing HSCs individually in a bleeding harness consisting of a board with a rubber strap used to stabilize and position each animal to expose its arthrodial membrane. The surface of the arthrodial membrane was disinfected with 10% betadine, and a sterile 14-gauge, 2-inch trocar needle was inserted through the arthrodial membrane into the cardiac sinus. The pressure in the cardiac sinus pushed hemolymph through the needle and into a beaker. Following industry standards, animals were bled for 8 min. To simulate aerial exposure during transport from the bleeding facility back to the ocean, HSCs in the treatment group were held out of water for 2 h after hemolymph extraction and then placed in the same recovery tank (described below) as the control crabs.

After the biomedically bled HSCs had been sampled and aerial exposure had begun, four animals were removed from each of the three ponds to constitute the unbled control group. Individual HSCs were weighed and prosoma width was measured. One millilitre of hemolymph was sampled from the large cardiac sinus with a syringe, as described above, for measurements of hemocyte density and hemocyanin concentration. These animals did not undergo a period of aerial exposure but were placed immediately in the recovery tank.

The recovery tank was a shaded outdoor 4.6-m circular tank (≈16,000 L) of aerated natural flow-through seawater under ambient temperature and salinity regimes. The recovery tank was monitored continuously for salinity, temperature, dissolved oxygen, and pH. In the recovery tank, however, unlike in the experimental ponds, HSCs were fed thawed shrimp once every other day to satiation. On recovery days 0, 2, 6, and 12, both treatment and control HSCs ($n = 24$ total) were weighed, and 1 mL of hemolymph was sampled as above and analyzed for hemocyte density and hemocyanin concentration. On recovery day 12 after hemolymph sampling, these 24 HSCs were returned to the ocean. This process was repeated every two weeks for a total time of eight weeks, until all HSCs were removed from experimental ponds (total bled $n = 48$; total control $n = 48$).

Variables (body weight, hemocyte density, hemocyanin concentration) were measured in all HSCs immediately after removal from experimental ponds and prior to treatment (day 0) and then after 2, 6, and 12 days of recovery. Each 1 mL hemolymph sample was divided into two aliquots. Total circulating hemocytes were enumerated by diluting 0.1 mL of hemolymph with 0.9 mL anticoagulant solution (1.74% NaCl, 100 mM glucose, 34 mM trisodium citrate, 26 mM citric acid monohydrate, 4 mM EDTA, pH 4.5; Jill Arnold, Directory of Laboratory Services, National Aquarium, Baltimore, Maryland, USA, pers. comm.) and then fixing the diluted sample with 0.1 mL 10% neutral buffered formalin. The hemocytes per volume of hemolymph were counted with a hemocytometer (Hausser Scientific; Mix and Sparks, 1980; $n = 3$ technical replicates) and hemocyte densities expressed as total hemocytes mL^{-1} hemolymph. Total hemocyte densities in HSCs at day 0 prior to biomedical bleeding ranged from about 7 to 21 × 10^6 mL^{-1}. These values are slightly lower but within an order of magnitude of values reported by others (Yeager and Tauber, 1935; Levin and Bang, 1968). Similarly, Coates et al. (2012) reported hemocyte densities of 26 to 46 × 10^6 hemocytes mL^{-1} for animals acclimated to 15 °C, but hemocyte densities declined from a mean of 29 ± 1.7 SD × 10^6 to 10 ± 1.4 SD × 10^6 hemocytes mL^{-1} hemolymph in animals shifted from 15 °C to 23 °C, overlapping values reported in the present study.

To quantify hemocyanin concentration, the second hemolymph aliquot was allowed to clot; the clot was homogenized and pelleted by centrifugation for 6 min at 600 ×g, 4 °C; supernatant was stored at 4 °C. A volume of hemolymph supernatant was diluted 1:100 in 10 mM EDTA, 2.5% NaCl (pH 10), and absorbance was measured at 338 nm on a UV-VIS spectrophotometer (Eppendorf; $n = 2$ technical replicates). Hemocyanin concentration was calculated using an extinction coefficient of 2.23 (Nickerson and Van Holde, 1971) for a 1 g 100 mL^{-1} dilution of HSC hemocyanin. In the present study, conducted at approximately 26 °C, hemocyanin concentrations of HSCs at day 0 prior to biomedical bleeding ranged from an average of 2.12 to 6.92 g 100 mL^{-1} (Table 1). Using the same assay method, Mangum (1976) measured generally higher mean hemocyanin values (9–12 g 100 mL^{-1}) in adult HSCs held at slightly cooler temperatures (22–24 °C), while Coates et al. (2012), using a different method, found higher average hemocyanin concentrations (8.0 ± 2.4 SD g 100 mL^{-1}) in smaller (~1.0 kg) HSCs held at 15 °C.

Differences in variables were tested across pond holding times using a one-way ANOVA on animals before they were biomedically bled; at this stage there was no difference in the way the animals in the bleeding treatment and the unbled control treatment were handled, so these data were combined and only the factor of time in the ponds was considered. Holding time did not significantly affect body weight ($P = .125$, ANOVA) or hemocyte densities ($H = 6.940$, $P = .079$, Kruskal-Wallis one-way ANOVA on ranks) (Table 1). These results for HSCs held at mean temperatures of 25.8–27.5 °C stand in contrast to Coates et al. (2012) who reported a 65–71% decline in hemocyte densities of HSCs held at 23 °C for eight weeks, with smaller but significant declines in animals held at colder temperatures over the same period. Their results after six weeks also showed significant reductions in hemocyte density, but only at 23 °C and not at the lower temperatures. This lower temperature of collection and acclimation compared with the present study may be an important difference. Furthermore, the declines in hemocyte densities observed by Coates et al. (2012) may be related to the high stocking density of HSCs employed in their study (i.e., ≤3 m^{-2})

Table 1
Hemocyte densities and hemocyanin concentration of female horseshoe crabs sampled at 0, 2, 6, and 12 days from the recovery tank, after having been held in experimental ponds for 2, 4, 6, or 8 weeks prior to biomedical bleeding (biomedically bled HSCs) or maintained as unbled controls. Horseshoe crabs that died before hemolymph sampling on recovery day 12 were excluded.

	Hemocyte Density (hemocytes × 10^6 mL^{-1} hemolymph)								Hemocyanin Concentration (g 100 mL^{-1})							
	Held in Ponds for 2 Weeks								Held in Ponds for 2 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	12.79	11.23	11.14	10.32	20.71	21.38	15.52	9.96	5.26	3.50	3.41	3.73	6.11	4.94	5.29	6.16
SE	1.42	2.41	2.01	2.87	3.11	3.07	3.33	2.37	0.92	0.51	0.58	0.56	0.77	0.79	0.86	0.99
n	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	Held in Ponds for 4 Weeks								Held in Ponds for 4 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	19.84	12.85	9.18	8.25	18.46	14.52	11.92	7.89	6.92	4.29	4.79	3.92	5.08	4.07	3.61	3.05
SE	2.48	2.78	1.85	2.79	3.50	3.10	1.64	2.82	1.02	0.67	0.76	0.63	0.72	0.61	0.52	0.46
n	12	12	12	12	11	11	11	11	12	12	12	12	11	11	11	11
	Held in Ponds for 6 Weeks								Held in Ponds for 6 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	17.77	8.95	8.87	9.88	13.60	13.94	13.43	10.74	5.44	3.79	2.99	3.75	5.49	4.95	3.90	4.54
SE	3.29	1.61	1.43	2.07	3.11	2.28	1.78	3.00	0.67	0.52	0.51	0.48	1.00	0.87	0.77	0.76
n	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
	Held in Ponds for 8 Weeks								Held in Ponds for 8 Weeks							
	Biomedically Bled				Unbled Control				Biomedically Bled				Unbled Control			
Days	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**	**0**	**2**	**6**	**12**
Mean	16.97	11.06	10.85	7.40	7.44	11.09	9.52	7.02	4.55	4.04	2.34	4.14	2.12	3.04	2.61	2.99
SE	1.99	2.80	1.52	1.12	1.16	1.70	1.55	1.41	0.91	0.63	0.57	0.64	0.52	0.51	0.43	0.53
n	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11

compared with the present study ($\leq 0.034\ \text{m}^{-2}$).

Time held in ponds had a significant effect on hemocyanin concentration ($H = 12.862$, $P = .005$, Kruskal-Wallis one-way ANOVA on ranks) (Table 1); pairwise comparisons using Dunn's Method revealed significant differences between weeks 2 and 8 ($P = .020$), 4 and 8 ($P = .011$), and 6 and 8 ($P = .037$). Hemocyanin concentrations after 8 weeks were 55% to 61% of the values in weeks 2, 4, and 6. These observations were consistent with the findings of Coates et al. (2012), who showed that HSC hemocyanin concentrations negatively correlated with holding time and temperature, with greatest declines of 69.3% and 65% in HSCs held at the warmest test temperature (23 °C) for 56 days. These results suggest that the physiological condition of HSCs deteriorated as a consequence of prolonged holding in captivity, especially at warmer water temperatures.

Since the physiological status of HSCs at week 8 was different from those at weeks 2, 4, and 6 before treatment began, week 8 animals were excluded from the analyses of the effects of the biomedical bleeding treatment and recovery. To examine the effects of the time HSCs were held in the ponds up to 6 weeks, biomedical bleeding, and time in the recovery tanks (0, 2, 6, and 12 days) on hemocyte densities, hemocyanin concentrations, and body weight, three-way fixed-effects ANOVA were performed, with recovery time treated as the repeated measure. Statistically significant three-way interactions ($P < .05$) were further assessed for two-way interactions using Bonferroni-adjusted alpha values ($P < .0125$). In the absence of a significant three-way interaction, data were assessed for simple two-way interactions and where present, simple main effects were tested in SPSS. Outliers were not included in statistical analyses. Statistical tests on hemocyanin concentrations were performed on raw data (as summarized in Table 1) and in the case of hemocyte densities, data were square root-transformed prior to analysis. To illustrate physiological change, hemocyte densities and hemocyanin concentrations of individual animals (Table 1) were normalized to their values on recovery day 0 (Fig. 1).

Hemocyte density declined during the recovery period ($P < .001$; Table 1; Fig. 1A), with higher values on day 2 and lower values on day 12. Neither holding time up to 6 weeks nor treatment had a significant effect on hemocyte density. There were no significant interactive effects of the three factors on hemocyte densities (($F_{6,\ 174}$) = 1.038, $P = .402$), however, there was a significant two-way interaction of treatment and days of recovery (($F_{3,174}$) = 3.168, $P = .026$). At all three pond holding times, controls had higher average hemocyte densities than bled animals at days 2 and 6 of recovery (Table 1). This pattern was not evident at 0 or 12 days of recovery, at which times unbled control animals from only one of the three pond holding times had lower average hemocyte densities than bled animals. All other interactions were not significant ($P > .05$).

Similar to hemocyte density, hemocyanin concentration declined over recovery days ($P < .001$; Table 1; Fig. 1B), but neither time in the experimental holding ponds up to 6 weeks nor treatment had a significant overall effect on hemocyanin concentration. There was no significant three-way interaction on hemocyanin concentration ($F_{4,122} = 1.353$, $P = .254$). There were, however, two significant two-way interactions that influenced hemocyanin concentration: holding time up to 6 weeks and days of recovery ($F_{4,\ 122} = 7.876$, $P < .001$), and treatment and days of recovery ($F_{2,\ 122} = 9.111$, $P < .001$). There were no significant simple main effects associated with either of the two-way interactions ($P > 0.05$). Compared to the present study, others have reported more substantial effects of biomedical bleeding on hemocyanin concentration. Anderson et al. (2013) found a rapid (one-week post-bleeding) and sustained decline (up to six weeks post-bleeding) in hemocyanin concentration of HSCs after biomedical bleeding compared to control animals under laboratory conditions. Additionally, no indication of hemolymph protein recovery has been observed at 17 days (James-Pirri et al., 2012) or even six weeks (Anderson et al., 2013) after biomedical bleeding when compared to control animals. Unlike the present study, however, previous studies did not use ponds to mimic pre-bleeding holding conditions and most conducted bleeding experiments using HSCs recently collected from the wild.

There was an overall, experiment-wide mortality rate of 11% ($n = 11$, of 101 crabs), with 5% mortality occurring in the ponds before and 6% mortality after the bleeding treatments. The same number of mortalities occurred among bled ($n = 2$) and control ($n = 2$) HSCs held in ponds for up to 6 weeks, with two additional mortalities among

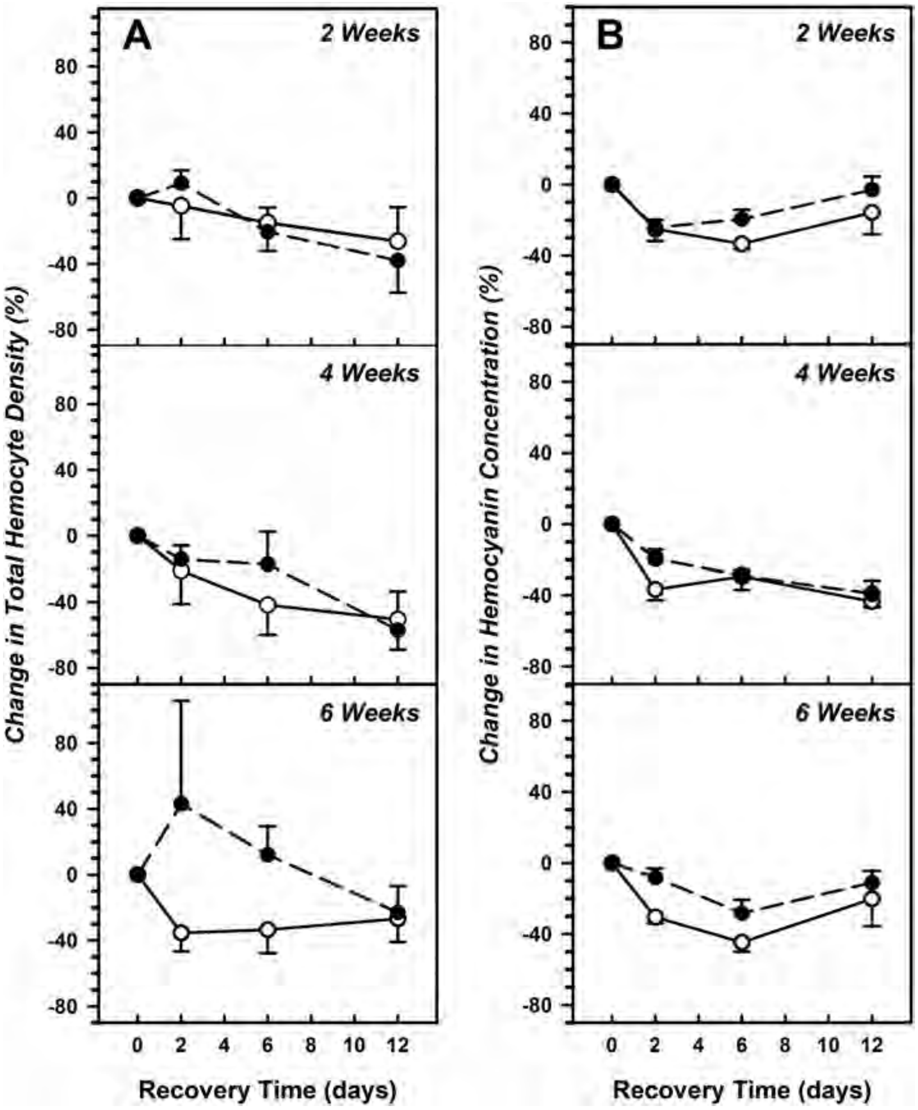

Fig. 1. Mean percent change (± SE) of hemocyte densities (A) and hemocyanin concentration (B) in biomedically bled (open circles, solid lines) and control (closed circles, dashed lines) female horseshoe crabs sampled at 0, 2, 6, and 12 days from the recovery tank, after being removed from the experimental ponds. Horseshoe crabs were held for 2, 4, or 6 weeks in experimental ponds prior to biomedical bleeding or maintained as unbled controls in May and June 2016. Horseshoe crabs that died before hemolymph sampling on recovery day 12 were excluded.

animals held for 8 weeks. The overall mortality rate was lower than the ASMFC's assumed mortality rate (15%) for HSCs undergoing biomedical bleeding (Eyler et al., 2018). Although the present study is unique in that it incorporates holding ponds representative of industry prebleeding conditions, this mortality rate was still well-within the range of rates (~8–30%) found in other studies of biomedical bleeding (Walls and Berkson, 2003; Hurton and Berkson, 2006; Leschen and Correia, 2010).

The initial body weights of HSCs held in ponds for 2, 4, and 6 weeks ranged from 2.35 to 4.65 kg ($\bar{x}$ = 3.30, n = 70, SE = 0.066). Body weight was positively correlated with prosoma width (linear regression: log body weight (kg) = −6.296 + (2.75 × log prosoma width (mm)), r^2 = 0.78; n = 70); Fig. S1). Body weight was analyzed using a three-way fixed effects ANOVA with recovery time treated as a repeated measure. There was a main effect of recovery day on body weight (P < .001), but there was no significant three-way interaction of the test factors on body weight. Pond holding time and days of recovery had a significant two-way interaction (($F_{1,54}$) = 2.641, P = .018). This analysis supported the observed decrease in the average body weight of animals (controls and bled) with longer pond holding times at each recovery day tested. Average body weights for animals held in ponds for 2, 4, and 6 weeks respectively were 3.33, 3.24, and 3.20 kg at day 0 of recovery, 3.29, 3.21, and 3.21 kg at day 2 of recovery, 3.32, 3.25, and 3.19 kg at day 6 of recovery, and 3.32, 3.25, and 3.19 kg at day 12 of recovery. These differences were small however, and all pair-wise interactions between pond holding time and day of recovery were not statistically significant.

There was a positive relationship between HSC body weight and the volume of hemolymph biomedically extracted for 8 min, with heavier animals yielding more hemolymph. This held true when HSCs that died after bleeding were included in the analysis (linear regression: hemolymph volume extracted (g) = −70.007 + (0.104 × body weight (kg)); r^2 = 0.34, $F_{1,33}$ = 17, P < .001) and when they were not included (linear regression: hemolymph volume extracted (g) = −94.794 + (0.1092 × body weight (kg)); r^2 = 0.40, $F_{1,31}$ = 20.35, P < .001). The total volume of hemolymph in an HSC is approximately 25% of its body weight (Hurton et al., 2005; i.e., a 3000 g HSC would have 750 mL hemolymph). Total hemolymph volumes were calculated based on body weight (Hurton et al., 2005), and volumes extracted were converted to percentages of the calculated total hemolymph volumes. The average volume of hemolymph extracted from individual HSCs was 263.2 mL (32% of the total hemolymph volume), ranging from 18.6 to 437.1 mL (3–51% of total hemolymph volume). Novitsky (1984) previously estimated that 30% of HSC blood volume could be safely extracted during the biomedical bleeding process, while Hurton and Berkson (2006) found that mortalities increased with extraction volumes up to 40% of total hemolymph. Indeed, in this study the two bled HSCs that died had 47.2% and 50.9% of their total hemolymph volume extracted. Our results indicate that as much as half of an individual's total hemolymph volume might be extracted during a standardized eight-minute biomedical bleeding, a substantial loss of hemolymph during an already stressful process (Leschen and Correia, 2010; James-Pirri et al., 2012). Current protocols for biomedical bleeding, however, do not measure the total or extracted volume of hemolymph in individual animals.

To our knowledge, this is the first published study to explicitly incorporate holding ponds into mortality and physiological assessments of HSCs used for biomedical bleeding. Hemocyte densities and hemocyanin concentrations of HSCs were not significantly affected by being held in ponds up to six weeks, but reduced physiological status (i.e., hemocyanin concentration) was documented at eight weeks. While duration of recovery had significant main effects on physiological measures, the interactive effects reported here indicate that synergistic impacts of the biomedical harvest on HSC health are important to consider. Overall, this study supports previous findings that HSC physiological status may be impacted by biomedical harvest.

Ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.110554.

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EXHIBIT E

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH CAROLINA COASTAL CONSERVATION LEAGUE,

Plaintiffs,

v.

ROBERT H. BOYLES, JR., in his official Capacity as Director of the South Carolina Department of Natural Resources; BLAIK KEPPLER, in her official capacity as Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; MELVIN BELL, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources; and CHARLES RIVER LABORATORIES INTERNATIONAL, INC.,

Defendants.

C.A. No. 2:22-cv-00112-RMG

DNR DEFENDANTS' RESPONSES TO PLAINTIFFS' FIRST SET OF REQUESTS FOR ADMISSION

Pursuant to Rule 36 of the Federal Rules of Civil Procedure, Defendants Robert H. Boyles, Jr., in his official Capacity as Director of the South Carolina Department of Natural Resources ("DNR" or the "Department"); Blaik Keppler, in her official capacity as Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; and Melvin Bell, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources (collectively, "DNR Defendants") herein respond to Plaintiff's First Requests for Admission as follows:

RESPONSES TO REQUESTS FOR ADMISSION

1. Please admit that biomedical bleeding or processing is the only non-research use for which horseshoe crabs may lawfully be taken, transported, or held in ponds in South Carolina.

RESPONSE: Admitted.

2. Please admit that there is only one entity or person permitted to biomedically bleed or process horseshoe crabs in South Carolina.

RESPONSE: Admitted.

3. Please admit that horseshoe crabs have died in containment ponds in South Carolina.

    a. Please admit that horseshoe crabs have died as a result of being held in containment ponds in South Carolina.

RESPONSE: Admitted as to 3. As to 3a, the DNR Defendants lack information or belief as to whether "horseshoe crabs have died as a result of being held in containment ponds in South Carolina" and therefore deny same.

4. Please admit that horseshoe crabs have been injured in containment ponds in South Carolina.

    a. Please admit that horseshoe crabs have been injured as a result of being held in containment ponds in South Carolina.

RESPONSE: As to 4 and 4a, the DNR Defendants lack information or belief as to whether "horseshoe crabs have been injured in containment ponds in South Carolina" and whether "horseshoe crabs have been injured as a result of being held in containment ponds" and therefore deny same.

5. Please admit that horseshoe crabs have spawned in containment ponds in South Carolina.

RESPONSE: The DNR Defendants lack information or belief as to whether "horseshoe crabs have spawned in containment ponds in South Carolina" and therefore deny same.

6. Please admit that red knots do not consume horseshoe crab eggs located in or adjacent to containment ponds in South Carolina.

RESPONSE: The DNR Defendants lack information or belief as to whether "red knots do not consume horseshoe crab eggs located in or adjacent to containment ponds in South Carolina" and therefore deny the same.

7. Please admit that containment ponds are physically separated from tidal salt waters.

RESPONSE: Although the containment ponds are not contained within tidal salt waters, all containment ponds have water exchange in some manner with tidal salt waters upon information and belief. For this reason, the DNR Defendants deny Request 7 as stated.

8. Please admit that no measures are in place at containment ponds to reduce water temperatures.

RESPONSE: The water exchange mechanisms referenced in Request 7 can act to reduce water temperatures. In addition, upon information and belief, the pond operators have installed aeration equipment at the containment ponds which can also reduce water temperatures. For these reasons, the DNR Defendants deny Request 8 as stated.

9. Please admit that horseshoe crabs are not fed in containment ponds.

RESPONSE: Admitted, however, the lack of feeding is designed to preserve dissolved oxygen levels in the containment ponds thereby improving conditions for the horseshoe crabs during the limited periods they are permitted to be held in the containment ponds. In addition, although the horseshoe crabs are not fed by the pond operators, there are potential natural food sources within the containment ponds upon information and belief.

10. Please admit that horseshoe crabs have died during or shortly after biomedical bleeding or processing in South Carolina.

a. Please admit that horseshoe crabs have died as a result of biomedical bleeding or processing in South Carolina.

RESPONSE: As to 10 and 10a, the DNR Defendants lack information or belief as to whether "horseshoe crabs have died during or shortly after biomedical bleeding or processing in South Carolina" or whether "horseshoe crabs have died as a result of biomedical bleeding or processing in South Carolina" and therefore deny same.

11. Please admit that horseshoe crabs held in containment ponds suffer greater health consequences from subsequent biomedical bleeding or processing.

RESPONSE: The DNR Defendants lack information or belief as to whether "horseshoe crabs held in containment ponds suffer greater health consequences from subsequent biomedical bleeding or processing" and therefore deny same.

12. Please admit that SCDNR permits for horseshoe crab harvesting, possession, transportation, holding and/or biomedical processing generally expire around the end of each harvest season.

RESPONSE: Admitted.

13. Please admit that SCDNR re-issues or issues new permits for horseshoe crab harvesting, possession, transportation, holding and/or biomedical processing each harvest season.

RESPONSE: Admitted.

14. Please admit that the conditions in SCDNR-issued permits for horseshoe crab harvesting, possession, transportation, holding and/or biomedical processing are subject to change each harvest season.

RESPONSE: Admitted.

15. Please admit that SCDNR has changed the conditions in SCDNR-issued permits for horseshoe crab harvesting, possession, transportation, holding, and/or biomedical processing from harvest season-to-season in one or more prior years.

RESPONSE: Admitted.

16. Please admit that SCDNR has never before imposed the specific conditions contained in the "Horseshoe Crab Possession (Pre/Post-Biomedical Processing)" permit, ECF No. 42-1 at 3–4, on any prior permit governing similar activities—namely, the conditions that apply to "female crabs" and the conditions stated in paragraphs 5 and 6 of this permit.

RESPONSE: Defendants admit that the permit language on the identified permit differed in some respects from that of prior years, including the referenced portions of Paragraphs 5 and 6.

17. Please admit that SCDNR has never before imposed the specific conditions contained in the "Horseshoe Crab Hand Harvest & Transport Permit HH22-00," ECF No. 42-1 at 5–6, on any prior permit governing similar activities—namely, the conditions that apply to "female crabs" and the time restrictions and reporting requirements in paragraph 1 of this permit.

RESPONSE: Defendants admit that the permit language on the identified permit differed in some respects from that of prior years, including the referenced portions of Paragraph 1.

s/ Sarah P. Spruill
Sarah P. Spruill, Fed. ID. No. 8054
HAYNSWORTH SINKLER BOYD, P.A.
ONE North Main, 2nd Floor
Greenville, SC 29601-2772
Telephone: 864.240.3200
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Attorneys for Defendants
Robert H. Boyles, Jr., in his official Capacity as Director of the South Carolina Department of Natural Resources; Blaik Keppler, in her official capacity as Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; and Melvin Bell, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources

Dated: October 11, 2022
Greenville, South Carolina

EXHIBIT F

ELI LILLY AND COMPANY

2018 UNGC COMMUNICATION ON PROGRESS

CONTENTS

UNGC PRINCIPLES ADDRESSED:

1 Businesses should support and respect the protection of internationally proclaimed human rights; and

2 make sure that they are not complicit in human rights abuses.

UNGC PRINCIPLES ADDRESSED:

3 Businesses should uphold the freedom of association and the effective recognition of the right to collective bargaining;

4 the elimination of all forms of forced and compulsory labor;

5 the effective abolition of child labor; and

6 the elimination of discrimination in respect of employment and occupation.

UNGC PRINCIPLES ADDRESSED:

7 Businesses should support a precautionary approach to environmental challenges;

8 undertake initiatives to promote greater environmental responsibility; and

9 encourage the development and diffusion of environmentally friendly technologies.

UNGC PRINCIPLES ADDRESSED:

10 Businesses should work against corruption in all its forms, including extortion and bribery.

Green Chemistry in Action: Replacing Horseshoe Crabs in Endotoxin Testing


JAY BOLDEN
Lilly Microbiologist

Lilly microbiologist Jay Bolden is an expert in bacterial endotoxin detection—a process used both by Lilly and the entire pharmaceutical industry to ensure the safety of their products. He never expected his hobby as a birder to intersect with a novel way to test for harmful bacteria in the lab. Yet Jay has helped Lilly to forge a new path, and in the process, **he has helped to protect a threatened marine species and the larger ecosystem that depends on it.**

For each batch of injectable medicines and medical devices manufactured across the globe, companies must prove that they have been checked—and tested free of—potentially life-threatening endotoxins. Water and raw materials used in manufacture must be tested as well. Around the world, this process is repeated approximately 70 million times each year, the linchpin of quality testing for the pharmaceutical industry. In this process, the humble horseshoe crab plays a vital role: its cloudy blue blood can be used to make an assay, known as LAL, that clots readily in **the presence of endotoxins that could prove fatal if exposed to a person's bloodstream or spinal fluid**.

To satisfy demand, LAL manufacturers capture, bleed, and release an estimated 500,000 horseshoe crabs along the eastern seaboard of the United States. Estimates of how many crabs die as a result are hard to pin down, with estimates ranging from five to 30 percent. In Asia, manufacturers use local horseshoe crab populations to make an alternative variant of the testing assay, TAL, which is used mainly by pharmaceutical manufacturers in the region. Most of the crabs harvested in Asia are ultimately killed after they are bled, and **in 2019, the International Union for Conservation of Nature and Natural Resources listed the Asian tri-spine horseshoe crab as an endangered species.**

Compounding adversities for the horseshoe crab are decades of overharvesting for use as eel and whelk bait, and fertilizer, not to mention devastating habitat loss linked to the commercial development of seashore communities and climate change impacts, including more intense storms and rising sea levels that threaten their spawning grounds. In the Delaware Bay, home to the world's largest population of spawning horseshoe crabs, the population has crashed—**declining by 90 percent over the past two decades.** As a keystone species, the crab is vital to area food webs, and its decline has adversely impacted many other animals, notably shorebirds that rely on consuming horseshoe crab eggs to complete their migrations. At least one bird species that feeds on the crab's eggs, the red knot, is now listed as threatened.

Working at Lilly, Jay had been aware of an alternative method to test for endotoxins using a synthetic alternative—recombinant factor C, or rFC—developed just over 20 years ago. However, lacking an official entry in the United States Pharmacopeia (USP), and facing regulatory uncertainty in the eyes of health authorities, it required additional validation testing to prove safety for a successful FDA approval. Across the industry there was reluctance to undertake this extra validation, as well as concerns about widespread access to the alternative test agent.

Jay began to wonder if he could make a difference. He had seen his first red knot in the Delaware Bay in 2005, during one of many birding adventures. But now, the bird and its plight held more of a personal connection. In 2014, when Jay met Dr. Jack Levin, one of the two scientists who discovered the LAL reaction in the first place, he recalled Dr. Levin saying that **if ever LAL were ever to be unseated from its central role as the main endotoxin test agent, it would be because of the birding community.**

The turning point came in 2013, when Lilly began planning an insulin-manufacturing facility in China, where the native horseshoe crab species has been in decline. Concerned about potential future supply problems with LAL—and knowing that Lilly is committed to reducing the use of animals in research and testing wherever possible—Jay lobbied and won support from two governance committees at Lilly, getting permission to validate the rFC approach.

Lilly drew a line in the sand in 2016, applying the rFC test to all new products being developed internally. We were the first company to submit an application for drug approval to the FDA—Emgality®—to prevent migraine headaches—where the final drug will be tested using rFC. In a watershed moment, the FDA approved Emgality in 2018, making it the first medicine approved for the market release using rFC. **By 2020, Lilly intends to transition 90 percent of our endotoxin tests to the synthetic compound.**

Upon implementation of the rFC test across all Lilly manufacturing sites, **on the order of several thousand horseshoe crabs will be saved in the first year and every year thereafter.** Jay hopes that the ripple effect will be much larger, if the broader pharmaceutical industry can be persuaded to adopt the rFC test as a more efficient and cost-effective approach that doesn't require the ethical tradeoffs of harvesting live animals.

EXHIBIT G

Increased Protection for Coastal Wildlife

DNR

Coastal Birds Project
Felicia Sanders

Red Knot Migration


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SC barrier islands have 1000s of migratory shorebirds. SCDNR is currently working on Red Knots, federally listed, and largest flock known on Atlantic in SC. SCDNR tracking their migration. 5000 miles from Brazil to SC. Resting and feeding undisturbed is crucial to make it to their nesting grounds and survival.

EXHIBIT H

From:	Robert H. Boyles, Jr. <BoylesR@dnr.sc.gov>
Sent:	Tuesday, March 29, 2022 1:03 PM
To:	Christian Hunt
Cc:	Shannon Bobertz
Subject:	Re: A Call for Intervention: The Decline of South Carolina's Horseshoe Crab Population
Attachments:	A Call for Intervention - The Decline of South Carolina's Horseshoe Crab Population (2022).pdf

Mr Hunt

Thank you for sharing this. I have forwarded to our staff for review and comment.

Have a great day.

rhbjr

Sent from a mobile device

> On Mar 29, 2022, at 12:33 PM, Christian Hunt <CHunt@defenders.org> wrote:
>
> Director Boyles,
>
> I trust this finds you well and healthy. I have attached a comprehensive report detailing the decline of South Carolina's horseshoe crab population. In it you will find harvest numbers and locations (including at least five poached properties), a synthesis of DNR staff warnings and observations, and management recommendations, among other things, which I hope you will consider. The piece will be sent to state legislators and various other state and federal agencies. Please let me know if you have any questions.
>
> Sincerely,
>
> **Christian Hunt**
> *Southeast Program Representative*
>
> DEFENDERS OF WILDLIFE
> TEL: 828-417-0862
> Facebook | Twitter | Instagram | Medium
>
>

A Call for Intervention

The Decline of South Carolina's Horseshoe Crab Population

DEFENDERS OF WILDLIFE

Defenders of Wildlife is a national, nonprofit membership organization dedicated to the protection of all native wild animals and plants in their natural communities.

Defenders has worked to conserve migratory shorebirds and the resources they rely on for decades, including in South Carolina. We played a major role in securing Endangered Species Act listing as threatened for the rufa red knot, a subspecies (*rufa*) that relies on horseshoe crab egg availability to survive its northbound migration. We also compelled the U.S. Fish and Wildlife Service to propose critical habitat for the red knot and successfully litigated over the illicit harvesting of horseshoe crabs from Cape Romain National Wildlife Refuge, near Charleston, South Carolina. Defenders is an active member of the Horseshoe Crab Recovery Coalition and advocates before state and federal agencies for expanded protections for horseshoe crabs.

Author
Christian Hunt, J.D., Defenders of Wildlife
Southeast Program Representative

Reviewers
Carl Brzorad, J.D., Southern Environmental Law Center
Emily Cedzo, M.E.M., South Carolina Coastal Conservation League
Deborah Cramer, Author, *The Narrow Edge: A Tiny Bird, an Ancient Crab, and an Epic Journey*
Lindsay Dubin, J.D., Defenders of Wildlife
Lawrence Niles, Ph.D., Wildlife Restoration Partnerships

Thanks to the following who provided additional insights
Ben Prater, M.E.M., Defenders of Wildlife
Rikki Parker, J.D., South Carolina Coastal Conservation League

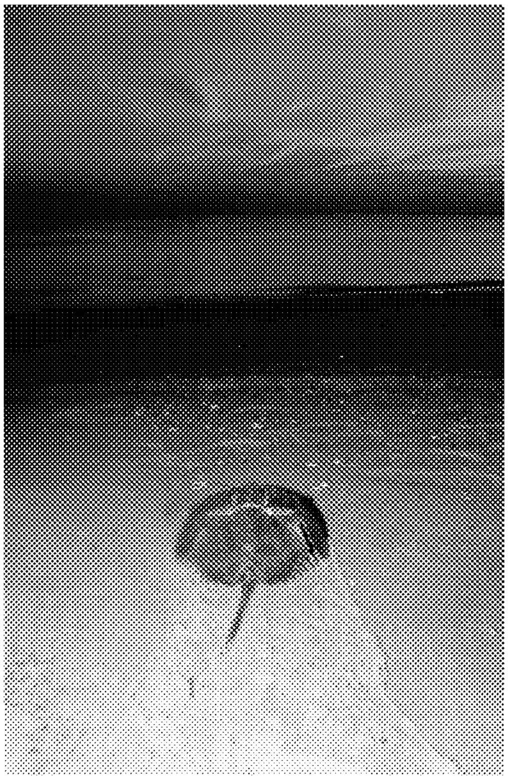
Cover Photo: Horseshoe Crabs on Beach © Ariane Mueller

Overview

The American horseshoe crab (*Limulus polyphemus*) is a unique, primordial animal. Having evolved little over hundreds of millions of years, this "living fossil" is more closely related to spiders and scorpions than crabs (Walls et al., 2002).

The horseshoe crab can be found from Maine to the Yucatan Peninsula in Mexico (South Carolina Department of Natural Resources [SCDNR], 2019b) and spends most of the year dwelling on the ocean bottom until changes in temperature draw adults to spawning beaches in early spring (Walls et al., 2002). Female crabs deposit their eggs among males in excavated nests within the intertidal zone (Brockmann & Penn, 1992).

In an ancient synchrony, migratory shorebirds time their arrival to coincide with horseshoe crab spawning events. In one of the country's most anticipated wildlife spectacles, red knots and other migrant species converge to feed on horseshoe crab eggs before continuing the journey to their Arctic breeding grounds (Botton et al., 1994). Red knots rely on horseshoe crab eggs in Delaware Bay, South Carolina and Georgia (Botton et al., 1994; Smith et al., 2019; SCDNR, 2013).


© DAVID NOE

Like the bison or passenger pigeon before it, the horseshoe crab was once considered an inexhaustible resource, with spawning aggregations so dense they could be "shovelled up and collected by the wagon load" (New Jersey Geological Survey, 1857, p. 106). Millions were annually harvested from Delaware Bay, site of the country's largest population, to feed the livestock and fertilizer industries (Cramer, 2016). Factories were established near prime spawning beaches, with crabs speared, stacked and ground into fertilizer paste (*The Great King Crab Invasion*, 1871). By the early 1900s, the Delaware Bay population had been depleted (Cramer, 2016; *Knot Then, Knot Now, Knot Later*, 2012).

Today, the species is still managed as a commodity. The horseshoe crab is harvested and bled by the biomedical industry and used as bait by eel and whelk fishermen (Atlantic States Marine Fisheries Commission [ASMFC], 2019). The ASMFC, a compact of state wildlife agencies and industry interests, oversees the management of horseshoe crabs. By developing horseshoe crab benchmark stock assessments, implementing restrictions on the number of crabs harvested for bait and imposing state quotas, the ASMFC has taken important steps to redress historical overharvesting.

Such regulations, however, are applied to bait fishermen only (ASMFC, 2019) and have failed to recover mature female horseshoe crabs, the preferred target of industry (Bi et al., 2020). At roughly 8,000 per square meter, horseshoe crab egg densities in Delaware Bay have shrunk by roughly 80% in the past three decades (Niles, 2021). Similar declines have followed in South Carolina (Niles, 2021; Niles et al., 2021).

Unlike bait fishermen, the biomedical industry has largely avoided regulation, and harvesters are uniquely unencumbered by time, place or quota restrictions (ASMFC, 2019). Despite annually exceeding coastwide kill thresholds meant to trigger intervention, no actions have followed (ASMFC, 2019). Best management practices are suggested but not enforced. Harvesters are generally monitored no more than once per year (SCDNR, 2019c). Little data is publicly shared, moreover, with harvest locations and kill rates kept confidential.

It is within this context that horseshoe crab poaching has occurred on private lands and national wildlife refuges in South Carolina, where as many as 150,000 crabs are harvested annually (Kinnard, 2021). With nearly a third of harvested female crabs potentially killed per year (Leschen & Correia, 2010), unregulated harvesting has set the stage for declines in horseshoe crab abundance, particularly among smaller, less resilient populations.

This report offers SCDNR[1]—the agency overseeing the state's horseshoe crab harvest—a set of management suggestions necessary to restore oversight and accountability, mitigate the continued decline of the species and foreclose potential conflicts of interest.

Specifically, we recommend that SCDNR:[2]

1. Restrict the timing and manner of harvest practices.
2. Explicitly articulate all relevant closures in the hand harvest permit.
3. Create and implement a horseshoe crab-specific trawl survey.
4. Prohibit the use of horseshoe crab containment ponds, where crabs are held for weeks or months without food before bleeding.
5. Sever financial ties to Charles River Laboratories, the Charleston-based company that produces the pharmaceutical product derived from horseshoe crab blood.

---

[1] For purposes of this report, SCDNR can be stratified into three groups: management or leadership; crustacean researchers; and shorebird biologists.

[2] While these actions are far from comprehensive, they provide the first step toward recovering horseshoe crabs and migratory shorebirds in South Carolina.

Red knots fuel up on horseshoe crab eggs before continuing the journey to their Arctic breeding grounds.

The Keystone Crab

Once considered a "trash fish" (Walls et al., 2002, p. 41), the horseshoe crab has enjoyed a scientific renaissance and is now recognized as a keystone species essential to the survival of many other species.

The horseshoe crab's importance to shorebirds is well-established. Each spring, red knots, ruddy turnstones, semipalmated sandpipers and other notable shorebirds descend on eastern coastal foraging areas to feed on horseshoe crab eggs before resuming their journeys to Arctic breeding grounds (Clark et al., 1993). Horseshoe crab eggs constitute the majority of the gut contents of these birds (Tsipoura & Burger, 1999).

Horseshoe crab eggs are the favored food source for the federally listed red knot and provide the highest energy accumulation rates in the birds worldwide (Piersma et al., 2005). Each knot must consume roughly 400,000 eggs to fuel the second leg of its journey (Cramer, 2018). With breeding season performance, recruitment, and population dynamics correlated to body condition, the birds that fail to acquire such reserves are less likely to survive and reproduce (Duijns et. al., 2017).

Horseshoe crabs lay their eggs 10 to 20 centimeters underground (Botton et al., 2010), making them generally out of reach to shorebirds. Only with repeated spawning by multiple crabs are their eggs distributed onto or just below the surface where they are accessible to the birds (Smith et al., 2002; Smith, 2007). Areas supporting higher densities of horseshoe crabs attract more birds because eggs are likelier to be within their reach (Niles, 2021).

During the 2000s, the overharvest of horseshoe crabs in Delaware Bay was considered the "key causal factor" in the population decline of the red knot (U.S. Fish and Wildlife Service [USFWS], 2021a, p. 9).

Red knots also rely on horseshoe crab eggs in South Carolina and Georgia (Smith et al., 2019; SCDNR, 2013). One of the single largest flocks—as many as 8,000—has been documented in South Carolina (SCDNR, 2013; SCDNR, 2018c; Smith et al., 2019).

While there, red knots feed on less energy rich coquina clams until the horseshoe crab spawn occurs, at which point they switch diet and disperse to priority crab spawning areas, such as Harbor Island and Deveaux Bank (SCDNR, 2013; SCDNR, 2018c).

©GREGORY BREESE/USFWS

Horseshoe crab eggs are a food source for many species, but an indispensable one for red knots.

In Cape Romain National Wildlife Refuge, the foraging distribution of migratory shorebirds, including the red knot, is spatially correlated to horseshoe crab egg abundance (Takahashi, 2016). The eggs produced in Cape Romain provide sustenance to not only the red knot, but also to the ruddy turnstone, short-billed dowitcher, semipalmated sandpiper, sanderling and dunlin, with 95% of tested shorebird fecal samples showing traces of eggs (Takahashi, 2016).

Two-thirds of the red knots that forage on South Carolina resources bypass Delaware Bay altogether (SCDNR, 2018c), underpinning the state's unique importance as a staging area for long-distance migrants (Smith et al., 2019).

A wide diversity of marine species also relies on horseshoe crab eggs and larvae, including Atlantic silverside, flounder, striped bass, perch and eel (Shuster, 1982a; Antonucci et al., n.d.). Adult crabs are preyed on by hammerhead and tiger sharks (Cramer, 2016). The species is also considered the preferred prey of the loggerhead sea turtle, with the management of the stock directly tied to alterations in loggerhead foraging patterns (Seney & Musick, 2007).

Following the depletion of the horseshoe crab in Virginia, the loggerhead shifted its diet to secondary prey such as blue crab. After blue crab abundance declined, the loggerhead then resorted to scavenging on net-entangled or discarded fish (Seney & Musick, 2007).

South Carolina's Biomedical Industry

Roughly 700,000 horseshoe crabs are annually harvested and live bled by the biomedical industry (ASMFC, 2020). Limulus Amebocyte Lysate (LAL), the product derived from the horseshoe crab, is manufactured by a handful of companies including Charles River Laboratories operating in Charleston, South Carolina (ASMFC, 2019).

The production of LAL ensures medical devices, such as implants and other intravenous devices, are free of bacterial contaminants (Burgenson, 2020) and supports a multi-million-dollar industry, even though synthetic alternatives are available.

The biomedical industry's annual kill rate can vary according to external stressors such as handling practices, transport, time out of water, etc. (Hurton & Berkson, 2006; Leschen & Correia, 2010).

Bleeding can result in decreased activity levels, failure to respond to tidal rhythms and reduced hemocyanin counts, which could diminish immune function (Anderson et al., 2013). Bleeding can impair mating behaviors (Owings et al., 2019), decreasing the survival of the species. Stress caused by exposure to high temperatures can impact quality and overall health (Coates et al., 2012). Mortality rates can be as high as 30% among bled females (Leschen & Correia, 2010).

The ASMFC adopted a 15% intermediate mortality rate for bled and released crabs (ASMFC, 2019). Best management practices—a set of handling guidelines designed by industry—are assumed to reduce the negative costs of bleeding (ASMFC, 2019).

A Humane Alternative to LAL

Synthetic substitute spares horseshoe crabs

By virtue of its extreme sensitivity, the horseshoe crab derivative LAL has long been considered the most reliable method for bacterial testing to ensure the safety of various pharmaceutical products (Burgenson, 2020).

In recent years, the demand for endotoxin testing has increased (ASMFC, 2020), and so too has the pressure on the wild horseshoe crab stock, inspiring the use of alternative technologies (Burgenson, 2020; Eli Lilly, 2018).

A synthetic alternative–recombinant Factor C (rFC)–precludes the need for LAL and the industry's reliance on wild crab populations. In 2012, the U.S. Food and Drug Administration approved its use if testing methods provide equivalent or better results (U.S. Food and Drug Administration, 2012).

For its equal if not superior reliability, rFC promises an eventual industry-wide transition (Piehler et al., 2020; Bolden & Smith, 2017; Abate et al., 2017). The pharmaceutical giant, Eli Lilly, has already shifted roughly 90% of its testing to the synthetic (Eli Lilly, 2018). The U.S. government contracted to purchase at least 100,000 doses of two COVID-19 drugs tested using rFC (Eisner, 2021b). The synthetic has also been deemed safe for use in Europe (Balfour, 2020).

By discouraging authorities from streamlining its adoption, however, Charles River has obstructed the synthetic's implementation (O'Hare, 2019), while casting doubt on its efficacy (Baldwin, 2021; O'Hare, 2019; Jordan, 2021).

A major European journal recently published an Expression of Concern, chastising Charles River's employees for inappropriately generalizing about the synthetic in 2021 (Brandl, 2021; Eisner, 2021b).

Today, Charles River relies on cartridge technology designed to reduce, but not yet replace, the use of horseshoe crab blood. That technology requires 5% of the LAL (Collins, 2017), yet is purportedly sold for significantly more than the traditional cost, incentivizing continued bleeding (Undisclosed, 2021).

Harvest Numbers

From 2004 to 2020, the East Coast biomedical harvest increased by 138% (ASMFC, 2020; ASMFC, 2005). Based on existing records, much of this growth has been concentrated in South Carolina.

When South Carolina reporting requirements began in 1991, fewer than 5,000 crabs were removed from spawning beaches (Thompson, 1999). Two years later, the number had spiked to roughly 30,000 animals and had nearly doubled again by 1996 (Thompson, 1999). By 2001, 90,000 crabs were being harvested annually (Wenner et al., 2002). Two decades later, that number had further ballooned to an astounding 150,000 animals (Kinnard, 2021). Roughly 25% of the entire biomedical take now occurs in South Carolina (ASMFC, 2019).

In South Carolina, best management practices are generally monitored no more than once per year and rarely practically applied (SCDNR, 2019c). Horseshoe crabs are regularly retrieved by the telson (tail) (Crolley, 2019; Horan, 2019; SCDNR, 2019a) in a manner known to harm the animals (SCDNR, 2019b); indelicately stacked to capacity in uncovered boats (Horan, 2019; Eisner, 2022); and harvested from beaches over 100 miles from the bleeding facility (Smith et al., 2019; Horan, 2019), confounding the true kill rate of the overall bleeding process.

Thousands of crabs are held in man-made ponds for weeks or months before bleeding, without food or regulated pond conditions, exacerbating the negative impacts of bleeding (Linesch, 2017; Hamilton et al., 2019).

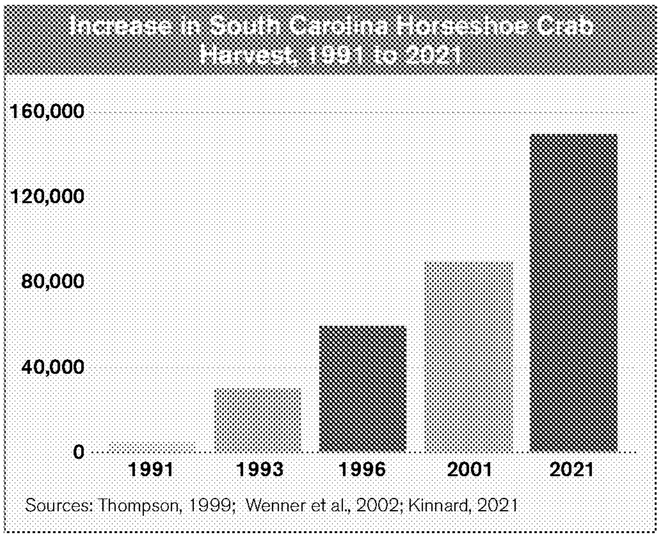
Under the most careful of handling conditions, roughly 22,000 crabs are likely killed per harvest season in South Carolina (ASMFC, 2019). Under a 30%

Technicians bleed horseshoe crabs at a Charles River Laboratories bleeding facility.

mortality threshold, the kill rate could reach nearly 50,000 per year.

In the early days of harvesting, the impacts on horseshoe crab survival were largely unknown, although the basic principles of precautionary wildlife management were well-established. According to a 1999 study, the exploitation of breeding stocks, without a management plan, could lead to abrupt and significant declines in South Carolina's horseshoe crab population, meriting the crab's listing as a threatened or endangered species (Thompson, 1999). Effective management would therefore require "knowledge of population fitness, distribution, [and] critical habitat requirements" (Thompson, 1999, p. 6), as well as active management on the part of SCDNR (Thompson, 1999).

Similar sentiments were expressed years later, when SCDNR scientists warned that an "unchecked" harvest could result in population declines that would not be apparent for nearly a decade (Wenner et al., 2002, p. 3). The magnitude of the take at that time—over 90,000 animals in 2001—was "of great concern" (Wenner, et al., p. 3) and conducted in a manner unconducive to successful spawning, with crabs removed from beaches during their most sensitive life stage (Wenner at al., 2002).



Increase in South Carolina Horseshoe Crab Harvest, 1991 to 2021

Sources: Thompson, 1999; Wenner et al., 2002; Kinnard, 2021

Over the next decade, research intensified but ultimately yielded few substantive insights into the health of localized horseshoe crab populations. By 2012, SCDNR compliance reports tied "worrisome" sampling declines to increases in harvesting (SCDNR, 2012b, p. 3).

Three years later, researchers again noted the potential for "profound" long-term impacts (SCDNR, 2015a, p. 2), with upwards of as many as 150,000 animals removed from spawning beaches per year (Kinnard, 2021).

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A harvester adds a horseshoe crab grabbed by the telson (tail)–a practice that can harm the crabs–to his haul.

After the federal listing of the red knot, SCDNR biologists, among others, expressed the need for horseshoe crab protections (Sanders et al., 2019) and mitigation of human disturbances (SCDNR, 2019e).

Rather than acting on these warnings, SCDNR leadership encouraged the phenomenal growth of industry, while conceding that its surveys were unreliable[3] (SCDNR, 2015b; ASMFC, 1998), and the status of the population structure largely unclear (SCDNR, 2015a).

Today, the male-to-female ratio and locations of juveniles, among other basic data, remain largely unknown.

Even so, *every* crab, regardless of gender, can be harvested in the midst of spawning events, on virtually all South Carolina properties. No caps are imposed on the number killed, and as many as 50,000 are potentially lost each year. Thousands are detained in ponds (Kingsley-Smith, 2017) precluding reproduction. Management decisions are rarely if ever subject to public comment or input. Even harvest data—locations, year-by-year trends, etc.—are withheld from the public.

Between 1993 and 2021, this harvest pressure increased by roughly 400% (Thompson, 1999; Wenner et al., 2002; Kinnard, 2021). Relative to population size, South Carolina's stock may suffer today from the greatest biomedical harvest pressure coastwide (Kinnard, 2021; ASMFC, 2019).

Reports of declines and/or nonexistent spawning have since become commonplace.

Purported Declines

The American horseshoe crab is currently listed by the International Union for Conservation of Nature as "Vulnerable" to extinction (Smith et al., 2016). The IUCN also listed *Tachypleus tridentatus*, a once abundant Asian horseshoe crab, as "Endangered" (Laurie et. al., 2019).

With the Asian population on the decline, increased harvest pressure may shift to North America (Burgenson, 2020), putting added pressure on South Carolina. Whether the population can sustain increased harvesting is unknown.

According to a recent study, the horseshoe crab appears genetically stable (Cushman et al., 2019) and presumably in little need of intervention in South Carolina. Because that study was the first of its kind, it could not quantify the impact that bleeding may have had on the species (Eisner, 2021b).

On-the-ground SCDNR staff, however, are now seeing fewer crabs on spawning beaches (SCDNR, 2019e). In 2018, SCDNR surveys yielded few sightings on priority spawning grounds such as Marsh Island and Hilton Head Island (SCDNR, 2018a), both heavily harvested habitats (SCDNR, 2015a; USFWS, 2019). For two years straight (2017-2018), South Carolina altogether failed to produce a meaningful spawn (SCDNR, 2018b).

On Harbor Island, crabs have been "drastically dropping in numbers," according to local experts (Albert, 2019). Since 2004, the "crabs tagged numbered in the hundreds until 2018 when we only found three individuals. This year we only found two. This sudden decline in crabs has affected the number of migratory birds arriving to refuel for the final stages of their migrations. Birds such as the red knot, once numerous, have been seldom seen.... Having observed both species for the past 10 years it is easy to see a serious problem" (Albert, 2019).

In Cape Romain National Wildlife Refuge, knots have declined and are rarely, if ever, able to feed on horseshoe crab eggs (Niles, 2021), which were once found in suitable quantities on several islands, including Marsh, Bulls and Little Bulls (Takahashi, 2016), depriving them of a critical food source. Once seen in abundance, few if any crabs have been documented in Cape Romain in recent years (Crolley, 2021).

According to a commercial boatman who has spent two days a week on the water since 2009, there has been "a distinct decline in all wildlife in Beaufort County, South Carolina, but especially shorebirds, dolphin [sic] and horseshoe crabs. The latter being especially concern-

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[3] The same surveys were used by the ASMFC in the development of the latest benchmark stock assessment, which described the trend of the Southeast population as "good." The ASMFC historically discounted nontargeted survey methods of this nature for their inherent shortcomings and potential biases (ASMFC, 1998). See p. 19 for more information.

ing because the horseshoe crab eggs have been the main food source for many migrating shorebirds" (Horan, 2021).

Where hundreds of crabs used to be tagged on important spawning beaches, a former SCDNR biologist now claims to see only four or five at any given point (Eisner, 2022).

Finally, harvesting on Turtle Island Wildlife Management Area, a once-prolific spawning beach, has depleted the local population, according to USFWS (2021c). No spawning has been documented since intensive harvesting occurred in 2019 (USFWS, 2021c), whereas spawning just across the state line in Georgia has remained consistent (Eisner, 2022).

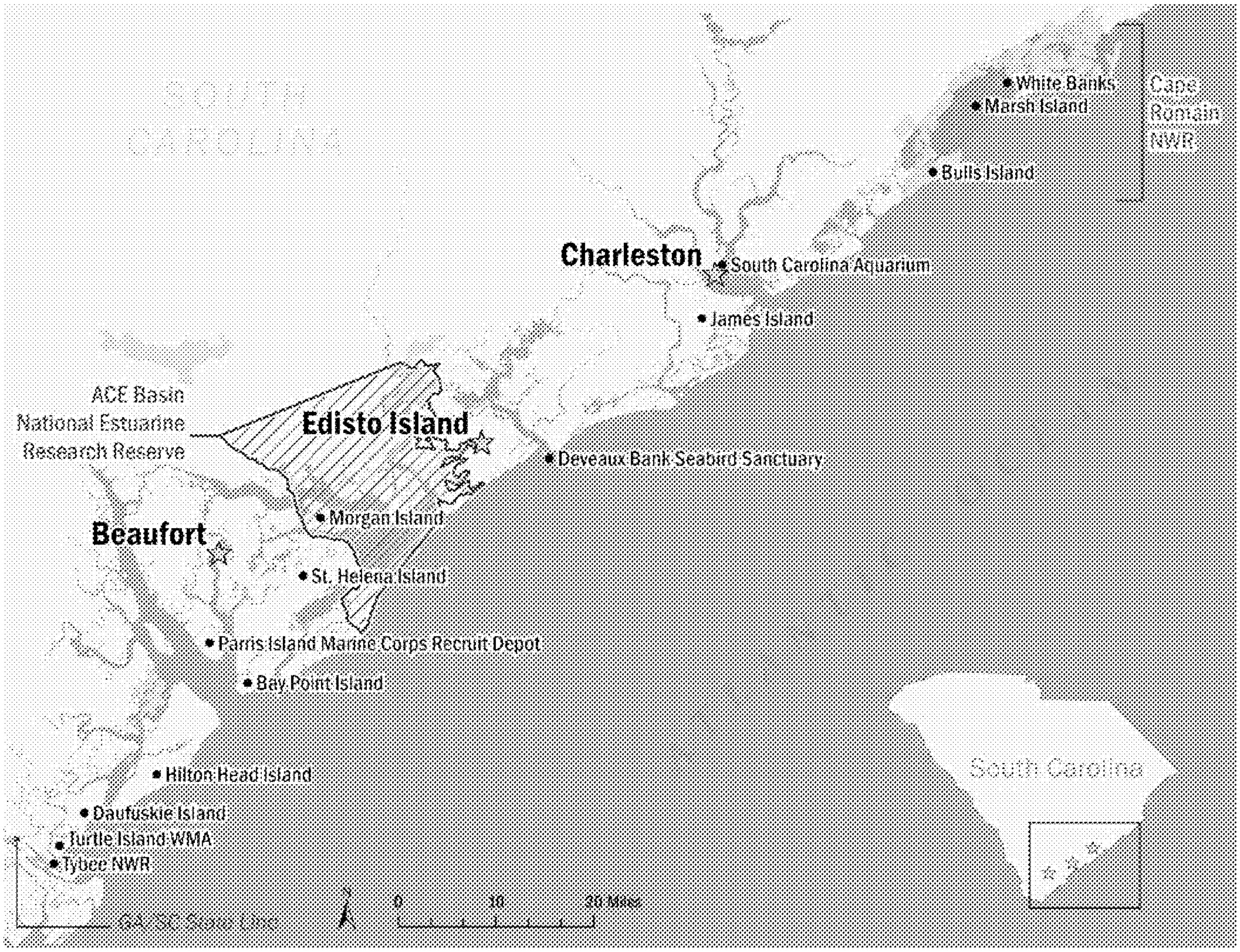
Such declines, reported throughout virtually the entire range in South Carolina, may force red knots to relocate from the state (Niles, 2021), with only Delaware Bay now capable of supporting meaningful egg densities (Niles et al., 2021).

According to biologists with the Georgia Department of Natural Resources (GDNR), "we may be witnessing a collapse" in South Carolina (Eisner, 2022).

Harvest Locations

Generally operating within informal territories, fishermen repeatedly harvest the same population throughout the summer. To prevent recapture, crabs are generally returned to the same focal region, but not the discrete habitat from which they were harvested (SCDNR, 2019d).

Dozens of state-permitted fishermen gather crabs on behalf of Charles River (Rhodes, 2012). Some of these

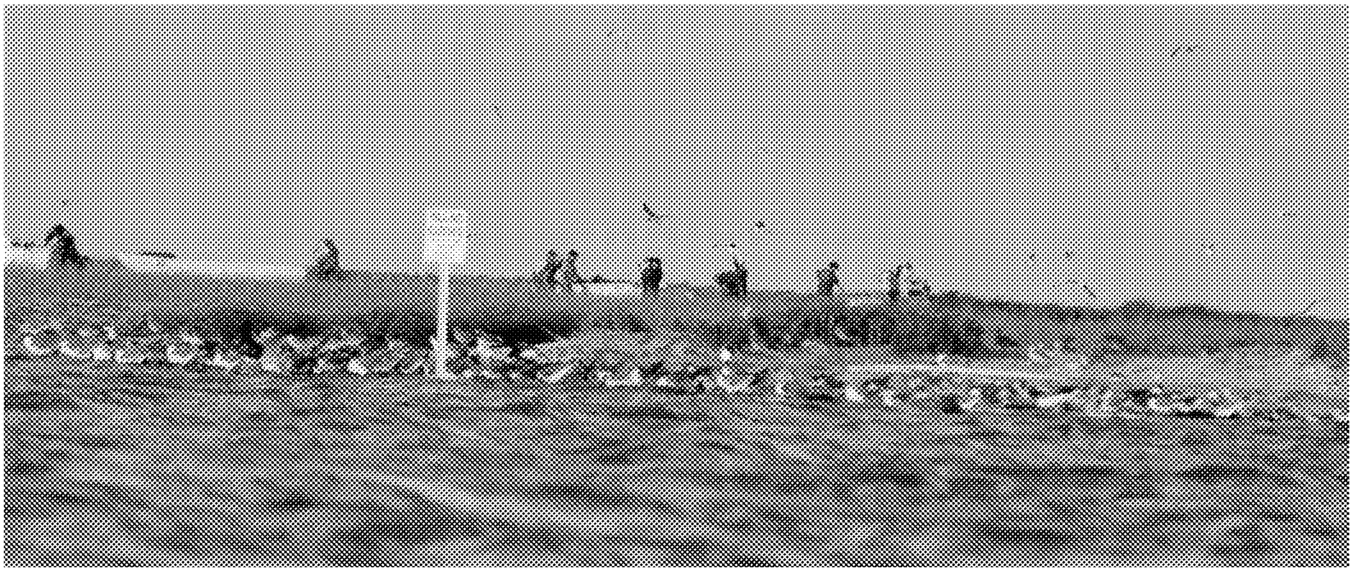


harvested crabs must be transported roughly 30 miles, others far more, to reach the Charleston-based bleeding facility. A handful of harvesters hold crabs in earthen ponds for bleeding at a later date (Hamilton et al., 2019).

Harvesting generally occurs in three focal regions in South Carolina: Beaufort, Edisto and Cape Romain (Egger, 2021). Of the specific harvest locations in these regions, the seven singled out below are notable for either their critical importance to shorebirds, documentation of poaching or a combination thereof.

Poaching has likely occurred on at least five of these properties and potentially more areas in South Carolina. (Horseshoe crabs are also harvested on Hilton Head Island, Daufuskie Island, St. Helena Island, the beach adjacent to the South Carolina Aquarium and James Island, among other places [SCDNR, 2015a; USFWS, 2021d]).

Curtailing harvesting on all of these areas can be achieved through various procedural and substantive mechanisms, such as state-enacted closures, revisions to the horseshoe crab hand harvest permit and active oversight and policing. (See section on management suggestions, page 17).



USFWS

Horseshoe crab poachers work Marsh Island in Cape Romain National Wildlife Refuge.

Cape Romain National Wildlife Refuge

Renowned for its world-class shorebird habitat, Cape Romain is among the most important wintering and migration areas on the Atlantic Coast, supporting hundreds of thousands of birds (Dodd & Spinks, 2001). The refuge has a higher critical value than both Delaware Bay and all other Pacific, Atlantic and Gulf Coast Western Hemisphere Shorebird Reserve Network sites once overall species richness and presence of declining species are considered (Dodd & Spinks, 2001).

Cape Romain's Marsh Island is the state's only site with documented brown pelican nesting every year since recording began (USFWS, 2019). Over five decades, Marsh Island has supported 18 nesting species of seabirds, shorebirds and wading birds (USFWS, 2019). The island also supports the highest number of red knots on the refuge (Wallover et al., 2015), as well as the state's largest assemblage of roosting and foraging marbled godwits (USFWS, 2019).

Human disturbance is considered a significant cause of shorebird declines, impacting their ability to successfully breed, roost and forage (Pfister et al., 1992; Burger, 1994). Disturbance causes increased flushing from nests and decreased chick survival (USFWS, 2010). Even occupying boats and kayaks *near* nesting islands poses a threat (USFWS, 2010). Mitigating human disturbance is thus of critical conservation importance (USFWS, 2010).

To that end, USFWS closes Marsh Island, White Banks and Sandy Point to all entry annually from February 15 to September 15, with the closed area extending down to the low mean water mark (50 CFR 26.34(mm)(1)(v); USFWS, 2019).

Notwithstanding these restrictions, a state-licensed fisherman and his crew bypassed island closure signs for roughly a decade, harvesting tens of thousands of crabs in violation of federal law (USFWS, 2019; Dawsey, 2014).

In 2013, SCDNR was "in agreement" that the Marsh Island harvest was not in compliance with federal regulations (USFWS, 2019, p. 3). USFWS also notified Charles River of its violations (USFWS, 2019; Eisner, 2021a). While closures were temporarily honored (Wagner, 2014), poaching resumed shortly thereafter (USFWS, 2019; Boyles, 2018).

During that time, USFWS pled for help from SCDNR in protecting the refuge, meeting with leaders on six occasions (Boyles, 2018). Ultimately, SCDNR denied additional meeting requests (Boyles, 2018); refused to explicitly incorporate the closures into hand harvest permits (Bell, 2014; Bell, 2020; SCDNR, 2019c); and issued continued permits, later claiming USFWS could not close any areas of the refuge to human entry (SCDNR, 2021b).

Defenders of Wildlife and the Southern Environmental Law Center sued USFWS over the unregulated harvesting in Cape Romain, but voluntarily dismissed their case after USFWS announced its intention to regulate the harvest in accordance with federal law.

SCDNR, meanwhile, appears poised to reissue permits to the same poacher (SCDNR, 2021b; Bell, 2020), setting the stage for continued conflict.

Tybee National Wildlife Refuge

Located near the Georgia-South Carolina state line, Tybee National Wildlife Refuge sits at the southern end of Charles River's operating range. Managed primarily for nesting shorebirds (USFWS, 2011), Tybee has supported thousands of red knots and suffers from heavy human disturbance (Smith et al., 2019).

While protected on paper, high levels of harvest have been documented in the refuge (Smith et al., 2019). According to biologists from multiple agencies and states, the magnitude of the horseshoe crab take was "unexpected" and likely "unsustainable" (Smith et al., 2019, p. 15, 18), with thousands taken during peak migration events.

With the refuge closed to public use (USFWS, 2011), the state-permitted harvest on Tybee occurs in violation of federal law, degrading a key foraging area for red knots and other shorebirds.

Turtle Island Wildlife Management Area

In 2019, thousands of red knots were documented on Turtle Island Wildlife Management Area (Smith et al., 2019). Ruddy turnstones, long-distance migrants like red knots, were also seen consuming horseshoe crab eggs before departing to fly straight to the Arctic (SCDNR, 2020a). Various imperiled species such as American oystercatchers and piping plovers use the island as well (*Turtle Island*, 2018).

The migratory birds that visit Turtle Island also rely on Georgia resources in places such as Tybee Bar and Little Tybee Island, apparently utilizing the larger area as one staging site (Smith et al., 2019; SCDNR, 2018c). Conservation actions (or lack thereof) on Turtle Island can thus reinforce or undermine conservation efforts in Georgia.

With that in mind, multiple agencies, including USFWS and GDNR, attempted to mitigate the impacts of the Turtle Island harvest (Smith et al., 2019). Since 2019, however, spawning has not been documented on Turtle Island (USFWS, 2021c), irreparably diminishing a once-prolific South Carolina habitat.

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A harvester collects horseshoe crabs on Turtle Island Wildlife Management Area. Despite efforts to mitigate the impacts of harvesting on the area, no horseshoe crab spawning has been documented on Turtle Island since 2019, the year this scene was recorded.

Bay Point Island

One of the last undeveloped barrier islands in South Carolina, Bay Point Island is considered an Important Bird Area by Audubon (*Morse Creek Inlet/Bay Point Island*, 2018). Hundreds of knots have regularly been documented during migration (Sanders, 2020; SCDNR, 2021b), with as many as 8,000 other birds recorded during winter (*Morse Creek Inlet/Bay Point Island*, 2018).

Despite being privately owned, thousands of crabs are harvested from Bay Point Island and confined to a Beaufort-area containment pond (*Morse Creek Inlet/Bay Point Island*, 2018; Parker, 2021).[4] According to Charles River's agents, development proposed by the island's owner threatens the continued viability of their harvest (Parker, 2021).

In the absence of an agreement otherwise, the harvest on Bay Point Island may occur illicitly.

Deveaux Bank Seabird Sanctuary

Of the 187 miles of South Carolina coastline, Deveaux Bank, a state-managed seabird sanctuary, constitutes less than 1 mile, yet attracts tens of thousands of birds, including red knots, black skimmers and virtually every coastal bird species of "greatest conservation need" in South Carolina (*Deveaux Bank*, 2017; Cramer, 2021).

Deveaux Bank is considered by some without equal, supporting almost 20,000 roosting whimbrels (Sanders et al., 2021), the east's largest brown pelican colony (*Deveaux Bank*, 2017; Cramer, 2021) and thousands of red knots (Smith et al., 2019). Few sites anywhere in the region support the diversity or abundance found on Deveaux.

Many of Deveaux's iconic species—piping plover, whimbrel, red knot—are vulnerable to human disturbance (Gibson et al., 2018; Wilke & Johnston González,

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[4] The harvester's name has been intentionally withheld. While it is argued that harvesters operate in the intertidal zone on properties restricted above high tide, such as Bay Point Island, SCDNR correspondences indicate that harvesters move above high tide, then beneath it, depending on the presence of observers.

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2010; USFWS, 2021a). Full intertidal closures are considered necessary to protect Deveaux's peerless wildlife (Eggert, 2012).

With limited restrictions, boaters, recreationists and anglers nonetheless make regular incursions onto Deveaux (Cramer, 2021; *Deveaux Bank*, 2017). Horseshoe crab harvesting also occurs on an annual basis (SCDNR, 2015a), often at night when birds are most easily disturbed.

Because horseshoe crabs spawn over months, harvesters return to Deveaux, repeatedly disturbing birds throughout early summer, diminishing its already limited protections.

Morgan Island

Located within the ACE Basin National Estuarine Research Reserve, Morgan Island hosts a population of monkeys used for medical research purposes. The property itself is leased by SCDNR to Charles River for roughly $1.5 million per year (Beaufort County, South Carolina, 2017; Eisner, 2022).

By virtue of the lease agreement, the taking of wildlife is strictly prohibited (Beaufort County, South Carolina, 2017; Eisner, 2022). Former SCDNR staff have nonetheless documented harvesters on Morgan Island, operating above low tide and beyond no entry signs (Eisner, 2022).

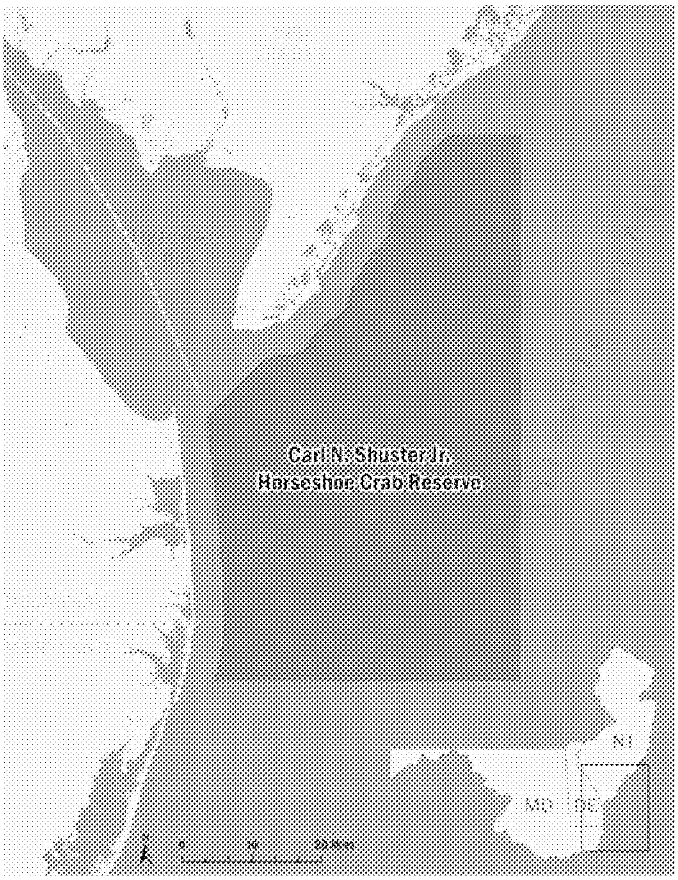
Parris Island Marine Corps Recruit Depot

Owned and operated by the U.S. Marine Corps (USMC), Parris Island serves as a recruit depot for marine training purposes. Although federal ownership extends to the channel (Presidential Proclamation, 1918; Redacted Author, 2021), harvesters have operated on Parris Island for years (SCDNR, 2015a; Horton, 2021), boating to the island and removing crabs without permission. At least one poacher has been intercepted by Parris Island law enforcement, though harvesting has largely continued unabated (Redacted Author, 2021; Horton, 2021).

USMC has discussed changes to the hand harvest permit with SCDNR (Redacted Author, 2021; Horton, 2021)—namely, the specific inclusion of Parris Island as a restricted area—but, as of December 2021, the agency had yet to commit (Horton, 2021).

Case Study: Carl N. Shuster Jr. Horseshoe Crab Reserve

In 2001, the National Marine Fisheries Service established 30 nautical miles off the mouth of Delaware Bay from south of Atlantic City, New Jersey, to just north of Ocean City, Maryland, as a horseshoe crab sanctuary. Named for pioneering horseshoe crab researcher Carl Shuster, the reserve limited possession and prohibited fishing of horseshoe crabs adjacent the bay (Restricted Gear Areas, 2001).



Shortly after the designation of the reserve, however, a biomedical company received an exemption, allowing the harvest of up to 10,000 crabs per year (Atlantic Coastal Fisheries Cooperative Management Act Provisions, 2016). In turn, harvesters tagged 15% of crabs and provided morphological data on the catch. The exemption was reissued on multiple occasions (Atlantic Coastal Fisheries Cooperative Management Act Provisions, 2016), arguably undermining the reserve's founding purposes.

In a similar vein, Charles River has requested access to the ACE[1] Basin National Estuarine Research Reserve in South Carolina. As one of the last potential viable spawning sites, the reserve's islands were set aside to monitor spawning, larval abundance, and survival (Jenkins, 2016), providing a control similar to the Carl Shuster Reserve.

SCDNR met with Charles River to discuss the prospect in July 2021 (Kinnard, 2021; SCDNR, 2021a). Shortly thereafter, the company requested universal access to all crabs under the state's jurisdiction, "*in exchange*" for $500,000 of cash resources and added data gathering (Flynn, 2021, p. 2; Eisner, 2022) (emphasis added).

Potential changes of this nature would be enshrined in the SCDNR horseshoe crab hand harvest permit.

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[1] ACE stands for the Ashepoo, Combahee and Edisto rivers.

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South Carolina Gov. Henry McMaster watches a video of the bleeding of horseshoe crabs during a visit to Charles River Laboratories in Charleston on August 6, 2021.

A Conflict of Management

When Charles River began harvesting operations, the company successfully advocated for legislation that prohibited the use of horseshoe crabs as bait. The resulting bill, drafted by Jim Cooper of Charles River, effectively privatized the use of a public trust resource, banning other commercial uses (Cooper, 2019). Since then, Charles River assumed a key role on the advisory panel of the ASMFC, regularly downplaying the impacts of the bleeding process (Cooper, 2019; ASMFC, 2019) and guiding the management of the species.

The horseshoe crab is listed today by SCDNR as one of two marine invertebrates of the highest conservation priority (SCDNR, 2014).

While various research efforts have followed (SCDNR, 2015a; SCDNR, 2015b), often with Charles River's funding (Eisner, 2020b), few if any tangible restrictions designed to prevent declines have been implemented (SCDNR, 2019c). Management suggestions from shorebird biologists have also gone largely unheeded.

According to SCDNR biologists, "efforts should be made to identify and protect foraging areas" in South Carolina (SCDNR, 2013, p. 6), since red knots often bypass Delaware Bay altogether (SCDNR, 2018c). One of the "first steps" toward that end must be the safeguarding of horseshoe crabs and coquina clams (Sanders et al., 2019, p. 1). Because red knots are unable to habituate to human presence, disturbances must also be mitigated (SCDNR, 2012a).

Today, most *if not all* priority red knot foraging areas suffer from heavy harvest disturbance, with fisherman and birds now vying for the same resources, at the same time of year, often during the agency's own monitoring efforts (Smith et al., 2019; SCDNR, 2015a). These interactions, according to SCDNR, reduce the number of red knots and the time they spend consuming prey (2012a).

Such conflicts in management—encouraging unencumbered harvesting, while trying to protect shorebird resources—have degraded even the most high-value habitats in South Carolina

For instance, Cape Romain supports 70% of South Carolina's royal and sandwich tern nests alone, as well as half the American oystercatcher population (*Cape Romain Bird Steward*, n.d.). In 2020, DNR hired a shorebird steward to work with the federal government in protecting the refuge from human disturbance (*Cape Romain Bird Steward*, n.d.).

Three days after Charles River met with the state, however, South Carolina intervened in litigation (Moore, 2021), arguing that USFWS could not close any portions of the refuge. The same areas patrolled by the shorebird steward were those DNR leadership refused to protect from harvesting (*Cape Romain Bird Steward*, n.d.; Boyles, 2018; SCDNR, 2021b).

The state also encouraged the designation of critical habitat—a framework meant to facilitate the red knot's recovery—while demanding that some of those same priority areas be opened to commercial harvesting (SCDNR, 2021b).

In some cases, efforts to limit recreational disturbance have succeeded. Specific calls for reform to horseshoe crab harvesting practices have been rebuffed, however (Smith et al., 2019; Hunt et al., 2020; Bell, 2020). Federal efforts to mitigate its impacts have also failed (Boyles, 2018; USFWS, 2019).

Shorebird biologists from multiple states now consider runaway horseshoe crab harvesting in South Carolina the *"most significant shorebird conservation issue moving forward in the region"* for its inherent disturbances and potential to deprive shorebirds of sustenance (Smith et al., 2019, p. 17) (emphasis added).

Recommended Management Actions

To address the issues raised in this report, we recommend SCDNR commit to the following precautionary management actions.

1. Restrict the timing and manner of harvest practices.

Horseshoe crabs are harvested by hand during tidal events before they have an opportunity to spawn, potentially impeding reproduction of the species (Wenner et al., 2002). To mitigate these dangers, SCDNR studied alternative collection methods in the early 2000s. The resulting data cautioned against harvesting during the high tide (Wenner et al., 2002).

While enforcement challenges would preclude the effective implementation of hourly restrictions, the principle applies: To ensure continued spawning, SCDNR must enact a series of full-day closures to prevent the interception of the crabs before they arrive on spawning beaches.

Enacting five-day closures around the new and full moons of April, May and June, for instance, could yield significant conservation gains.[5] The success of such an approach, however, would require the closing of containment ponds, where crabs are held throughout summer.

Harvesting should furthermore be prohibited on *all* known foraging red knot locations during the months of migration. State biologists have repeatedly called for the protection of crab eggs (SCDNR, 2013), the shielding of knots from human disturbance (SCDNR, 2012a), and the maintenance of inviolate habitats (Sanders et al., 2019). Without such measures, continued declines—in potential violation of the Endangered Species Act—are expected.

These foraging locations include, but not are not limited to (see USFWS, 2021b, for additional locations):

- ACE Basin National Estuarine Research Reserve (various islands)
- Bay Point Island
- Bird Key-Stono
- Cape Romain National Wildlife Refuge
- Capers Island
- Daufuskie Island
- Deveaux Bank
- Fripp Island

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[5] In Massachusetts, harvesting is prohibited on spawning beaches two days before the new and full moon and two days after, from April through June (*2021 horseshoe crab lunar spawning closure dates*, 2021).

- Harbor Island
- Hilton Head Island
- Seabrook Island
- Turtle Island Wildlife Management Area
- Tybee National Wildlife Refuge

Finally, crustacean researchers and shorebird biologists should collaboratively design a joint management framework focused on maintaining adequate spawns, providing undisturbed foraging for migratory birds and protecting additional priority roosting and nesting habitats. Publicly available records indicate that such collaboration has been lacking, with SCDNR researchers and biologists operating within their respective siloes and communicating little about these issues.

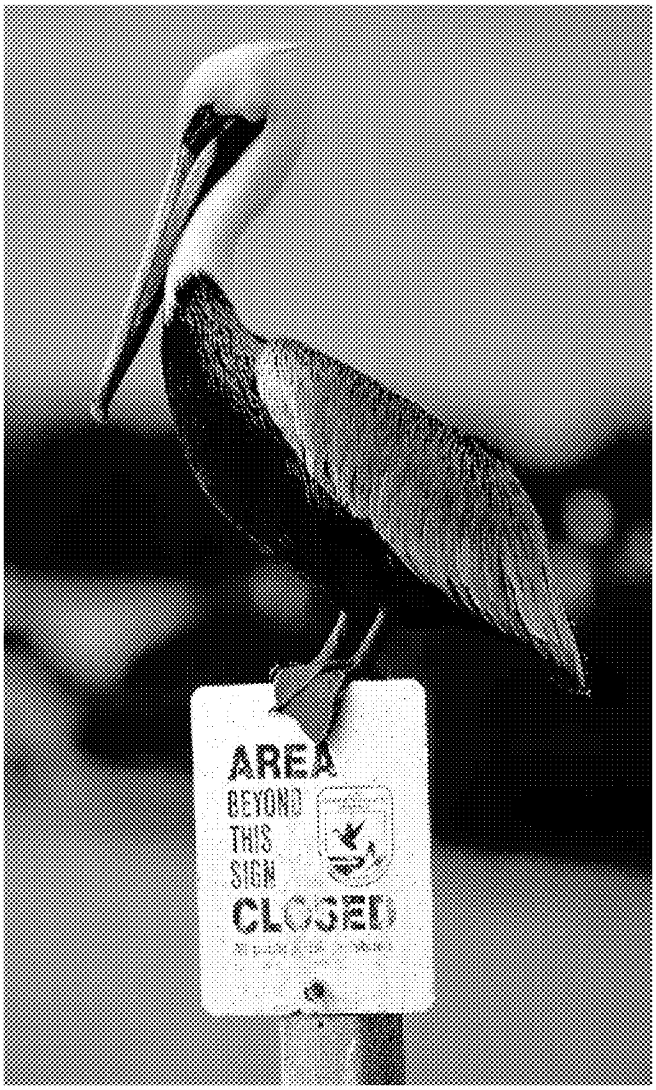
2. Explicitly articulate all relevant closures in the hand harvest permit.

Before the harvest season commences each year, SCDNR reissues saltwater fishing permits, articulating handling, transportation and return-to-water requirements. The permit generally covers a three-to-four-month window and must be renewed annually (SCDNR, 2019c).

The current permit explicitly notes closures within the ACE Basin National Estuarine Research Reserve but falls short of describing closures elsewhere (SCDNR, 2019c). Although SCDNR has the authority to list closures in the permit (SC Code § 50-5-1330, 2019), the onus is on harvesters to determine, on a property-by-property basis, which habitats are off-limits (SCDNR, 2019c). The practical effect is that few if any areas are considered restricted.

Federal land managers and nonprofits have advocated to no avail for explicit permit revisions (Hunt et al., 2020; Bell, 2014; Boyles, 2018). Nevertheless, we are persisting and encouraging the listing of *all* closed areas (national wildlife refuges, state parks, etc.), particularly those where poaching is known to occur.

We further suggest that harvest privileges be withheld from at least two poachers, whose names we can provide, to restore a sense of competitive equity for those operating within the law. Doing so would foreclose poaching on at least three, and possibly more, properties.



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Finally, in place of the permit's suggested handling practices, the vagueness of which precludes effective guidance (SCDNR, 2019c), the hand harvest permit should:

- Require the use of refrigerated trucks for transporting crabs, which are uniquely vulnerable to temperature-related impacts in South Carolina (Coates et al., 2012).
- Impose a 24-hour window in which to harvest, bleed and return crabs to water.
- Prohibit the handling of crabs by the telson.
- Mandate the marking of bled crabs to prevent recapture.

3. Create and implement a horseshoe crab-specific trawl survey.

By virtue of their passive, incidental nature, surveys used to assess horseshoe crab population trends in South Carolina are fundamentally limited (SCDNR 2015b) and of questionable reliability. None of SCDNR's surveys are designed solely for horseshoe crabs.

The ASMFC has long discounted coastwide trawl, dredge and other surveys of this kind, noting that while useful for general trends within specific areas, "each is complicated by factors that may bias the data, such as sampling error, inappropriate equipment or incomplete sampling effort" (ASMFC, 1998, p. 22).

Only one survey—the horseshoe crab-specific Virginia Tech trawl survey—has been considered fully reliable by the ASMFC (ASMFC, 2013; Niles et al., 2021), although it has yet to be replicated elsewhere.

In 2019, the ASMFC nonetheless developed a regional benchmark stock assessment utilizing state surveys of various methodologies. The ASMFC was unable to estimate male-to-female ratios or determine the structure or spawning success of discrete populations in South Carolina (ASMFC, 2019). Despite these shortcomings, the ASMFC's assessment is used by industry to justify unregulated harvesting in South Carolina (Jordan, 2021), while anecdotal reports of declines are dismissed.

In place of these surveys, SCDNR should design its own horseshoe crab-specific survey, with support from university, state and nonprofit partners.

Robust egg monitoring programs should also be consistently implemented. Horseshoe crab egg densities offer a clear connection to recovery trends for crabs and birds alike, while providing a backstop against which to examine trawl surveys (i.e., trends in trawl survey results should be closely mirrored by egg densities [buried egg clusters and surface eggs]).

In the absence of such data, SCDNR cannot defensibly condone harvesting on the scale seen today.

4. Prohibit the use of horseshoe crab containment ponds.

Crabs harvested from the Beaufort area are often deposited in earthen containment ponds, where they are stored for later bleeding. Unique to South Carolina, the practice deprives shorebirds of eggs, with crabs often stored for the entire summer when they would otherwise be spawning on beaches (SCDNR, 2020b).[6]

With numerous operators (Bell, 2015), containment ponds can encompass nearly 50 acres, holding anywhere from 10,000 to 15,000 crabs, according to Charles River (Egger, 2021).

SCDNR does not limit the number of crabs held in ponds or the duration held (SCDNR, 2020b). Crabs are not fed during detention, nor are specific habitat conditions required of operators (SCDNR, 2020b). Every year, thousands spawn in vain and die, trapped in the unsanitary, overcrowded conditions (Hamilton et al., 2019).

Because females produce upwards of 80,000 eggs per season (Taft, 2016), *billions* of eggs can be lost in ponds, endangering the long-term survival of the species.

Twenty years ago, roughly 10% of harvested crabs were held in containment ponds (Wenner et al., 2002). Today,

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Horseshoe crabs waiting to be bled are held in containment ponds like this, a practice only South Carolina permits.

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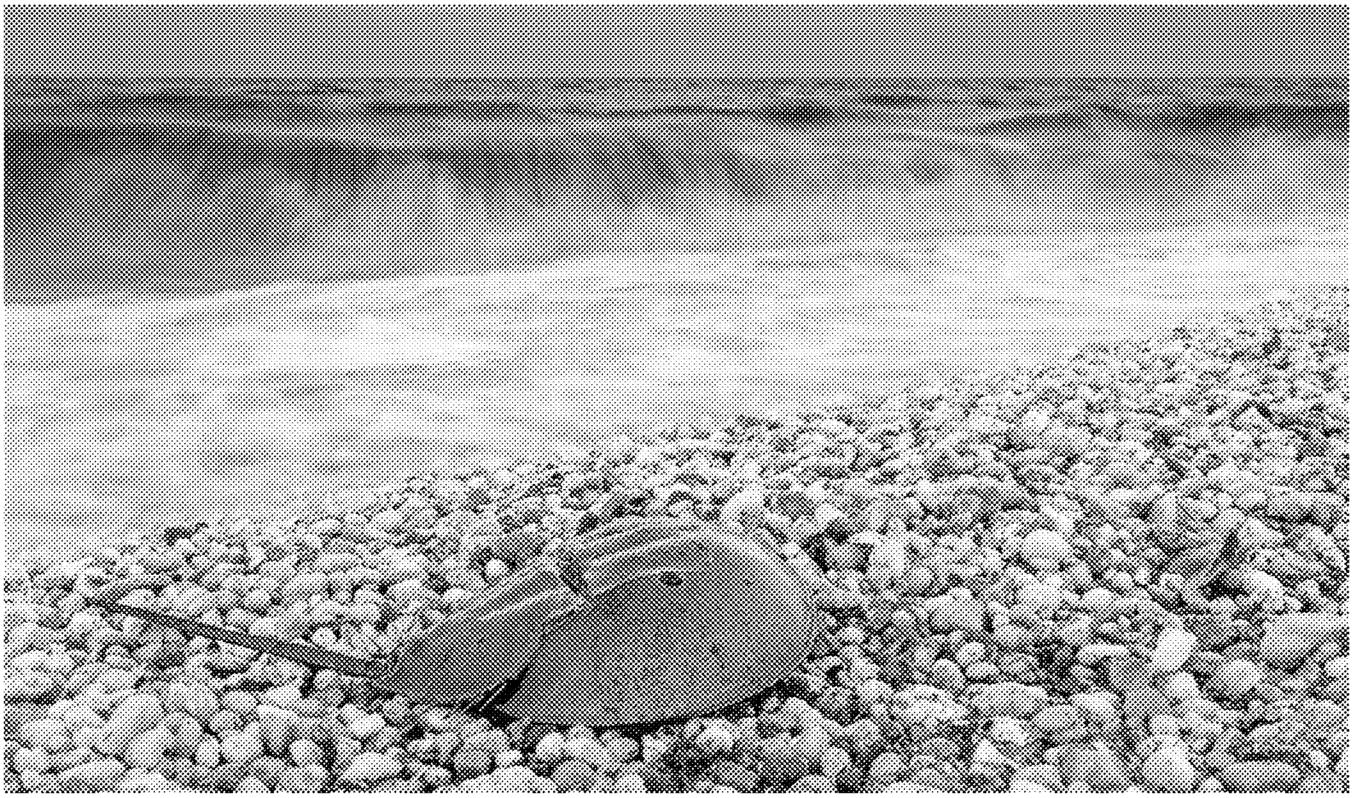
[6] In January 2022, Defenders of Wildlife and the South Carolina Coastal Conservation League, represented by the Southern Environmental Law Center, sued SCDNR and Charles River Laboratories for violating the Endangered Species Act over the practice of detaining horseshoe crabs in containment ponds.



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the majority of crabs are detained, with 75% of all harvested crabs held before bleeding (Kingsley-Smith, 2017).

Only South Carolina allows such plainly unsustainable practices, and SCDNR should forgo possession permits and instead require that harvesters transport crabs for bleeding *immediately* after removal.

5. Sever financial ties with Charles River Laboratories.

By leasing Morgan Island, a SCDNR-managed property, to Charles River, in exchange for as much as 20% of the horseshoe crab division's revenue (Eisner, 2021a), an incentive exists to allow unregulated harvesting in South Carolina. SCDNR is expected to receive roughly $1.5 million from Charles River in 2022 alone, with continued options for renewal (Beaufort County, South Carolina, 2017)

Charles River has further ingratiated itself with SCDNR by paying for research, sponsoring NGO conservation events (Eisner, 2022) and funding the agency's educational partners—including the South Carolina Aquarium (Thill, 2017).

Presumably for these reasons, the "horseshoe crab fishery in South Carolina is [considered] a sensitive one" (Kingsley-Smith, 2017, p. 1) that stands apart as uniquely politicized, according to SCDNR researchers (Kingsley-Smith, 2017, p. 1). New SCDNR staff are told to "keep at the front of your mind" internal political dynamics before unfavorably describing biomedical operations (Kingsley-Smith, 2017, p.1). Implicitly, public acknowledgements of declines are prohibited. Public management decisions are also potentially influenced through corporate offers of cash resources (Eisner, 2022; Flynn, 2021).

SCDNR cannot manage the horseshoe crab fishery in the public interest if it is financially entangled with the industry it is charged with regulating. To address the appearance, if not reality, of impropriety, SCDNR must forego all financial ties (leases, research funding, sponsorships, etc.) with Charles River and pursue funding for the Marine Resources Division through other public mechanisms. Management proposals submitted by Charles River to SCDNR should also be disclosed for public comment in a manner that solicits and welcomes public input.

Conclusion: Commit to Recovery

On average, shorebird populations have shrunk by an estimated 70% across North America in the past 50 years, with Arctic-breeding species the most severely diminished (Munro, 2017). Among these species is the rufa red knot, a bird once considered abundantly common in the Lowcountry (Bent, 1928). That abundance, according to USFWS, "remains depleted" (2021c, p. 24).

South Carolina nonetheless remains a critically important stopover. While the management suggestions offered in this report are not exhaustive, they could slow the continued degradation of this vital habitat.

To that end, SCDNR's leadership must impose *enforceable* management restrictions and faithfully enforce them, depoliticize and implement staff suggestions, engage the public in management decisions and uphold legal protections, while establishing distance between the agency and industry.

Most fundamental to this effort is the reconciling of nearly unregulated harvesting and shorebird conservation. By fighting for both, SCDNR staff are burdened with incompatible management goals, the tension of which is manifested on the most studied and high priority habitats.

Without these interventions, the red knot and horseshoe crab are likely to continue their decline, undermining South Carolina's conservation legacy.

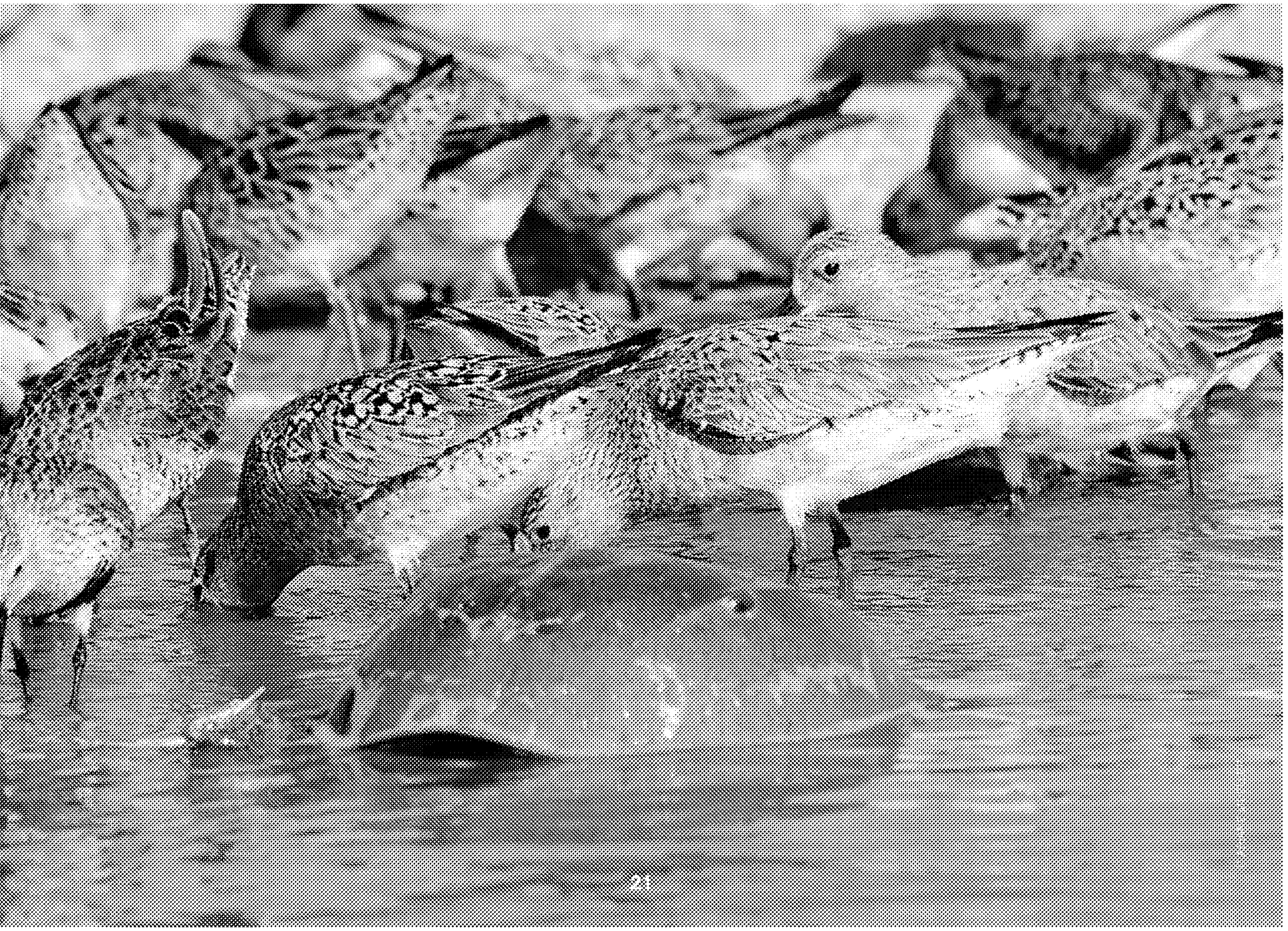

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EXHIBIT I

From: Spruill, Sarah <sspruill@hsblawfirm.com>
Sent: Tuesday, March 14, 2023 10:26 AM
To: Catherine Wannamaker; Lewis DeHope; Carl Brzorad
Subject: Permit timing

As of today, the Department's goal is to begin mailing permits for the 2023 season on Friday.

I will get you sample copies of the finalized permits as soon as I have them.

HAYNSWORTH
SINKLER BOYD

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EXHIBIT J

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH CAROLINA COASTAL CONSERVATION LEAGUE, Plaintiffs, v. ROBERT H. BOYLES, JR., in his official Capacity as Director of the South Carolina Department of Natural Resources; BLAIK KEPPLER, in her official capacity as Acting Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; MELVIN BELL, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources; and CHARLES RIVER LABORATORIES INTERNATIONAL, INC., Defendants.	)	Civ. No. 2:22-CV-112-RMG **DECLARATION OF CHRIS CROLLEY**

I, Chris Crolley, declare as follows:

1. My name is Chris Crolley. I am over the age of eighteen and suffer from no legal incapacity. This declaration is based on my personal knowledge, information, and belief.

2. I live in Awendaw, South Carolina, and I have lived in South Carolina all my life.

3. I am a member of Defenders of Wildlife ("Defenders"). I became a member of Defenders in 2021. I became a member because I support the organization's mission to protect imperiled wildlife and natural habitat.

4. I have loved the diverse habitats of South Carolina's Lowcountry all my life, so much so that I have made a career out of exploring and sharing my knowledge of the local ecosystems. This region is one of the most biodiverse on the planet, and I believe its protection is paramount.

5. I am a professional naturalist and a boat captain. Currently, I am the CEO of Coastal Expeditions, a guiding company that takes people on boat tours through the wetland and coastal areas of South Carolina and teaches them about conserving our local ecosystems. I have worked for Coastal Expeditions since 1994, and have run boat trips all along the East Coast from Newfoundland Island to the Caribbean.

6. Coastal Expeditions has its flagship location at Shem Creek and outposts on Kiawah Island, Bulls Island, St. Phillips Island, and Isle of Palms. We also offer blackwater river tours around Francis Marion National Forest, Santee Delta, ACE Basin, and beyond. We offer many different paddle, hike, and boat trips based out of these locations. For example, we offer a Kiawah River paddle tour, kayaking through the salt marsh and tidal flats around Kiawah Island and viewing the abundant wildlife in the area, including bottlenose dolphins and lots of bird species. We also offer combination paddle and hiking tours around that area for tourists to see eagles, alligators, wild turkeys, painted buntings, and the beautiful pink roseate spoonbill.

7. Coastal Expeditions is also associated with the Coastal Expeditions Foundation, a charitable group founded in 2018 with the mission of being part of the protection and conservation of the beautiful places we share on our tour groups. The Foundation funds sea turtle conservation in Cape Romain National Wildlife Refuge, sponsors educational nature field trips for local young people, and runs the Veterans Sea Kayak Resilience Program, giving local

veterans the skills they need to prevent suicide by connecting them to their environment and each other.

8. Among many other trips, Coastal Expeditions offers a Bulls Island Ferry tour for birding and hiking in Cape Romain National Wildlife Refuge. Our “Bulls Island Beach Drop” takes trip participants to a secluded and pristine beach in Cape Romain, where a naturalist is available to show them local wildlife and their habitats. Local native wildlife species include loggerhead sea turtles, bald eagles, bottlenose dolphins, horseshoe crabs, and nearly 300 bird species, including rare red knots, several species of terns, ospreys, and a plethora of other shorebirds. It is wonderful to see red knots on our trips, but they have been on the decline in the area, so it is far from certain that I will see one, even on the beaches during the spring season when they typically stop in the area.

9. In addition to the Beach Drops, Coastal Expeditions runs trips to Captain Sam’s Spit on the south side of Kiawah Island, where there is a flock of red knots that still comes to the area. Red knots are on the decline and it is getting harder and harder to see them, but it is still possible to see some at Captain Sam’s Spit, albeit in smaller numbers than in the past.

10. In my free time, I enjoy being in nature and am an avid birder. Through my work, and for personal enjoyment, I visit many of the islands and refuges in the Lowcountry region throughout the year. I am lucky to have seen red knots many times over the years. I have made many trips specifically to see red knots over the years, including to Bulls Island and Cape Romain. I live near these areas, and travel with my family to both places to look for birds and enjoy the wildlife year-round.

11. Whenever I take a trip out to the islands and beaches of the Lowcountry, I look for and try to identify shorebirds, including red knots. Although red knots are getting harder to

observe in the area, I do always look for them and sometimes succeed in seeing them. For example, in 2022 I saw a small flock of red knots on Captain Sam's Spit and about 11 red knots on Bulls Island at Cape Romain National Wildlife Refuge. I am always happy to see these beautiful birds.

12. As an example of a recent trip to the areas where red knots can sometimes be observed, in the first week of March 2023 I took a trip to Price Inlet, just south of Bulls Island and north of Capers Island, at the southern end of Cape Romain. Capers Island is a state heritage preserve and has abundant wildlife. I saw huge flocks of shorebirds (likely dowagers) while I was there. Of course I also looked for red knots, as I always do, but it was a little early in the season to see red knots and I did not identify any. On this trip to Price Inlet I was with a group of Boy Scouts from Charlotte that I have been taking to this area for decades. I plan to return next year at about the same time with the same group, and will again look for wildlife and shorebirds, including red knots.

13. I have a whole slew of similar trips coming up soon. In spring 2023 alone, I have 25 Bulls Island Beach Drop trips scheduled, so I will be traveling to the southern end of Cape Romain numerous times during the red knot stopover season. I plan to participate in a similar number of Bulls Island Beach Drop trips in the spring of 2024 as well. These trips are part of a partnership with the South Carolina Department of Natural Resources ("SCDNR") to share South Carolina's migratory shorebirds with the public and teach the public how to ethically interact with these birds. Several of the trips are with groups of birders, and part of the fun of the trip will be to see and identify birds—American oystercatchers, Wilson's plovers, least terns, and hopefully red knots. I very much hope to see red knots during the Beach Drops, but as mentioned they have been on the decline and I am not as confident as I used to be that we will be able to see

them. We would absolutely have much more enjoyable trip if we saw red knots on a Beach Drop outing.

14. Because I have been visiting South Carolina beaches on a regular basis for many years, I know from personal experience that horseshoe crab populations in southern South Carolina beaches are on the decline. Around ten years ago, I witnessed hundreds of thousands of horseshoe crabs on South Carolina beaches, especially in Cape Romain. In 2020, I only saw <u>two</u> horseshoe crabs in Cape Romain. In 2021, I specifically went looking for horseshoe crabs in the refuge and only found <u>one</u>. This trend continued in 2022. During the spring 2023 spawning season, sadly I do not expect to see horseshoe crabs in the refuge in the large numbers that I used to. In addition, I used to see dead horseshoe crabs on the beaches as part of their natural lifecycle but do not even see this anymore because their numbers are so depleted.

15. Over the years, I have seen harvesters pile horseshoe crabs into skiffs like trash while the crabs are spawning. I have seen those harvesters bring the horseshoe crabs back in a pile and throw them overboard. Once it looked like there was a contest as to who could throw the crabs farther and make them flip over in the air. There is nothing humane about the process as far as I have seen, and the fact that horseshoe crabs are held in poorly regulated containment ponds adds insult to injury.

16. The depletion of the horseshoe crab population has harmed both my business and my personal enjoyment of seeing red knots during their annual migration. I used to run a charter to take birding photographers (some professionals) to see red knots gather where the horseshoe crabs are spawning, in north Cape Romain around White Banks and Marsh Island. We tried to continue the charter in 2017, 2018, and 2019, but I have not run that charter for the last few years

because there is not much to see. There used to be hundreds of different bird species shoulder-to-shoulder gorging on horseshoe crab eggs, but this phenomenon no longer happens.

17. Each year, I track the moon and the tide, and visit the places where horseshoe crabs are meant to be spawning. I will continue to do so into the future, both for myself and to investigate whether Coastal Expeditions might be able to run a charter trip for passengers to see the natural event. I will do so this year, but sadly expect to find that the decline in horseshoe crabs in the area has continued.

18. As mentioned above, I also intend to continue to visit all the areas where red knots land for the same reasons, including Cape Romain, Kiawah, Seabrook, and the greater Beaufort area. I have run tours and personally enjoyed these areas for years, and will continue to do so this year and for many years to come. However, again, I sadly expect that it will be hard to see red knots during their annual migration, which makes my outings less enjoyable personally as a birder, and less successful professionally, as my passengers who come hoping to see migratory birds will be unlikely to be able to see red knots.

19. I fear that the horseshoe crab harvest and the use of containment ponds will continue to harm my business and my enjoyment of South Carolina’s coastal biodiversity. Over my lifetime, I have seen firsthand the depletion of the horseshoe crab population and the subsequent impacts on shorebirds. In fact, although my nine-year-old child has had the opportunity to see horseshoe crabs molting and shedding their shells, my four-year-old has never seen one. It makes me sad to know that that chance may never come. It is also disheartening to realize and to see firsthand how shorebirds are declining in this area.

20. I have become aware that SCDNR has been permitting the use of containment ponds in addition to harvesting during the horseshoe crabs’ breeding season. I have witnessed the

inhumane treatment of horseshoe crabs by harvesters, and the lack of apparent oversight or monitoring of the commercial take and containment of horseshoe crabs is baffling.

21. Based on my understanding of the unsustainable harvest of horseshoe crabs in South Carolina for biomedical purposes, including the harvesting that I have witnessed directly, I believe that the activities of Charles River Laboratories and SCDNR are causing the decline in horseshoe crabs that I have personally observed. I believe that these activities are in turn causing the decline in red knots that I have noticed.

22. The agencies that were created to regulate and protect our wildlife, should put measures in place so that horseshoe crab and red knot populations will have an opportunity to recover before the damage can no longer be undone. Then maybe, my younger children will get to witness the spawning events that my older children and I have been able to see. I will have a better chance of seeing larger flocks of red knots each migratory season and of sharing that joy with my children and with those who come to see nature with Coastal Expeditions.

23. I hope that the Court will hold SCDNR and Charles River accountable, and will end the irresponsible harvesting and containment permitting and practices that are contributing to the decline of iconic species. An order from this Court prohibiting SCDNR and Charles River Labs from holding horseshoe crabs in containment ponds would likely contribute to the recovery of horseshoe crab and red knot populations, thereby increasing my chances of again seeing and enjoying both horseshoe crabs and red knots on South Carolina’s beaches.

I declare under penalty of perjury that the foregoing declaration is true and correct. Executed on March 9, 2023 in Awendaw, SC.

By: Cal Crolley

EXHIBIT K

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH CAROLINA COASTAL CONSERVATION LEAGUE, Plaintiffs, v. ROBERT H. BOYLES, JR., in his official Capacity as Director of the South Carolina Department of Natural Resources; BLAIK KEPPLER, in her official capacity as Acting Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; MELVIN BELL, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources; and CHARLES RIVER LABORATORIES INTERNATIONAL, INC., Defendants.	Civ. No. 2:22-CV-112-RMG **DECLARATION OF DANIEL PROHASKA**

I, Daniel Prohaska, declare as follows:

1. My name is Daniel Prohaska. I am over the age of eighteen and suffer from no legal incapacity. This declaration is based on my personal knowledge, information, and belief.

2. I live in Summerville, South Carolina.

3. I am a member of Defenders of Wildlife and have been a member since 2021. I became a member because I support the organization's mission to protect and restore imperiled wildlife and their natural habitats, including here in the South Carolina Lowcountry.

4. I currently run a nonprofit that works to expand access to vision-related health care in South Carolina for low income families by improving their quality of life.

5. In my free time, I am a passionate birder and conservationist and a frequent visitor of South Carolina's parks and natural areas.

6. I have been a birder and conservationist ever since my father took me to educational programs at the South Carolina Center for Birds of Prey ("Center") and the Sewee Center in Awendaw as a young child. I was instantly hooked after observing native birds of prey up-close, and volunteered with the Center for eight years through high school and college. During that time, I went on many nature excursions and was introduced to important shorebird habitats, such as Bulls Island and the Cape Romain National Wildlife Refuge. Again, I was hooked—this time by the natural landscapes and breadth of biodiversity, especially shorebird species, right in Charleston's backyard.

7. My story came full circle when I took a job in the development office at the Center in 2016. During my tenure, which ended in February 2020, I worked closely with Friends of Coastal South Carolina, an organization dedicated to promoting citizen stewardship and supporting the Lowcountry's public lands, to facilitate educational programs for local schoolchildren in Cape Romain. Our "Year of the Bird" program in 2018 aimed to incorporate education on birds in underserved schools and organize trips for those students to Bulls Island through Coastal Expeditions. These programs left the students with perceptible enthusiasm and admiration for the refuge system, native wildlife, and avian conservation. Though I no longer work at the Center, I remain an active volunteer with their injured birds transport team rehabilitating a variety of shorebirds statewide.

8. Through my work with the Center, I learned a lot about the threats that red knots and other avian residents of South Carolina face. Although I have not yet made a positive identification of a red knot, the species is on my list of birds that I hope to see one day.

9. My experiences with the Center and as a birder, more generally, have instilled in me the need to preserve habitat for birds to feed and nest free of human disturbances. A healthy bird population is an indicator of a healthy ecosystem. However, in recent years, I have become aware that the South Carolina Department of Natural Resources (SCDNR) authorizes commercial fishermen to harvest and contain horseshoe crabs during the time of year when the red knot feeds on horseshoe crab eggs. I understand that these eggs are a critical source of nutrition for the red knot's annual migration to the Arctic, and without enough eggs to fuel their journey—due to the horseshoe crab harvest and containment—many birds will likely perish.

10. In October 2021, as part of Leadership South Carolina's state leadership development program, I traveled to Camp St. Christopher on Seabrook Island to assist the SCDNR with some ecosystem and biodiversity studies, mostly focused on fiddler crab population densities. Due to my interest in the issue and the impacts to shorebirds, I took the opportunity to ask SCDNR representatives why the agency continues to allow the harvesting of horseshoe crabs. While I did not feel that a satisfactory answer was given, I asked in an effort to promote accountability for this issue.

11. Both horseshoe crabs and red knots have special importance for me. Growing up in South Carolina, horseshoe crabs were some of the first marine animals I encountered. I remember seeing and touching them in "touch tanks" at zoos and environmental education centers as a young child. I have also seen them on the beaches over the years. I recall seeing one washed up on the

beach on a recent family trip, in fact, in 2021 or 2022. I love seeing horseshoe crabs on the beaches of my home state.

12. Red knots are special migratory birds that indicate ecosystem health and have an important environmental role. I believe there is a reason that each animal was created, to fill its niche in the environment. Removing an animal like a red knot or horseshoe crab from its niche leaves a void, with repercussions that we cannot begin to understand. I plan to teach my young son about red knots and other threatened and endangered species of birds. These birds tell the story of our natural history and also tell our human story, about the kind of world we are leaving to future generations. It is very important to me that we do all we can to protect and conserve special animals like these.

13. I do all I can in my personal life to get out in nature and see wildlife and birds. I have taken regular birding outings over the years up and down the South Carolina coast, from Huntington Beach State Park near Myrtle Beach to Edisto Beach State Park near Seabrook and Hunting Island near Beaufort. Whenever I get the chance, I enjoy birding by kayak through the region's tidal estuaries and salt marshes, and have frequently visited Garris Landing with my binoculars to check out birds and other wildlife in Cape Romain. My wife and I also enjoy visiting Kiawah Island to use the trails and beaches there and observe shorebirds in their natural habitat. I have also traveled out with our friends in Beaufort on Battery Creek to explore the ecosystems and waterways of the area.

14. In the last two years specifically, my family and I have been on a number of beach trips in South Carolina that have given me the opportunity to go birding and look for red knots and other shorebirds. We went to Kiawah Island, Edisto Island, Hunting Island, and Huntington Beach in summer 2021. In spring and summer 2022, we visited Myrtle Beach State Park and Isle of

Palms. We also visited Sullivans Island more than once in spring/summer 2022. On every one of these trips, walking on the beach and looking for birds is part of the trip and is one of my favorite things to do. I have a birding journal that contains my "life list" and carry my birding guides and binoculars with me. I am always looking for red knots, though of course they are difficult to see now that they are becoming rare. I have never seen a red knot on the beach, but would be thrilled if I did and always keep my eye out for them.

15. In 2023, I hope to get to the beach as early in the year as possible, as the weather is getting nicer. I also have a plan to visit Isle of Palms with my family and my in-laws in May 2023. On both of those trips, I will walk the beaches and look for birds, hoping to see as many shorebirds as I can. I will definitely be looking for red knots and very much hope to see one. I will also look for horseshoe crabs on these beach walks. I would be delighted to see a red knot (and also to see horseshoe crabs) on these upcoming trips.

16. In spring 2024, I plan to take one of Coastal Expeditions' new trips in the Beaufort, SC area to explore the barrier islands. I plan to visit St. Phillips Island, which offers great birding and may provide me with an opportunity to observe red knots during the spring migration. In addition, a trip to the Daufuskie Island/Turtle Island area is on my bucket list. There is a charter boat that travels from Hilton Head Island to the surrounding barrier islands and gives tourists the chance to take in local cultural sites and abundant wildlife. I hope to take this trip in 2024, giving me another opportunity to look for and try to see red knots.

17. I fear that the horseshoe crab harvest and the use of containment ponds diminishes my opportunity to ever witness red knots firsthand in the wild. I also fear that harvesting and containing horseshoe crabs, including while they are spawning, is diminishing the horseshoe crab

population and harming red knots and other shorebird species through physical degradation of bird habitat.

18. Growing up in South Carolina, I remember going to the beaches and seeing horseshoe crabs was a common occurrence. This has become significantly less common in my lifetime, particularly in the Beaufort area. In my opinion, horseshoe crabs are emblematic of South Carolina's beaches. As a person who has roots in this community and enjoys the natural wonders of this region, I want future generations to be able to witness the incredible phenomenon when horseshoe crabs and shorebirds descend on the beaches each year.

19. In my opinion, the SCDNR should not be permitting any commercial activity like horseshoe crab containment in a manner that harms shorebirds, especially a threatened species like the red knot. By killing or otherwise imperiling birds, this activity impairs my interests as a birder and my years-long work with the Center to educate people about birds and their conservation needs and to rehabilitate injured individuals.

20. I hope that the Court will put a stop to the SCDNR's irresponsible harvesting and containment authorization, so that I can enjoy birding in the Lowcountry again with the knowledge that shorebirds and horseshoe crabs are receiving the protections they deserve.

I declare under penalty of perjury that the foregoing is true and correct. Executed on March 10, 2023 in Summerville, South Carolina.

By: ______________________

EXHIBIT L

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and
SOUTH CAROLINA COASTAL
CONSERVATION LEAGUE,

Plaintiffs,

v.

ROBERT H. BOYLES, JR., in his official Capacity as Director of the South Carolina Department of Natural Resources; BLAIK KEPPLER, in her official capacity as Acting Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; MELVIN BELL, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources; and CHARLES RIVER LABORATORIES INTERNATIONAL, INC.,

Defendants.

Civ. No. 2:22-CV-112-RMG

STANDING DECLARATION OF DANA BEACH

I, Dana Beach, hereby declare and state as follows:

1. My name is Dana Beach. I am over the age of eighteen (18) and suffer from no legal incapacity. This declaration is based on my personal knowledge, information, and belief.

2. I live on Wadmalaw Island. I founded the Coastal Conservation League ("League") in 1989 and served as its executive director until 2017. I am currently a member of both the League and Defenders of Wildlife, and the League and Defenders of Wildlife represent my interests in this matter.

3. I am 66 years old and have lived in the Charleston area with my wife since 1984. I have dedicated my career to conserving the natural resources and wildlife of the Lowcountry, including threatened and endangered species, such as the *rufa* red knot.

4. I have been an amateur bird enthusiast ever since I took a course from the preeminent ornithologist John Bull at the American Museum of Natural History in 1981. That interest grew into a lifelong passion when I moved to Charleston.

5. My wife and I have been frequent guests on birding trips organized by ornithologists from The Citadel, Clemson University, and the National Audubon Society. We also became actively involved in the Charleston Natural History Society, a chapter of the National Audubon Society, and participated in the group's annual Christmas Bird Counts and other year-round bird counts.

6. Many of these birding trips have been in search of the red knot. Each spring, I make regular visits to Cape Romain, Deveaux Bank, and other South Carolina beaches to photograph and record bird sightings from a distance, with a particular emphasis on viewing and recording red knots. I intend to seek out red knots again this spring, as well as into the future.

7. My book *Deveaux* about the Deveaux Bank rookery near Seabrook Island dedicates a full chapter to red knots, and I have closely tracked its declining populations globally and in the state. The red knot decline has become apparent to me based on my own birding trips with fewer large flocks of red knots descending upon South Carolina's beaches than in years past.

8. With fewer red knots visiting South Carolina's beaches, my enjoyment in viewing this fascinating species has likewise been diminished. I am concerned that a continued population decrease will further diminish my enjoyment, or that the possible extinction of the

species will prevent me from viewing red knots altogether. This would be a significant harm to me as a resident of South Carolina with a lifelong interest in – and history of – observing this very special bird.

9. Through my research, I have learned that red knots are critically dependent on horseshoe crab eggs to fuel their migration to their Arctic breeding grounds each spring. Without a sufficient supply of nutrient-rich horseshoe crab eggs on South Carolina beaches, many birds will not complete that migration, putting this already threatened species at greater risk of extinction.

10. While red knots have historically been drawn to Delaware Bay with its once abundant egg supply, industrial-scale horseshoe crab harvesting has significantly depleted that supply. As red knots now make fewer stops in Delaware Bay, South Carolina has become an even more important migratory stop.

11. I have visited Deveaux Bank and Botany Island by boat over the past 37 years roughly 26 times a year in all seasons. In the late 1980s and 19990s horseshoe crabs often covered the beaches during the egg laying season in the spring. Numbers were so large that it was occasionally difficult to walk on the beach without diverted around the crabs. At other times during the year, the crabs were often present on the beaches and in the waters, but in lower numbers.

12. Over the past decade or so, horseshoe crab numbers have declined on these beaches dramatically. Today, even during the breeding season, only a few crabs have been present on Deveaux and Botany on a given day.

13. I am concerned that worsening conditions for red knots in South Carolina will cause the same result as Delaware Bay – fewer birds will visit the state because of a decreased

supply of horseshoe crab eggs, and my enjoyment in viewing these amazing birds will be diminished.

14. I understand that South Carolina is the only state in which horseshoe crabs are permitted to be removed from beaches and placed in containment ponds, at the precise moment that the crabs are laying the eggs upon which red knots rely. By preventing these horseshoe crabs from laying their eggs on beaches, horseshoe crab harvesters are denying red knots the vital food source on which they depend to survive their long migration to the Arctic. If red knots are denied this critical food source, there is an increased likelihood that they either die before they reach the Arctic, do not successfully reproduce, or decline to return to South Carolina’s beaches. Each of these outcomes substantially diminishes my future prospects for enjoying and photographing a robust red knot population stopping on South Carolina beaches.

15. I am also concerned about the physical disturbances that horseshoe crab harvesters are inflicting on South Carolina’s beaches, and consequently, on bird species. Many South Carolina beaches are closed to all human traffic during the spring and summer months to protect feeding and nesting birds, and the beaches that remain open still depend on minimal human interference for the best chances of survival for birds like the red knot.

16. The use and authorization of these containment ponds injures me and my interests. The removal of eggs from beaches where red knots can consume them, to containment ponds where red knots cannot, is harming and likely resulting in the death of red knots by depleting their food source and damaging their habitat. This practice reduces the numbers of red knots in South Carolina, and in turn, diminishes my opportunities to view and photograph red knots in the wild.

17. My injuries would be substantially resolved if the Court were to halt the use of horseshoe crab containment ponds in South Carolina. The end of containment ponds would result in more spawning horseshoe crabs on beaches (vs in containment ponds) and would increase the food supply for red knots. Thus, more red knots would survive their migration, reproduce, and return to the beaches of South Carolina for me to use and enjoy in the future.

I declare under penalty of perjury that the foregoing is true and correct.

Dana Beach

Dana Beach
6890 Maybank Highway
Wadmalaw Island, SC 29487

March 11, 2023

EXHIBIT M

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF SOUTH CAROLINA
CHARLESTON DIVISION

DEFENDERS OF WILDLIFE and SOUTH CAROLINA COASTAL CONSERVATION LEAGUE, Plaintiffs, v. ROBERT H. BOYLES, JR., in his official Capacity as Director of the South Carolina Department of Natural Resources; BLAIK KEPPLER, in her official capacity as Acting Deputy Director of the Marine Resources Division of the South Carolina Department of Natural Resources; MELVIN BELL, in his official capacity as Director of the Office of Fisheries Management of the South Carolina Department of Natural Resources; and CHARLES RIVER LABORATORIES INTERNATIONAL, INC., Defendants.	Civ. No. 2:22-CV-112-RMG **DECLARATION OF CHRISTIAN HUNT**

I, Christian Hunt, declare as follows:

1. My name is Christian Hunt. I am over the age of eighteen and suffer from no legal incapacity. This declaration is based on my personal knowledge, information, and belief.

2. I have devoted my career to advancing environmental protection and species conservation. I hold a law degree from Charlotte Law, where I studied environmental law, with a particular emphasis on climate change and the law and the Endangered Species Act. I also hold a B.S. in Geography from the University of North Carolina-Charlotte.

3. I have worked at Defenders of Wildlife since 2016. I'm based in Charlotte, North Carolina. I have been a member of Defenders since my hire date in 2016.

4. Defenders is a non-profit organization headquartered in Washington, D.C., with over 2.1 million members and supporters nationwide and about 16,550 in South Carolina. Defenders is dedicated to protecting native animals, plants, and the habitats they depend on, including on the South Carolina coast. Defenders advocates for approaches to wildlife conservation that help keep species from becoming threatened and endangered, using education, research, policy, and, where necessary, litigation to defend wildlife and their habitat.

5. At present, my title at Defenders of Wildlife is Senior Federal Lands Policy Analyst. I have been in that role since September 2022. In that role, I work to increase habitat protections and advance imperiled species recovery on the lands and waters of the National Wildlife Refuge System by partnering with agency decision-makers, influencing members of Congress, building coalitions, and coordinating community advocacy efforts.

6. Prior to becoming a Senior Federal Lands Policy Analyst, I was a Southeast Associate from 2016 to 2019, and a Southeast Representative from 2019 to 2022. In those roles, I worked with Defenders' partners on conservation initiatives to protect and restore the Southeast region's imperiled wildlife and their habitats. For example, I advocated to combat proposals for mining that threatened to harm the vibrant ecosystem at Okefenokee National Wildlife Refuge and worked to educate the public and decisionmakers about that issue. I worked to defend Rocky Fork State Park in Tennessee from a roadbuilding project that would have bisected valuable wildlife habitat. I also worked on various initiatives related to the imperiled red wolf, hellbender salamander, Florida panther, and other iconic Southeastern species. In addition, I worked to investigate the harvesting of horseshoe crabs at Cape Romain National Wildlife Refuge. I also

authored a policy report titled, *A Call for Intervention: The Decline of South Carolina's Horseshoe Crab*, which examined in detail the history of harvesting practices in South Carolina, including the use of horseshoe crab containment ponds. That report was published in spring of 2022.

7. Even though I have transitioned from Southeast Representative to Senior Federal Lands Policy Analyst, Defenders continues to prioritize its advocacy to protect red knots from the harms posed by horseshoe crab harvesting and horseshoe crab containment ponds in South Carolina. In my current role, I am continuing to cover this work for Defenders and will do so while Defenders works to hire a new Southeast Representative who can take the lead.

8. In late July 2021, I was working as Defenders' Southeast Representative, focusing on some of the issues described above. That month, a colleague of mine—another Southeast Representative at Defenders—asked me for assistance covering an issue that had arisen in Tennessee. That issue concerned the Volunteer Mine, a sand and gravel mining operation on the banks of the Duck River in Tennessee. The Duck River is an ecologically rich region, supporting native mussel species and more fish than all European rivers combined. The colleague was unable to devote the time needed to covering the issue, yet explained that the issue was important to Defenders' conservation mission and was eager to expand Defenders' involvement in the issue. I stepped in to help my colleague and to advance Defenders' mission of conserving imperiled species in this important bioregion.

9. During the months of August, September, October, and November, 2021, I stayed involved in the Duck River issues. The time I spent on the issue mostly consisted of participation in calls among Defenders of Wildlife and its allies regarding developments at the mine site. Throughout this time period, it was clear that more Defenders staff time was needed to help

bolster our advocacy and education efforts related to the Duck River and the Volunteer Mine. To develop a full campaign, I would have needed to stay involved and ideally increase the time I spent on the issue.

10. However, I ultimately was not able to increase my involvement in the Duck River issues and in fact eventually dropped the issues altogether to attend to urgent concerns related to the take of red knots in South Carolina. In late 2021 and the early days of 2022, my time and attention were increasingly diverted to the harvesting of horseshoe crabs from South Carolina beaches by Charles River Laboratories and its agents and the holding of those crabs in containment ponds, which interferes with spawning during the critical red knot migration season. Ultimately, those concerns culminated in the January 13, 2022 filing of the complaint in this case in federal court in Charleston. Given the amount of time required to assist with the case, prepare for, file, and monitor the lawsuit, there was ultimately no way that I could stay involved in the Duck River issues at all, let alone increase my involvement.

11. I believe that if it were not for the pressing need for education, advocacy, and litigation surrounding South Carolina horseshoe crab harvest and containment and its effects on red knots, I would have been able to participate more extensively in the Duck River mussel issues and develop a more robust campaign opposing the mining. At present I am not aware of anyone at Defenders of Wildlife working on those issues.

12. I care deeply about the health of Southeastern coastal ecosystems, including shorebirds in the Southeast U.S. I am passionate about the conservation of red knots and their horseshoe crab prey. Red knots are special to me because they are ecosystem indicators. It is my understanding, based on years of working on and studying Southeastern coastal ecosystems, that if a birder observes large gatherings of red knots, horseshoe crab eggs are typically present

nearby, which in turn means that loggerhead sea turtles and sharks also have access to their adult horseshoe crab prey base. Thriving red knot populations thus serve as a signal that larger ecosystems are functioning as they should.

13. I am a birder who focuses on looking for and trying to catch a glimpse of long-distance migratory birds. These include semipalmated sandpipers, ruddy turnstones, and red knots. These birds are fascinating to me because of their amazingly long migratory flights and because the health of their population is a reflection of the health of coastal ecosystems. All of these birds require horseshoe crab eggs to survive. They depend on thriving horseshoe crab populations.

14. From where I live in Charlotte, I visit the South Carolina beaches as often as I can. Several years ago, I went to Beaufort. I also go to Charleston fairly frequently, and recently visited the Charleston Aquarium to view their horseshoe crab exhibits and spawning beach. I always look for shorebirds when I am at the beach in South Carolina, including the red knot (although they are difficult to see because they are becoming so rare). I look for horseshoe crabs, too, every time I am exploring the beaches of South Carolina.

15. I visited the area of Cape Romain National Wildlife Refuge in the spring of 2021. On that trip, I traveled on a boat around the refuge and monitored the beaches for wildlife, including red knots. I viewed Bulls Island, Marsh Island, and White Banks. The wildlife was incredible. I saw dolphins from the boat, and of course numerous birds on the beaches and in the air. Marsh Island in particular was pulsing with birds. There was a huge diversity and abundance of birds that I’ve never seen anywhere else. That moment really brought home to me why this area, and Marsh Island in particular, is so special and important. Because this was a spring trip, I thought it might be possible to see a red knot during the spring red knot migration through the

area and of course I looked for them, but I did not see any. I was also looking for horseshoe crabs, but only saw one. The beaches were barren of horseshoe crabs.

16. I plan to return to the area, including the beaches around Cape Romain, in spring of 2024 to try once again to see red knots (and of course also horseshoe crabs). I will return to Bulls Island, Marsh Island, and White Banks to look for these special animals along the shoreline. To the extent possible given conditions at the time, I will also visit Turtle Island and Tybee National Wildlife Refuge, to see if I might be able to see a red knot or horseshoe crab at those locations as well. On this trip, like the previous one, I plan to view from a boat the spawning beaches where horseshoe crabs and red knots are known to be present (or at least should be present) to look for these special species.

17. I have been sad to learn that the practices of Charles River Labs and the South Carolina Department of Natural Resources are placing at risk my ability to see red knots and horseshoe crabs on the beaches of South Carolina. I understand that Charles River and its agents are being allowed to take horseshoe crabs from the beaches en masse and hold them in containment ponds; they may not survive the transportation and containment process and may not be on the beaches to spawn at the time the red knots arrive from their long-distance migratory flights. I find it bewildering that South Carolina’s vibrant coastal ecosystems are being harmed in this way. It is depressing to realize that if these practices are allowed to continue I am unlikely to see red knots or even horseshoe crabs on my spring 2024 trip to South Carolina. If the practices continue, I may never see a red knot in South Carolina no matter how many trips I take.

18. If the Court were to disallow the use and authorization of horseshoe crab containment in South Carolina, that would address the issue by—among other things—better ensuring that horseshoe crabs are not removed from red knots’ feeding beaches for extended

periods, and harmed in the process, during the key red knot migratory season. The closure of the containment facilities could mitigate the harms from the horseshoe crab harvest and increase the likelihood that it is actually possible for birders and nature lovers like me to see a red knot or horseshoe crab on the beach in the spring. This would put my mind at ease and would make my upcoming spring 2024 trip to the beaches of southern SC, as well as future trips, much more fun and enjoyable.

I declare under penalty of perjury that the foregoing is true and correct. Executed on March 10, 2023, at Charlotte, North Carolina.

By: ______________________

EXHIBIT N

COMMONWEALTH of VIRGINIA

Marine Resources Commission
380 Fenwick Road
Building 96
Fort Monroe, Virginia 23651

Justin D. Worrell
Acting Commissioner

Scientific Collection Permit
§ 28.2-205 Code of Virginia

Date:

Applicant Name: David Glover **Title:** Facility Director **Affiliation:** Charles River Laboratories	**Applicant Phone:** 843-327-4662 **Phone #2:** 843-402-4900 **Email:** david.glover@crl.com **Address:** 1023 Wappoo Rd Charleston, SC 29407
Vessel/ID #: "Go For It", 644807	**Project Personnel:** Yvonne Bowden (MRC ID: 008707, 757-894-0818, 7405 Emma Ln Chincoteague VA, 23336), TW Bowden (MRC ID: 0214, 757-894-0818, 7405 Emma Ln Chincoteague VA, 23336)

Purpose of Permit: To contract project personnel to harvest horseshoe crabs for biomedical purposes for Charles River Laboratories

Collection Area: Virginia Territorial Sea greater than 3 miles	**Sampling gear to be used:** Otter Trawl

Special Permit Conditions:

1) Only legal, licensed fishing gear shall be used by project personnel, and personnel shall notify the VMRC Operations Station by phone (**1-800-541-4646**) or by fax (**757-247-8026**), 24 hours prior to sampling.
2) A copy of this collection permit must be available for presentation, by any designated project personnel, to any Police Officer during all phases of collection activities.
3) Only Horseshoe crabs may be taken. After bleeding for biomedical purposes at the facility located at 10073 Lankford Highway, Temperanceville VA 23442, all horseshoe crabs shall be returned to the waters from which they were collected.
4) Only vessels and personnel listed on this permit are authorized to make collections.
5) The applicant must allow VMRC staff access to the bleeding facility to inspect for adherence to the applicant's HSC Harvest and Bleeding Permit Application.
6) The quantities of horseshoe crabs and additional trip level information shall be reported to this agency on the Biomedical Horseshoe Crab Harvest Reporting Form by **December 31, 2022**. This information must be received prior to reissuance of permit.
7) Collection will only occur in the Virginia Territorial Sea, greater than 3 miles.
8) Applicant or project personnel shall notify VMRC if any deviations from the HSC Harvest and Bleeding Permit Application occur, such as;
   - Increase or decrease in estimated total numbers of crabs to be harvested and bled
   - Change to the final disposition of crabs, I.E. live release vs. return to bait fishery
   - Change in harvest location or release site.

COMMONWEALTH of VIRGINIA

Marine Resources Commission
380 Fenwick Road
Building 96
Fort Monroe, Virginia 23651

Justin D. Worrell
Acting Commissioner

9) This experimental permit does not provide the applicants or project personnel the authority to harvest or possess any species that is currently listed as endangered, threatened, or prohibited (ie., sea turtles, sturgeon, marine mammals, etc) to possess by moratorium or regulation. Catch of any protected species (turtle, sturgeon, dolphin, etc) must be returned to the water immediately and the VMRC will be notified within 24 hours.

10) Any violation of these restrictions and requirements may be grounds for the Agency to revoke this permit.

Effective Dates of Permit: 3/18/2022 – 12/31/2022	**Permit: #22-032**

22 Mar 2022

Permittee Signature **Date**

3-22-2022

Commissioner **Date**

cc: Patrick Geer, Matthew Rogers, Jamie Green, LE Supervisors

APPLICATION FOR A PERMIT TO HARVEST AND BLEED HORSESHOE FOR LAL PRODUCTION

Email Application to:
Ethan Simpson
ethan.simpson@mrc.virginia.gov

OR

Mail to:
Attn: Ethan Simpson
Virginia Marine Resources Commission
380 Fenwick Road, Building 96,
Fort Monroe, VA 23651

Date of Application:	01 Mar 2022
Company Name:	Charles River Laboratories
Bleeding Facility Address:	10073 Lankford Hwy Temperanceville Va 23442
Mailing Address:	1023 Wappoo Rd Charleston, SC 29407
Name/Title of Applicant(s):	David Glover Facility Director
Telephone(s):	843-327-4662 843-402-4900
Email(s):	david.glover@crl.com

HARVESTER INFORMATION:

1) Description of how your company intends to harvest crabs for this operation (do you intend to harvest using your own means, or contract waterman to assist in the capture of live crabs. Include gear and vessel descriptions)

Charles River has contracted with Thomas Bowden to trawl for Horseshoe Crabs. They will be delivered to the bleeding site and returned to their waters. Thomas will utilize a 38' fiberglass vessel with one single net 60'. Horseshoe Crabs will be returned to native waters within 24 hours of removal from their native environment.

2) If applicable, please list any VA harvesters and their contact info. That you intend to hire/buy from for this operation. Note: These individuals will be subject to review by VMRC during application process.

Name	Yvonne Bowden (Teager Company LLC.)	**Name**	Thomas Bowden (Teager Company LLC)
MRC ID	008707	**MRC ID**	008707
Telephone	757-894-0818	**Telephone**	757-894-0818
Address	7405 Emma Ln Chincoteague, Va. 23336	**Address**	7405 Emma Ln Chincoteague, Va. 23336
Name		**Name**	
MRC ID		**MRC ID**	
Telephone		**Telephone**	
Address		**Address**	
Name		**Name**	
MRC ID		**MRC ID**	
Telephone		**Telephone**	
Address		**Address**	

Additional names may be submitted as a supplemental page to this application

3) Intended waterbody where horseshoe crabs are to be collected (be specific as possible):

Horseshoe Crabs will be collected 0-3 miles north of Assateague Beach, and 0-3 miles south of Assowoman bay.

4) Method of transportation and travel time between landing location and bleeding facility

A 6 wheel truck covered will be utilized to transport. The travel time will be 15 minutes to the bleeding facility. The site was chosen to keep the travel time to a minimum.

5A) Intended average weekly landings of crabs during operating season	~9000 per week
5B) Facilities maximum possible capacity of weekly landings	~11,500

FACILITY DESCRIPTION:

1) Description of holding environment prior to bleeding (temperature control, moisture, holding tanks, etc...), staging capacity, and time period between landing and bleeding

The facility located at 10073 Lankford Hwy is being upgraded to include HVAC for the bleeding area where the Horseshoe crabs will be processed. Horseshoe crabs are held in plastic crates on-board the fishing vessel for 3-5 hours until the catch is complete (about 1500- 1800 crabs/day). HSCs will be collected 0-3 miles north of Assateague Beach, and 0-3 miles south of Assowoman bay. They are then loaded onto a 6-wheel truck at the landing. After the 15-minute drive, Horseshoe crabs are loaded into the facility in totes, (each tote containing 10-15 HSCs). These totes are kept in a temperature-controlled environment from 5-7 hours until the entire shipment of HSC's is processed at the bleed facility.

2) Description/specifications of the bleeding facility (Square footage, number of crabs being bled concurrently, disposition of crabs post-bleed at facility):

Crabs will be held in plastic totes (each containing 10-15 crabs). Totes will be held in a temperature-controlled environment before and after bleeding.The facility, located at 10073 Lankford Hwy, is 6600sqft. It consists of a washing and bleeding area with a Class 1000 bleeding tent. The area is temperature controlled and crabs will remain in this space for the preprocessing and post-processing time for the whole batch (maximum 7 hours). Once the blood is collected it will be processed through different stages. It is anticipated to process ~1800 crabs per day. Once the entire batch of HSC's have been processed, they will be returned to the waters that they were collected in by the contracted fisherman. The area of release will be 0-3 miles north of Assateague Beach, and 0-3 miles south of Assowoman bay.

3) Description of holding environment post-bleed, prior to release (temperature control, moisture, holding tanks, etc...) staging capacity, and time period between bleeding and final disposition

Crabs will be held in plastic totes (each containing 10-15 crabs). Totes will be held in a temperature-controlled environment before and after bleeding. The holding environment will out of direct sunlight. The facility has the capacity to hold 1800 crabs post bled to be delivered back to the waters of collection by the contracted fisherman(0-3 miles north of Assateague Beach, and 0-3 miles south of Assowoman bay).

4) Final disposition of bled-crabs (Released alive or sold to bait fishery)

The Horseshoe Crabs will be returned alive to the waters they were collected in (0-3 miles north of Assateague Beach, and 0-3 miles south of Assowoman bay).

4A) If returned alive to VA waters, list the intended release location and describe how the crabs will be transported to said location. Also, describe how you intend to quantify post-bleed mortality before the crabs are released

The release location will be transported back to the dock with the 6 wheel truck, loaded to the 38' fiberglass vessel and broad casted back into their native waters (0-3 miles north of Assateague Beach, and 0-3 miles south of Assowoman bay).

4B) If sold/returned to the bait fishery, please list the intended buyer(s) of the crabs

N/A

5) Description of how to locate bleeding facility (Use road numbers and mileage between points if you do not have a 911 address)

10073 Lankford Hwy, located on the northbound side approximately 1/2 mile north of Va 695.

ANTICIPATED CAPACITY AND PRODUCTION:

1) During what months will your facility be actively bleeding horseshoe crabs?

   Anticipated to bleed from April to December.

2) What is the expected average weekly processing capacity of the bleeding portion of your facility (#'s of crabs)? Maximum weekly capacity?

   The average weekly capacity is 9000 crabs. Maximum 14000.

3) Provide an estimate of the total number of crabs to be harvested and processed by your facility in a given calendar year

   The estimate for the year 120000-180000 crabs

4) Provide an estimate of the total quantity of LAL to be produced by your facility in a given year

   anticipated yield is 900 liters.

5) At the time of this application, what is the intended disposition of any LAL products?

   All LAL products are produced under our FDA license and sold to drug and device companies to release their products. The test is used to ensure the absence of endotoxin.

EXHIBIT O

FEATURED STORY

f 🐦 in

Aug 27 2020

Horseshoe Crabs Continue to Make Waves Amidst a Global Pandemic

As nature's only source of Limulus amebocyte lysate (LAL), the gold standard for bacterial endotoxin detection, the blue-blooded Atlantic horseshoe crab is critical to the safety of injectable drugs.

Remarkably, just a single day's production of LAL can test the anticipated 5 billion doses of the most coveted COVID-19 vaccine. However, a lot of questions have arisen regarding how pharmaceutical manufacturers are going to keep up with production demands amid a global pandemic.

In 2019, The Atlantic States Marine Fisheries Commission (ASMFC) compiled a comprehensive two-year study on the status of the Atlantic horseshoe crab and how its population was stable and thriving through the Atlantic coastal waters.

Dr. James Cooper, who established the first commercial LAL in 1971, reveals the impact of advocacy for these 445 million-year-old creatures in his blog post, "**The LAL Industry's Remarkable Stewardship of Horseshoe Crabs (/insights/lal-industrys-remarkable-stewardship-horseshoe-crabs)**."

Amidst the misinformation from activists out in the field, the FDA has a zero-tolerance policy for endotoxin contamination, so it’s critical to ensure the methodologies and technology we use are up to par. Charles River's senior technical writer Rachel Kerr concurs. Writing for *American Pharmaceutical Review*, she **details the shortcomings (/resources/rely-data-not-lore-meet-rising-demand-apr)** found in recombinant endotoxin testing products and the pause we should take to ensure patient safety.

FEEDBACK

With several vaccines in development, the race to the COVID-19 vaccine finish line is a hot topic, however data-driven decisions and sound science need to remain the foundation of pharmaceutical manufacturing.

Just ask Foster Jordan, Charles River’s CVP for Microbial Solutions. Foster’s only had one job his whole life – working for South Carolina based Endosafe®, the FDA-licensed facility manufactures LAL. Over his long career, Jordan has immersed himself in the science of LAL and conservation of the crabs from which it comes.

Jordan discusses horseshoe crabs, recombinant endotoxin testing technologies, and what's at stake for the pharma industry on Eureka's **Sounds of Science podcast (https://eureka.criver.com/podcast/e26-from-rabbit-to-crab-to-the-lab/)**, but his contributions and insight can’t be overlooked. He's positive that when a vaccine becomes available, the horseshoe crab will continue to protect patients as it has for nearly 50 years.

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EXHIBIT P

An Atlantic horseshoe crab lies on the beach in Stone Harbor, New Jersey, not far from Delaware Bay.

PHOTOGRAPH BY JOEL SARTORE, NAT GEO IMAGE COLLECTION

ANIMALS CORONAVIRUS COVERAGE

Horseshoe crab blood is key to making a COVID-19 vaccine—but the ecosystem may suffer.

Conservationists worry the animals, which are vital food sources for many species along the U.S. East Coast, will decline in number.

BY **CARRIE ARNOLD**

PUBLISHED JULY 2, 2020 • 9 MIN READ

Each spring, guided by the full moon, hundreds of thousands of horseshoe crabs clamber onto beaches across the U.S. mid-Atlantic to lay their eggs. For hungry birds, it’s a cornucopia. For drug companies, it’s a crucial resource for making human medicines safe.

That’s because these animals’ milky-blue blood provides the only known natural source of limulus amebocyte lysate, a substance that detects a contaminant called endotoxin. If even tiny amounts of endotoxin—a type of bacterial toxin—make their way into vaccines, injectable drugs, or other sterile pharmaceuticals such as artificial knees and hips, the results can be deadly.

“All pharmaceutical companies around the world rely on these crabs. When you think about it, your mind is boggled by the reliance that we have on this primitive creature," says Barbara Brummer, state director for The Nature Conservancy in New Jersey.

Every year, pharmaceutical companies round up half a million Atlantic horseshoe crabs, bleed them, and return them to the ocean— after which many will die. This practice, combined with overharvesting of the crabs for fishing bait, has caused a decline in the species in the region in the past few decades.

In 1990, biologists estimated 1.24 million crabs spawned in Delaware Bay, a main egg-laying spot and prime collection point for the companies. By 2002, that number had dropped to 333,500. In recent years, numbers of Delaware Bay spawning crabs have hovered around the same amount, with the 2019 survey estimating about 335,211. (The pandemic canceled the 2020 crab count.)

Catching crabs and harvesting their blood is time-consuming, and the resulting lysate costs $60,000 per gallon. In 2016, a synthetic alternative to crab lysate, recombinant factor C (rFC), was approved as an alternative in Europe, and a handful of U.S. drug companies also began using it.

But on June 1, 2020, the American Pharmacopeia, which sets the scientific standards for drugs and other products in the U.S., declined to place rFC on equal footing with crab lysate, claiming that its safety is still unproven.

Starting in July, Swiss-based Lonza will begin manufacturing a COVID-19 vaccine for human clinical trials—and they'll have to use lysate in the vaccine if they plan to sell it in the U.S. (Here's how we'll know when a COVID-19 vaccine is ready.)

A ruddy turnstone eats a horseshoe crab in Delaware Bay, New Jersey. The animals are vital food sources for ruddy turnstones and other migratory birds.
PHOTOGRAPH BY DOUG WECHSLER, MINDEN PICTURES

Human health and safety, especially for something as high stakes as the coronavirus vaccine, is paramount, says Brummer. But she and other conservationists fear that without rFC or other alternatives available, the ongoing burden on horseshoe crab blood for COVID-19 vaccines and related therapeutics may imperil the crabs and the marine ecosystems that depend on them.

A written statement from Lonza says that testing the company's COVID-19 vaccine will not require more than a day's worth of lysate production from the three U.S. manufacturers.

One of those three—Charles River Laboratories, based in Massachusetts—gave National Geographic the same statistic. The laboratory's John Dubczak explained in an email that to make five billion doses of the COVID-19 vaccine, 600,000 tests will be performed, which will use the amount of lysate created in a single day.

"This places no undue burden on the [lysate] supply chain or horseshoe crab populations," said Dubczak, executive director of reagent development and pilot program operations.

Blue bloods

Nearly unchanged for hundreds of millions of years, horseshoe crabs have some unusual traits. Despite their name, these creatures are more closely related to spiders and scorpions than crabs. They also have nine eyes—two compound eyes and seven simple ones. (Read more about how horseshoe crabs evolved.)

In 1956, medical researcher Fred Bang noticed another strange characteristic: When horseshoe crab blood interacts with endotoxin, cells called amebocytes clot and form a solid mass. Bang realized that these amebocytes—part of the crab's ancient immune system—could detect deadly bacterial contaminants in the rapidly expanding array of pharmaceuticals designed to enter the human bloodstream.

Scientists eventually figured out how to use the amebocyte lysate to test drugs and vaccines, and in 1977, the U.S. Food and Drug Administration approved horseshoe crab lysate for this use.

Horseshoe crabs are bled at the Charles River Laboratory in Charleston, South Carolina.
PHOTOGRAPH BY TIMOTHY FADEK, CORBIS/GETTY

Since then, each May the helmet-shaped creatures are brought en masse to specialized labs along the U.S. East Coast, where technicians extract the blood from a vein near the heart before returning them to the sea. (Their blue blood comes from the metal copper in their oxygen-transporting proteins, called hemocyanin.)

In the 1980s and through the early 1990s, the process seemed sustainable. The pharmaceutical industry claimed that only three percent of the crabs they bled died. Population surveys showed that the crabs were plentiful, and conservationists didn't place much value on the species, says Larry Niles, a biologist with the Conserve Wildlife Foundation of New Jersey.

But by the early 2000s, the picture began to shift. Annual horseshoe crab counts during spawning season revealed smaller numbers, and a 2010 study found that as many as 30 percent of the bled crabs ultimately died—10 times as many as first estimated.

Lonza, the Swiss corporation, says it is “committed to protecting the welfare of the horseshoe crab,” for instance by “actively supporting conservation efforts.”

According to the statement from Lonza, Charles River Laboratories and another lysate maker, Associates of Cape Cod, Inc., raise horseshoe crabs in hatcheries and release them into the ocean. Lonza reports that in 2019, the Cape Cod company reintroduced 100,000 juvenile crabs into the waters around Massachusetts and Rhode Island.

Lonza’s statement says the company would also prefer to use lysate alternatives and has trademarked its own rFC, called PyroGene. But, as the American Pharmacopeia decision illustrates, “regulatory hurdles remain. We remain hopeful that the barriers preventing drug developers from using the synthetic alternatives are starting to fall,” the statement says.

Disrupting the food chain

Meanwhile, conservationists are monitoring the impact to the species that rely on horseshoe crab eggs as vital food sources.

Sport fish that once were numerous, such as striped bass and flounder, have plummeted in number in the region, in part due to fewer horseshoe crab eggs, Niles says. Diamondback terrapins, a type of reptile that’s vulnerable to extinction, also depend on this seasonal buffet.

Both Niles and Brummer are particularly concerned about migratory shorebirds, such as red knots and ruddy turnstones, which stop at Delaware Bay on their 9,000-mile journey from iTierra del Fuego in Chile to Arctic breeding grounds. These birds need tremendous amounts of energy for their long-distance flights, and calorie-rich horseshoe crab eggs are the perfect fuel. (Read how climate change is shrinking red knots.)

During their two-week sojourn on Delaware Bay, red knots nearly double their body weight to prepare for the final leg of their voyage. This year, however, cool temperatures delayed crab spawning, and only 30,000 red knots stayed in the bay, a drop from an estimated 40,000 birds in 2019.

Niles cautions that a weakening of one link in the food chain can reverberate, with potentially disastrous consequences. The depletion of horseshoe crabs could ultimately eliminate benefits that tourists, fishermen, and others get from enjoying the bay.

“The value of a natural resource,” he says, “doesn't belong to companies that are exploiting it. It belongs to us.”

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COVID-19 and the Sustainability of the LAL Supply

Manufacturing Science Apr 22, 2021

by

Brett Hoffmeister, Associates of Cape Cod, Inc., Allen Burgenson, Lonza, and John Dubczak, Charles River Laboratories

The COVID-19 virus has been impacting humans worldwide for nearly a year at this writing. Over that time, there was a great deal of speculation regarding how the pandemic and vaccine production to combat the virus would affect the production of Limulus amebocyte lysate (LAL) and possibly outstrip naturally sourced LAL availability. There was also speculation regarding the impact the potential increase in demand of LAL might have on American horseshoe crab populations.

Multiple vaccines have been approved and millions of COVID-19 vaccines have been produced, which are being used to inoculate human beings around the world. Not a single dose has been delayed because of insufficient LAL supplies to vaccine manufacturers. Vaccine production is underway, with more expected to be released for use in the very near future.

As representatives of LAL manufacturers, we wish to assuage concerns and provide assurance that the demands of LAL testing in support of COVID-19 vaccines will not adversely impact its production, nor will it put product availability or the American horseshoe crab population at increased risk.

LAL is a reagent produced from the white blood cells of the Atlantic horseshoe crab (*Limulus polyphemus*), which is utilized by pharmaceutical and medical device manufacturers in a bacterial endotoxin test (BET) mandated by the U.S. Food and Drug Association.

Past and recent articles have given voice to concerns about the availability of the LAL supply to accommodate large-scale vaccine testing. Other media suggest that single-source reliance on one species is an untenable risk, with some having gone so far as to suggest that LAL manufacturing jeopardizes the horseshoe crab population. The media attention has provided renewed opportunity for some to recirculate inaccurate and misleading information to the public about the impact of biomedical uses of horseshoe crabs and the status of the crab population in the United States.

It is important to have a discussion of the relevant factors involved. By reviewing some of the questions and issues recently expressed by the media and other platforms, we wish to educate readers on the facts.

Is LAL-based product availability particularly fragile or easily interrupted by natural or manmade events?

No, the LAL industry has been manufacturing for over 40 years with no significant interruption of services resulting from hurricanes, floods, blizzards, oil spills or other disasters.

LAL manufacturers are geographically diverse, located along the East Coast of the United States: Associates of Cape Cod, Inc. (Massachusetts), Charles River Laboratories (South Carolina), Lonza (Maryland), and Wako Chemicals (Virginia).

This large geographical footprint helps avoid a natural or manmade disaster from interrupting product availability. All LAL manufacturers operate with contingency plans in place and maintain the inventory needed to meet customer demands. Millions of LAL tests are performed annually. Production of COVD-19 vaccines is underway around the world and serves as a good example of the robustness of the LAL BET supply chain.

This is what we do.

Are horseshoe crabs endangered?

No. It is the duty of the U.S. Fish and Wildlife Service (FWS) to determine if an animal in the United States is "threatened" or "endangered." The FWS has made no such claim about the status of the American horseshoe crab.

They are not endangered; in fact, it is estimated that there are tens of millions of adult crabs in the Delaware Bay region alone ***(1)***. In many areas, populations are growing considerably; however, in other parts of the world, horseshoe crabs are not so closely monitored. Tachypleus tridentatus, found in Southeast and East Asia, for instance, is used as food, fertilizer and manufacturing–for chitin and its LAL equivalent, TAL.

In the United States, American horseshoe crab harvest is regulated by state agencies and the Atlantic States Marine Fisheries Commission (ASMFC), which oversees coast-wide fishery.

The ASMFC is made up of members from the FWS, academia, fisheries managers, statisticians and scientists, and representatives of industry, government and others who work to regulate horseshoe crab fisheries and monitor populations on the East Coast of the United States. Current management of the fishery is robust and science-based. The most recent benchmark stock assessment (2019) determined that the overall number of American horseshoe crabs appears to be stable and is increasing in some areas ***(1,2,3)***. It is reasonable to say that there may be more horseshoe crabs today than there have been for decades.

Does biomedical use of horseshoe crabs threaten the population?

The simple answer is, "No."

The data show clearly that even a complete cessation of the biomedical fishery would have a minimal impact on the overall fishery mortality of horseshoe crabs. In fact, the population is so healthy that there is a coast-wide quota, to be lawfully harvested for bait, of nearly 1.6 million crabs. Actual landings based on market demand and state regulations are far less than that, at approximately 800,000 crabs annually ***(1)***. The biomedical mortality is roughly 10% of that of the bait industry. See **Figure 1**.

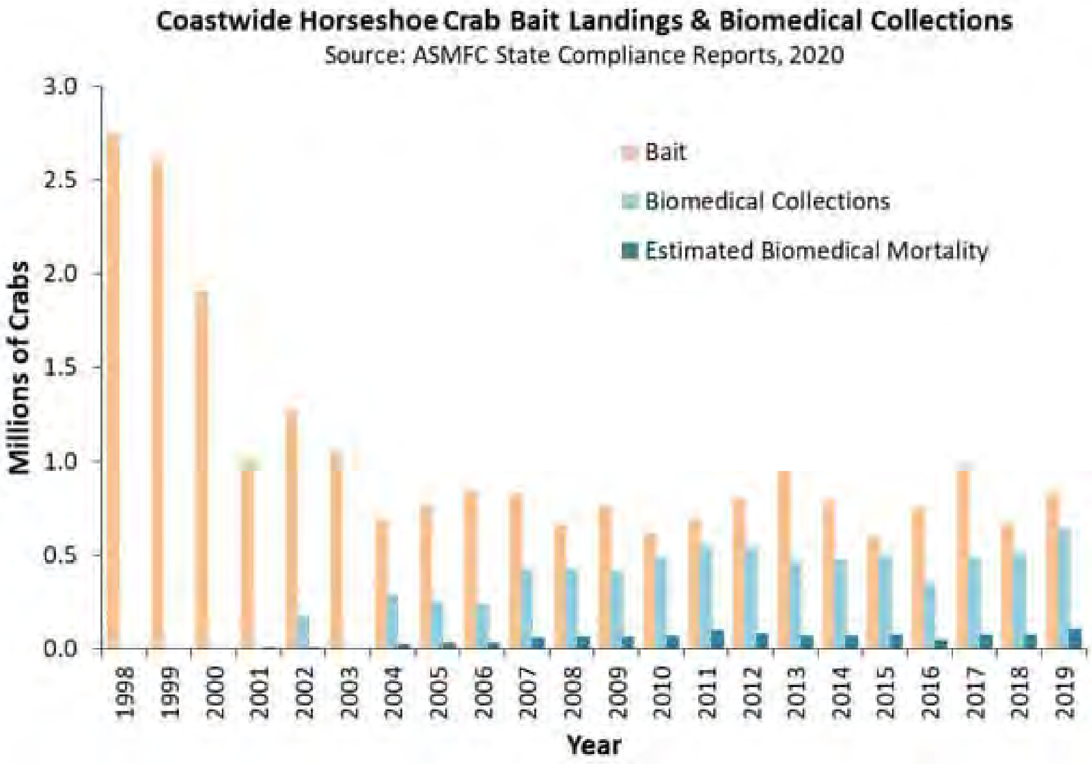

Figure 1 Coastwide Horseshoe Crab Bait Landings and Biomedical Collections

The 2019 stock assessment by the ASMFC states that the biomedical use of crabs has no impact on the population in the Delaware Bay region ***(1)***. It is estimated that there are tens of millions of horseshoe crabs in the Delaware Bay region alone ***(4)***.

In the areas where collection for LAL manufacturing exists, horseshoe crab populations are doing quite well and are stable and/or increasing. A recent study of nearly 175,000 crabs, of which 68,000 were bled at LAL manufacturers over multiple seasons, showed that long-term survival of those crabs, over multiple years, was as good or better than the survival rates of un-bled crabs ***(1)***.

Will COVID-19 vaccine production threaten the population of HSC because of increased need of LAL?

No.

The LAL test is an important quality control measure, required by law, for anything injected or implanted into the human body. It is already used millions of times annually on raw material, intermediates and final products, and only a very small amount of LAL is needed to perform these tests. Modern pharmaceutical manufacturing has significant scalability throughout the industry. It takes roughly the same amount of LAL to test 1,000 doses as it does to test 100,000 doses. This serves as a reminder that even a high and unexpected demand for vaccines and medical products can be managed with the proper safeguards and planning that are in place.

The demands of BET testing materials worldwide can and are being absorbed with available inventory and without a significant negative impact on the pharmaceutical industry or supply chain. This ability to scale up production of pharmaceuticals alleviates any sudden and unexpected increase in testing demands and need for significant increases in LAL inventory. There is no significant increase in the use of horseshoe crabs and no threat to the population because of COVID-19 vaccine production.

What are the threats horseshoe crabs face today?

According to the ASMFC *2019 Horseshoe Crab Benchmark Stock Assessment and Peer Review Report,* the following are the major sources of horseshoe crab mortality:

- Bait harvesting
- Bycatch from other fisheries
- Loss of habitat due to erosion prevention measures (rip-rap, seawalls, etc.) and human encroachment on spawning grounds
- Stranding after spawning (estimated 10% mortality of entire Delaware Bay population, annually)

Like any sea creature, horseshoe crabs are dependent on a suitable environment where they can live and reproduce. Water quality is an important factor, as is having suitable beaches in which to lay their eggs. Fertilizers, septic systems and other forms of pollution can greatly reduce the quality of the water on which the crabs depend. Sea walls, rip-rap and jetties can manipulate the natural movement of sand on beaches and affect spawning habitats. Beach nourishment, the practice of bringing in truckloads of sand to beaches to replenish what has been lost or to make them look nice, can bury millions of eggs before they hatch if not carefully planned. We all play a part in protecting this valuable resource.

Is there any oversight of the manufacturing and collection processes?

Yes, LAL manufacturing is a highly regulated, audited and complex process that provides a critical lifesaving assay for the pharmaceutical and medical device industry.

Manufacturers are regulated by the U.S. FDA and must comply with strict regulatory standards to certify product quality, efficacy and safety. In addition, routine audits of the process are conducted by the U.S. FDA, the International Organization for Standardization, fisheries managers and customers. Fishers collecting crabs for

LAL manufacturers are mandated to follow local regulations as a condition of permitting.

In 2011, the ASMFC partnered with LAL manufacturers, citizens groups, fishers and dealers to document industry best management practices ***(5)***. Many of these practices, such as a swift return to the water and careful handling practices, have been in place by manufacturers for more than 40 years and help to ensure product quality while minimizing the impact on individual crabs. This, in turn, helps ensure survivability of the animal and the population. In most East Coast states, there are regulations in place that help to protect horseshoe crab populations.

What do LAL manufacturers do to support conservation?

LAL manufacturers have practiced conservation measures since the beginning of this process, long before regulatory bodies began managing horseshoe crab fishery. In addition to the decades-long catch-and-release policy, LAL manufacturers work closely with fishery managers and have members on the advisory panel of the ASMFC. They have helped with or initiated such conservation measures as closing areas to bait fishing, participating in the “rent a crab” program that uses crabs from the bait industry, and supporting quotas and size limits. They support and have initiated the aquaculture of horseshoe crabs for release to the wild.

LAL manufacturers financially support organizations such as The Ecological Research & Development Group, aquariums and the Virginia Tech trawl survey. Volunteers participate in spawning surveys, tagging studies and the “Just flip ‘em!™” program, which saves thousands of crabs each year. Many employees also routinely work with universities, schools and citizen groups, helping to increase public awareness and educate people about these remarkable animals.

Conclusion

The proposition that populations of horseshoe crab are declining because of their use in biomedical testing ignores the fact that there is a healthy and stable population of crabs in the United States and that the impact of the LAL industry is minimal. The Review Panel, consisting of representatives from academia, the National Marine Fisheries Service and the Maine Department of Marine Resources, agreed with the ASMFC assessment team’s approach, but noted:

> *…some covariates such as season of harvest, size/condition of crabs, and location that are worth investigating. However, additional data and analyses are not likely to significantly alter assessment results due to the modest magnitude of biomedical mortality. As such, while an uncertainty, the biomedical mortality rate should receive less focus in future assessments* ***(6)***.

In conclusion, it is reasonable to state that the horseshoe crab population in the United States is viable and healthy, the biomedical industry does not impact this population negatively, and the supply of LAL is robust and healthy. Alarmists who suggest otherwise do so by ignoring the scientific facts and without any true knowledge of the LAL industry, horseshoe crab fishery or population data.

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ABOUT THE AUTHORS

Brett Hoffmeister is the LAL Production Manager with Associates of Cape Cod Inc. With over 18 years' experience in LAL manufacturing, Brett is also ACC's subject matter expert on horseshoe crab conservation and sustainability. Working closely with fisherman, dealers and regulators Brett has contributed to and developed a number of conservation initiatives at ACC, including ACC's Horseshoe

Crab Sustainability project, an aquaculture system launched in 2018 which has released over 800,000 juvenile horseshoe crabs into coastal waters of Massachusetts. Brett also manages internships with several colleges and universities, is a member of the Bio-tech advisory Committee at Bristol Community college and serves as Chair to the horseshoe crab advisory panel of the Atlantic States Marine Fisheries Commission.

Allen L. Burgenson is the Global Subject Matter Expert, Testing Solutions, with Lonza. Allen has over 35 years of experience in industries regulated by the U.S. FDA, including Foods, Drugs, Biologics, Medical Devices and Cosmetics. He has worked in R&D, QC, QA, Regulatory Affairs, and now Marketing as a SME for endotoxin detection. Allen is involved in several scientific organizations including as the Immediate Past Chair of the Horseshoe Crab Advisory Panel for the Atlantic States Marine Fisheries Commission (ASMFC) and as the immediate Past-President of the Capital Area Chapter of the Parenteral Drug Association (PDA). He currently serves on the Horseshoe Crab Working Group of the International Union for the Conservation of Nature.

John Dubczak is the Executive Director of Reagent Development and Pilot Plant Operations at Charles River Laboratory. John has 15 years of experience in research microbiology and 26 years in pharmaceutical manufacturing. He currently serves as Executive Director of Reagent Development and Pilot Plant Operations for Charles River's Microbial Solutions business. John's laboratory responsibilities have ranged from product development to sterility, particle and endotoxin testing. He has also been heavily involved with LAL raw material procurement, production and technical and customer service offerings. Prior to joining Charles River, John was a long-term employee of Baxter Healthcare Corp., where he developed Baxter's proprietary LAL formulation and manufacturing process. With seven years of large-volume parenteral manufacturing experience, he brings an in-depth understanding of issues surrounding all aspects of LAL testing.

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EXHIBIT R

Thank you Felicia.

I do wish we had been running video when the guys were walking across their boats, on top of a full load of crabs to get to the helm! Cant imagine the crabs surviving that.

Eric

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On Thu, Jun 27, 2019 at 6:19 AM Felicia Sanders <SandersF@dnr.sc.gov> wrote:
Eric,
Thank you so much! These are great. I have never heard of anyone videoing the harvest before so this is valuable. Turtle is the only beach I know of that still has a lot of crab spawning so also great documentation. I watched them harvest many times this spring and only saw them pick the crabs up by the telson or tail. In your video the guy close to you is picking them up by the carapace not the tail. He must know that is the proper procedure and that he was being filmed.... Thanks for sharing your work. Your photos you shared with me years ago at Harbor Island, of crabs and shorebirds, are valuable. The crabs don't spawn there any more or at least not many. Great to have proof of what happened in the past.
Felicia

From: eric horan (via Dropbox) <no-reply@dropbox.com>
Sent: Wednesday, June 26, 2019 3:45 PM
To: Felicia Sanders <SandersF@dnr.sc.gov>
Subject: eric horan shared "Felicia Sanders-DNR" with you

Hi Felicia,

eric horan (eh@horanphoto.com) invited you to edit the folder "**Felicia Sanders-DNR**" on Dropbox.

eric said:
"Sorry for the delay Felicia, I thought this was done after my offer to send but apparently they never loaded : ("

Go to folder

Enjoy!