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Impacts of biomedical bleeding on locomotion and mating behavior in the horseshoe crab *Limulus polyphemus*

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ABSTRACT: Every year, more than 600 000 horseshoe crabs are bled to produce *Limulus* amoebocyte lysate, which is used to detect Gram-negative bacteria in biomedical products. While numerous studies have shown that some horseshoe crabs die after being bled, less is known about what happens to those that are returned to their natural habitat. In this study, we used an array of VR2W acoustic receivers to track 10 bled and 10 control females during the mating season in the Great Bay Estuary, NH, USA. Animals were bled, or not, released where they had been initially captured, and tracked from 22 May to 26 June 2019. Bled and control females moved comparable distances at similar speeds during the weeks after they were released (controls: 90.3 m h^{-1}; bled: 89.7 m h^{-1}). The longer horseshoe crabs remained within the virtual positioning system array, the longer we were able to track them and the more beach approaches and mating attempts we were able to identify. When this relationship between the duration of time we were able to track a horseshoe crab and how many apparent mating attempts it expressed was taken into account, we found that bled females attempted to spawn half as often as control females, and this difference was significant. Overall, these data are consistent with previous findings indicating that females that are released back into their natural habitat after bleeding express similar levels of activity and seasonal movements but attempt to mate less than control animals, at least in the first few weeks after being bled.

KEY WORDS: Acoustic telemetry · Horseshoe crab · Mating · Spawning · Biomedical bleeding · Tracking · Estuary

1. INTRODUCTION

The blood of the American horseshoe crab *Limulus polyphemus* contains a substance that is very sensitive to Gram-negative bacteria, called *Limulus* amoebocyte lysate, or LAL (Novitsky 2009). It is used by the health industry to ensure that a wide range of products, including vaccines, are bacteria-free prior to implanting or injecting them into humans (Chen & Mozier 2013). Currently, to produce sufficient LAL, more than half a million horseshoe crabs are bled each year and then returned to their natural habitat (Krisfalusi-Gannon et al. 2018). As a result of blood loss and some of the stresses associated with their capture, transport, and holding, between 10 and 30% of the crabs die in the process (Rudloe 1983, Thompson 1998, Walls & Berkson 2000, 2003, Hurton & Berkson 2006, Leschen & Correia 2010, Anderson et al. 2013, Krisfalusi-Gannon et al. 2018), with higher mortality rates for females than males (up to ~29% for females and ~15% for males; Leschen & Correia 2010, James-Pirri et al. 2012).

Many of the horseshoe crabs that survive the bleeding procedure also experience some sublethal

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impacts. For example, in a laboratory study, Anderson et al. (2013) discovered that horseshoe crabs were less active after they were bled. More recently, a study by Owings et al. (2020) indicated that loss of the respiratory pigment hemocyanin might be a key factor contributing to both the lethal and sublethal impacts of the bleeding process. Horseshoe crabs with low hemocyanin levels prior to being bled were more likely to die during the process, and of those that survived, the ones with the lowest hemocyanin levels were the least active (Owings et al. 2020). Several field studies have also identified some sublethal impacts of the bleeding process, such as altered orientation abilities (Kurz & James-Pirri 2002) and a tendency for bled females to approach beaches to potentially spawn about half as often as controls (Owings et al. 2019). If bled females do, in fact, mate half as much as they normally would, this could reduce the overall reproductive output of populations that are fished heavily for LAL.

While 2 laboratory studies demonstrated that horseshoe crabs are less active after bleeding (Anderson et al. 2013, Owings et al. 2020), this change in their behavior has not been thoroughly investigated using individuals released back into their natural habitat. Therefore, the first goal of this study was to determine if biomedical bleeding led to decreased locomotion in crabs released back into their natural habitat. We addressed this question by using a high-resolution virtual positioning system (VPS) to track the movements of bled and control female horseshoe crabs fitted with acoustic transmitters for 1 mo during the mating season. The second goal of this study was to take advantage of the movement data provided by the VPS system to determine if the bleeding process has an impact on the mating behavior of female horseshoe crabs.

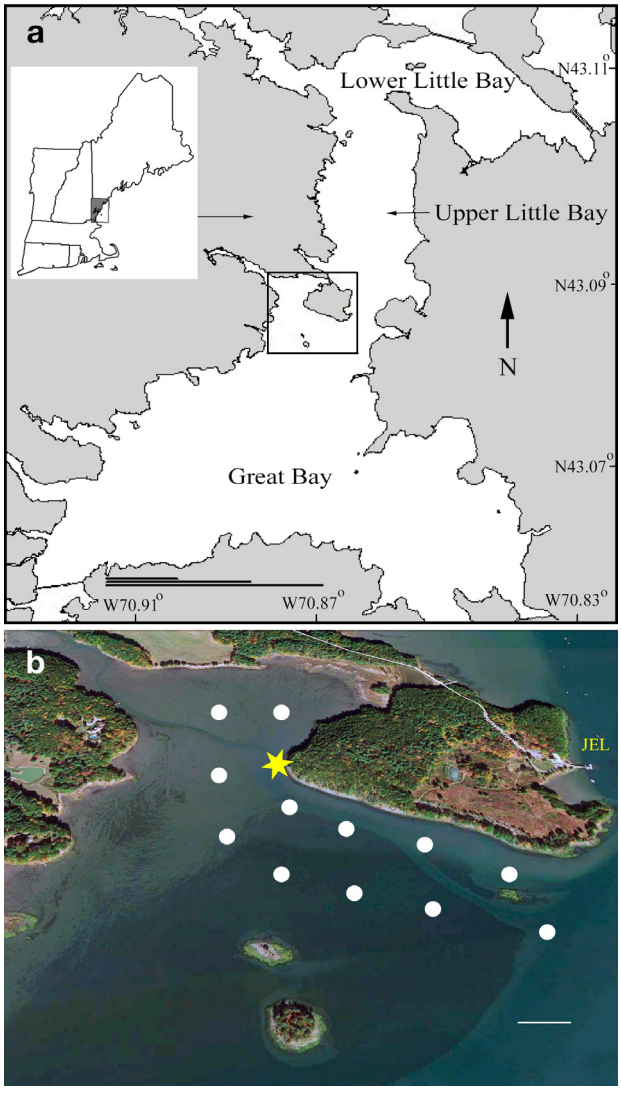

Fig. 1. Study area in the Great Bay Estuary, NH, USA, and arrangement of the VR2W acoustic receiver array. (a) Overview of the Great Bay Estuary. Box: study location along the coast of Adams Point. Black scale bars: 1, 2, and 3 km. (b) VPS array. White circles: VR2W receivers; yellow star: location where horseshoe crabs were captured and released. White scale bar = 100 m. JEL: Jackson Estuarine Laboratory

2. MATERIALS AND METHODS

2.1. Collection and preparation of horseshoe crabs

A total of 23 female horseshoe crabs were captured along the SW shore of Adams Point (Great Bay Estuary, Durham, NH, USA) on 21 May 2019 while they were close to shore preparing to spawn (Fig. 1). They were carried in 5 gallon (19 l) pails to the Jackson Estuarine Laboratory (JEL), which took about 15 min, randomly assigned to either the bled group or control group, and then measured to ensure both groups were made up of crabs of comparable sizes. Control crabs (n = 10) were 11.6 ± 0.8 cm (±SD) in carapace width, while those that were eventually bled (n = 13) were 11.5 ± 0.7 cm. The mean sizes of the 2 groups of females were not significantly different ($p = 0.76$). The blood volume of the females to be bled was estimated according to the methods used by Hurton et al. (2005), and then approximately 30% of their total blood volume was removed as described in Anderson et al. (2013) and Owings et al. (2019, 2020). Bled crabs were then placed in 2 large (50 gallon, 190 l) plastic bins inside the JEL (20°C) and covered with burlap soaked in seawater, while the control horseshoe crabs were held in tanks outside of JEL that were provided with flow-through water from the estuary.

The following day, 3 horseshoe crabs in the bled group appeared to be dying. They did not crawl when placed on the ground or try to turn over when placed upside down. Therefore, they were immediately put back in the estuary. This yielded an apparent 'mortality' rate of 23%, which is consistent with data from our previous bleeding studies (Anderson et al. 2013, Owings et al. 2019, 2020). While we could have fitted them with transmitters and released them as part of the bled group of crabs, it was apparent that they would not have survived more than 1 d and it would have reduced our bled sample group to only 7 crabs. The remaining 10 females from the bled group and the 10 control females were all fitted with Vemco V9 acoustic transmitters (69 kHz, 147 dB low-power output, 13 mm diameter, 27.5 mm length, 4.5 g in water, estimated battery life of ~530 d; VEMCO, Innovasea Systems), as described in Owings et al. (2019). The transmitters were programmed to 'ping' every ~2–5 min (pinging interval was random to avoid interference with signals from other transmitters in the same area). They were then carried in buckets back to where they had originally been collected and released at 15:00 h on 22 May 2019, which was 5 d after the full moon.

2.2. Hydrophone array

Between 9 and 15 May 2019, a total of 12 Vemco VR2W receivers were deployed in the Great Bay Estuary, near where the horseshoe crabs used in this study were collected and released (Fig. 1). They were secured in a vertical orientation (hydrophone end up) inside a lobster trap that was fitted with additional weights. The top of the receiver extended 6 inches (15 cm) above the top of the lobster trap. A Vemco 'synch tag' was also attached to each of the lobster trap moorings to help calibrate the distances between the receivers in the array. The receivers were laid out to maximize the opportunity to track horseshoe crabs as they approached known spawning beaches on Adams Point (Fig. 1). In general, they were 150 to 200 m apart and in approximately 2 to 8 m of water at low tide. This arrangement allowed us to detect horseshoe crabs over a total area of ~500 000 m^2 (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m699p065_supp.pdf). We did not carry out a range test before deploying the array because based on our previous acoustic telemetry studies in the same area, pingers could be detected from a distance of 200 to 300 m (Schaller et al. 2010, Watson et al. 2016, Owings et al. 2019). Two reference transmitters were also deployed within the array to help roughly calibrate the accuracy of the system, in terms of calculating how far individuals moved per hour. On 26 June, all VR2Ws were retrieved and their GPS coordinates were checked again to make sure they had not moved. The data stored on the VR2Ws were downloaded and sent to Vemco (Innovasea Systems) for processing along with details about the layout of the array and the individuals that were tagged.

2.3. Data collection and analyses

One bled and 5 control females only yielded good tracking data for 1–2 d, and therefore were not used for some of the analyses of mating and locomotion. The 5 remaining control females were tracked for an average of (±SD) 16.0 ± 6.03 d (max.: 27 d; min. 10 d), while the 9 bled crabs were tracked for 19.0 ± 7.84 d (max.: 30 d; min.: 7 d). While we obtained an average of (±SD) 774.1 ± 689.8 positional fixes for these 14 horseshoe crabs, after filtering the data to yield only the most accurate positions using a criterion horizontal positioning error value <11 (Meckley et al. 2014), we were left with 73.1 ± 17.7% of the original x,y positions, or an average of 592.9 ± 639 positions per individual (max.: 2040; min.: 26). We did not attempt to calculate the resolution of our VPS system, but Espinoza et al. (2011) determined that an array design similar to the one used in this study yielded a positional accuracy of 2.13 ± 1.31 m (±SD) within the array, with less precision when individuals were outside the array (5.12 ± 4.11 m).

We tested the effects of the bleeding treatment on the amount of time spent in the array using a general linear model with Poisson distribution and log-link function in R v.4.0.4 using the package 'lme4' (Bates et al. 2015). To focus on the most accurate and representative data with which to compare the movements of the 2 groups, we only used data from days during which individual horseshoe crabs had at least 30 positional fixes. On those 'good' days, we used the longest stretches of time during which the VPS array yielded consistent data to calculate their average walking speed. The distance moved between each positional fix was determined and these were summed for the time period being used. Then, all the distances and times for each good time period on a given day, for each individual, were summed and the total distance traveled was divided by the total time to yield a walking speed in m h^{-1}. Each horseshoe crab was only assigned one distance traveled or walking speed value per day. Furthermore, data

from the day they were released were not used because they were released in the middle of the afternoon, so the initial day of the trial would be less than 24 h and the data were likely not representative because the horseshoe crabs were still recovering from being handled. Because each horseshoe crab yielded data for a slightly different amount of time, most comparisons between them were carried out using the mean distance traveled per hour during the days when there were sufficient data for that individual.

To determine if bleeding had an impact on mating behavior, tracks of horseshoe crabs were played back in Google Earth (Google Earth Pro v.7.3.3) and scored for apparent mating attempts. All possible data were used for these analyses; we did not limit our analyses to days with sufficient data to calculate the distance moved. Mating attempts were considered as times when females approached to within 10 m of the shoreline during a 3 h window around peak high tide (2 h before, 1 h after). For each female, we recorded the total number of apparent mating events that took place during the entire 28 d study.

As the number of positional fixes received by the VPS array affected our ability to detect mating events, the impact of bleeding on mating was tested in R v.4.0.4 using ANCOVA, with the number of beach approaches (indicative of mating attempts) used as the response variable, treatment (bled or unbled) as the factor of interest, and the number of positional fixes obtained for a given female as the covariate. Data for the number of beach approaches and for the number of positional fixes were $\log_{10}(x + 1)$ transformed to meet the test's assumption of normality.

All other statistical analyses were performed with Prizm v.9.0 software (GraphPad). Unless stated otherwise, comparisons between data obtained from bled and control horseshoe crabs were carried out using unpaired Student's 2-tailed *t*-tests. Within the text, results are presented as means ± SEM, unless stated otherwise.

3. RESULTS

3.1. VPS array

The VPS array detected pings and calculated the positions of the 20 horseshoe crabs an average of 553.3 times (range: 48–2209; Table 1). Three of the control females rapidly moved away from the release site, while 11 females (4 controls and 7 bled) were tracked for at least 2 wk. Reference pinger 1 was detected 3064 times and Reference pinger 2 was detected 2815 times during the 30 d experiment, or 102.1 and 93.8 times d^{-1}, respectively. Reference pingers can be used as a rough indicator of the resolution of the system because they do not move. Due to the resolution of the VPS array and the fact that small errors accumulate, Reference pinger 1 'appeared to move' 4.3 m h^{-1} (103.9 m d^{-1}) during the 30 d it was deployed, while Reference pinger 2 'moved' 11.9 m h^{-1} (285.6 m d^{-1}). Therefore, even horseshoe crabs that did not actually move at all during a given hour might appear as if they moved 4 to 12 m during that time period.

Table 1. Telemetry, mating, and movement data for each horseshoe crab

No.	Days in the array	Total position fixes	Mating events	Distance moved (m d^{-1})
Control female				
1	20	502	4	940
2	10	48	0	4
4	9	334	3	603
5	14	856	7	668
6	<1	3	0	340
8	26	1327	9	770
9	14	94	2	170
10	<1	10	0	331
11	<1	6	0	400
20	12	2209	10	936
Bled female				
3	14	703	5	1066
7	6	413	2	940
12	22	274	2	670
13	18	331	1	800
14	23	328	2	95
15	2	81	1	620
16	26	2205	8	960
17	17	72	1	552
18	29	964	3	319
19	9	326	3	1142

3.2. *Limulus* tracking information

The VPS system detected and calculated the location of the 20 female horseshoe crabs 8974 times during the 30 d the receivers were deployed. However, because horseshoe crabs moved out of the range of the VPS array at a consistent rate (0.5–1 d^{-1}) (Table 1, Fig. S2), data were only obtained from the original 20 horseshoe crabs for an average (±SD) of 13.5 ± 8.7 d (max.: 30; min.: <1; Table 1). A generalized linear model using a Poisson distribution with log-link function found that there was a significant effect of bleeding on the time horseshoe crabs remained in the

array (null deviance: 152.68 on 19 df, residual deviance: 138.83 on 18 df, Akaike's information criterion: 218.76), with unbled crabs spending almost 50% less time (10.5 d) in the array than bled crabs (16.6 d; coefficient estimate: 0.458 ± 0.125, z = 3.673, $p < 0.001$). If the 3 control crabs for which there was <1 d of data are not included, an average (±SD) of 16.9 ± 7.8 d of tracking data (min.: 2; max.: 30) and 651.0 ± 680.5 (min.: 48; max.: 2209) positional fixes were obtained per female.

Horseshoe crabs in the Great Bay Estuary typically move about 5 km up into the estuary (south) each spring and then back down (north) in the late summer and fall to their overwintering areas (Schaller et al. 2010). Therefore, despite the size of the VPS array used here, which was able to detect tagged horseshoe crabs over an area of about 500 000 m^2 (Fig. S1), many of them left the area shortly after they were released (Fig. S2). During this study, there were other VR2Ws deployed in the Great Bay Estuary for a different investigation, and these receivers enabled us to confirm that the horseshoe crabs that left the array were still active and their pingers continued to transmit. Two of the additional receivers were located in Great Bay, about 2–3 km south and up-estuary from the middle of the VPS array, and one was located to the north, down-estuary, at the mouth of the Oyster River ~4 km away. Sixteen females were detected outside of the array an average (±SD) of 38.1 ± 27.6 d (max.: 104; min.: 9 d) after they were released, while the remaining 4 females moved out of the array but not to areas where they could be detected by any other receivers. While some of the females were heard by one of the outside receivers within days of leaving the array, others were not detected until over 1 mo later. The first 3 females to leave the VPS array were all controls that moved out of the range of detection during the first day. The last time control females were detected in 2019 by any VR2Ws was 105.1 ± 42.3 d (±SD; max.: 144 d; min.: 43 d) after they were released, which was mostly in the late summer and fall, while for bled females it was 123.7 ± 46.1 d (max.: 169 d; min.: 40 d) after the experiment started. These data demonstrate that most of the horseshoe crabs from both treatment groups were active enough to move several km after they were released and that the acoustic transmitters continued to send data well into the fall.

3.3. *Limulus* locomotion

When horseshoe crabs were walking, based on playbacks of their positional fixes in Google Earth or graphs of velocity vs. time of day, they typically moved about 3 to 5 m min^{-1} (180 to 300 m h^{-1}). The total distance all horseshoe crabs traveled during this study was, not surprisingly, highly correlated with the number of successful positional fixes that we obtained for each individual (R^2 = 0.859, Fig. S3). This is because each time a crab was detected, the distance it traveled from the previous location was determined and all these values were summed to determine the distance traveled during a given time period. To compensate for this detection bias, the primary measure we used to compare the control and bled groups of animals was the mean distance they moved per hour. Furthermore, as described in Section 2.3, on some days the number of data points obtained were not sufficient to use for analyses. Based on data obtained from horseshoe crabs that we successfully tracked for at least 3 d, control (n = 5) and bled (n = 8) females moved comparable distances per day (unpaired 2-tailed Mann-Whitney test, p = 0.83). The bled females moved 89.7 ± 48.7 (SD) m h^{-1}, while unbled females moved 90.3 ± 44.5 m h^{-1}.

3.4. Mating

During the 4 wk that horseshoe crabs were tracked, there were 63 cases (35 controls, 28 bled) when females approached beaches at high tide in an apparent effort to spawn (Fig. 2). The number of spawning events by a female was a positive asymptotic function of the number of position fixes (R^2 = 0.88) and was reduced significantly by the bleeding treatment (Table 2, Fig. 3). However, crabs that were most active did not necessarily approach beaches more often. In fact, there was a slight negative relationship between the distance traveled per week by horseshoe crabs and the number of times they approached a beach that week (slope of linear regression = –0.009, R^2 = 0.06). Moreover, this same trend was evident when both groups were examined separately (bled: slope = –0.006, R^2 = 0.06; controls: slope = –0.007, R^2 = 0.006).

Only a few individuals approached the shore in an apparent effort to spawn during the first few days after they were released (2 of 8 controls and 4 of 10 bled), but during the next 11 d, mating activity increased (Fig. S4). In total, 17 horseshoe crabs expressed a mean (±SD) of 3.71 ± 0.74 (n = 17, range: 0–10) spawning events during the 28 d period after they were released, or an average of 0.27 ± 0.05 mating attempts d^{-1} (range: 0–0.8). Six control females appeared to mate at least twice, and 3 of them ap-

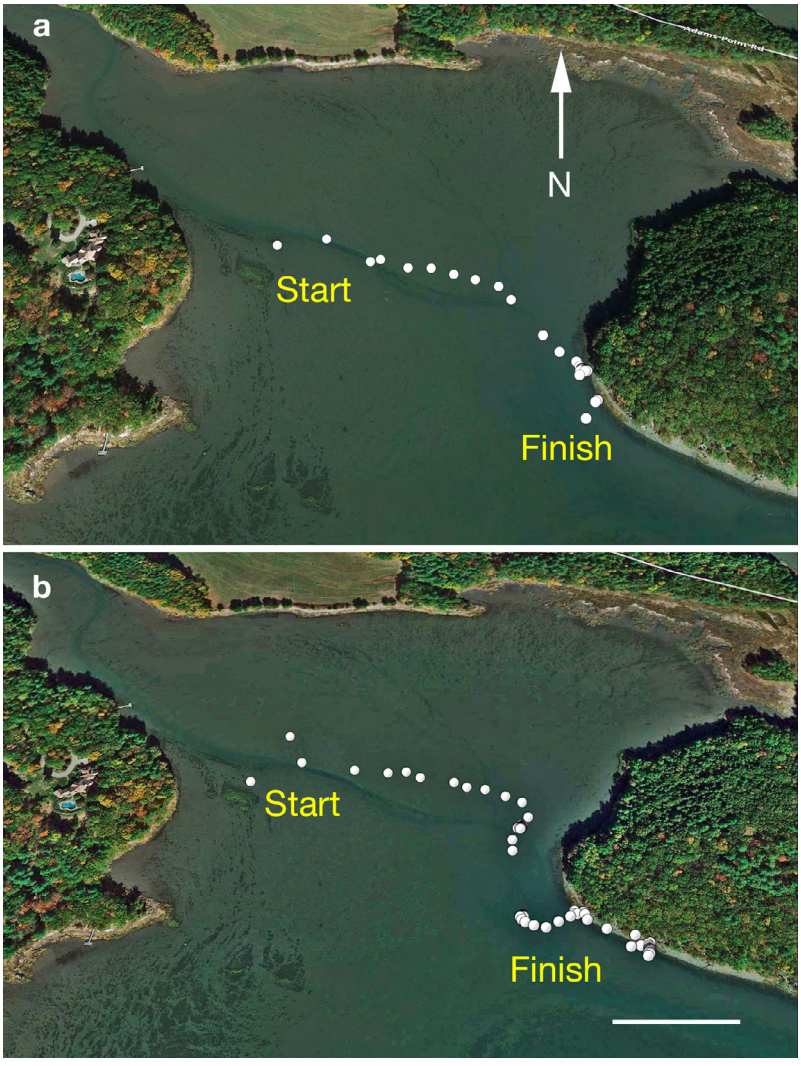

Fig. 2. Two examples of apparent attempts to spawn by female horseshoe crabs. (a) Path of a control female (#8) on 16 June 2019. The 3 h track shown started at 22:30 h and ended at 01:30 h near the beach where this horseshoe crab was initially collected while spawning. High tide occurred at 12:45 h. (b) The 3 h track taken by a bled female (#5) starting at 22:00 h on 28 May19 and ending at 01:00 h on 29 May 19. Peak high tide was at 22:30 h on this day. White scale bar = 100 m

proached beaches >6 times. In contrast, while 4 bled females approached beaches at least twice, none of them appeared to try to mate >5 times. Unsurprisingly, there was a significant positive effect of the number of VPS positional fixes received on the number of beach approaches (indicative of mating attempts) that were detected, as mating attempts could only be detected when crabs were within the array (Fig. 3). After accounting for this using an ANCOVA, we found that bled crabs made significantly fewer mating attempts than did control crabs (Table 2, Fig. 3). Based on the coefficient estimates from this analysis, bled females attempted to mate roughly half as often as control females (2.8 versus 5.0).

This tendency for bled females to spawn half as often as control females was also evident when the data were examined in 2 other ways. First, the percent of the females in each group that moved towards a beach at high tide as if they were attempting to spawn was determined for each day of available tracking data. These values were averaged every 3 d and, in all but one case, a higher percentage of control females attempted to mate during each 3 d period (Fig. S4). In the second case, we looked at the probability that females from each group would mate during a given high tide, based on a total of 105 good days of VPS data for the control females versus 166 d for bled females. Given that there are 2 tides each day, control females attempted to spawn on 16% (35 of 210) of the high tides for which we had data, while bled females only attempted to mate on 8% (28 of 332) of the high tides.

The average interval between apparent spawning events was 2.7 ± 0.07 d (range: 0.5 [or once at each high tide] to 13 d). Excluding the females that did not try to mate at all (4 control, 3 bled), 6 control females approached the spawning beaches every 2.4 ± 1.7 d (range: 2–15 d), while the 7 bled females did so every 4.4 ± 2.2 d (range 0.5–13 d). However, even though there was a tendency for control females to

Table 2. Generalized linear model testing the effects of bleeding treatment on the number of mating attempts made by horseshoe crabs ($\log_{10}[y+1]$ transformed), with the number of positional fixes received for each crab ($\log_{10}[x]$ transformed) used as a covariate to account for crabs remaining in the array to be detected. Adjusted $R^2 = 0.88$ with 17 df, $p < 0.001$

Parameter	Estimate	SE	*t*	p
Intercept	−0.367	0.085	−4.32	≪0.001
Treatment	−0.150	0.058	−2.57	<0.020
Fixes	0.409	0.037	11.13	≪0.001

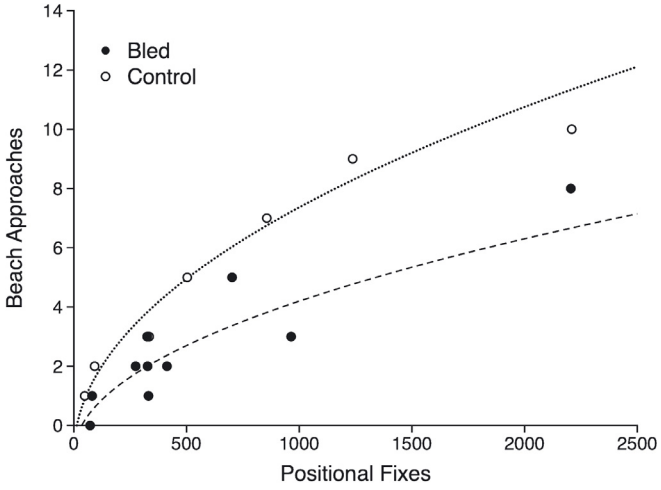

Fig. 3. Relationship between the number of positional fixes (data points) obtained for a given horseshoe crab and the number of times it approached a beach at high tide in an apparent attempt to spawn. For both bled and control females, there was a strong correlation (adjusted $R^2 = 0.88$; Table 2) between the time they were successfully tracked within the VPS array, as measured by the number of positional fixes obtained, and the number of times they apparently attempted to mate. The plotted curves were derived by back-transformation of the parameter estimates in Table 2. Controls: dotted line; $y_C = (0.28223)x^{0.49062} - 1$; treatment: dashed line; $y_T = (0.17537)x^{0.49062} - 1$. Both y_C and y_T had 1 subtracted to account for the addition of 1 before $\log_{10}$ transformation

have a shorter period of time between apparent spawning events than bled females, the difference was not statistically significant (unpaired 2-tailed *t*-test, $p = 0.076$).

4. DISCUSSION

While it is generally accepted that the biomedical bleeding process will result in the death of a certain percentage of the horseshoe crabs used, the procedure also appears to have some sublethal behavioral and physiological impacts (Kurz & James-Pirri 2002, Anderson et al. 2013, Krisfalusi-Gannon et al. 2018, Owings et al. 2019, 2020). For example, in a previous telemetry study, Owings et al. (2019) reported that bled females attempted to spawn about half as often as control females; the data from the present study are consistent with these findings. However, even though there was a statistically significant difference between the number of apparent mating attempts expressed by bled vs. control females, we strongly recommend further research into this phenomenon. In particular, future studies should use as many individuals as possible and track them using a method that makes it feasible to obtain consistent mating data over a longer period of time and a larger area. This might be accomplished by combining the use of a VPS array, like this study, with transmitters that send both acceleration and depth data, which was the method used by Owings et al. (2019).

Another sublethal impact of bleeding that has been reported is a reduction in activity or locomotion (Anderson et al. 2013, Owings et al. 2020). Both studies that reported these effects were carried out in the laboratory, and so one goal of the current investigation was to determine if this is also the case if horseshoe crabs are released back into their natural habitat. When we did this, we did not see any differences in the distance traveled per hour by bled females in comparison to control females. These findings could be different from the laboratory data for 3 reasons. First, in the current investigation, bled females experienced milder stressors than in the previous studies, including a shorter time out of water and less exposure to high temperatures. Second, it is likely that horseshoe crabs recover from the bleeding process faster when they are returned to their natural habitat, where they have access to optimal conditions and natural prey. As a result, they might restore both energy reserves and hemocyanin more quickly than when they are held in laboratory tanks. Finally, several of the most active control females left the array on the first day, and overall, control females spent 50% less time than bled females within the VPS array. Thus, some of the most active control females might have left the array and their movements outside the array were not included in our analyses.

4.1. Telemetry

While horseshoe crabs have been tracked using acoustic telemetry in several previous studies (James-Pirri 2010, Schaller et al. 2010, Watson & Chabot 2010), to our knowledge this is the first time a VPS array has been used. The major advantage of this approach is that it covers a larger area than the VRAP system we used in the past (Watson & Chabot 2010), which only employs 3 buoys. According to our calculations, the 12-receiver VPS array we used covered an area of ~500 000 m^2 (Fig. S1), while the 3-receiver VRAP array we previously used only covered 0.3% of that area (~1600 m^2). Therefore, the VPS system collects data over a much larger area and for a longer time period before tagged mobile animals, like horseshoe crabs, migrate out of the array. However, because the acoustic transmitters must be detected by 3

receivers at approximately the same time in order to triangulate a position, the area covered is still somewhat limited, and half of the animals in this study moved out of the array within 2 wk of being released (Fig. S2).

We have now used several different acoustic telemetry approaches to study the behavior of horseshoe crabs in their natural habitat, including manual tracking with one manual hydrophone/receiver, the VRAP system, a VPS array, and a combination of VR2S receivers and pingers that transmit acceleration and depth data. Each of these techniques has its advantages and disadvantages, and we strongly recommend trying to match the questions being addressed in a given study with the most appropriate telemetry method(s). To our knowledge, the Vemco VRAP and VPS systems are the only techniques available if the goal is to obtain nearly continuous fine-scale tracks of the movements of marine species. We have also used an HTI (Hydroacoustic Technology) system for this purpose, with fish in an aquaculture cage (Rillahan et al. 2011), but it is designed for use in freshwater and thus is limited in terms of the distance the high-frequency transmitters will send detectable signals in seawater. As stated above, the VRAP approach is limited in terms of the study area because it only uses 3 receivers, but it seems to have a higher resolution and the data are obtained in near real-time. In contrast, with the VPS approach, more buoys can be used to cover a wider area, but the data are not readily available until after the study has stopped and the positions calculated. This also means animals can leave the array and the user will not know about it immediately unless some manual tracking is also employed in the same area.

In a previous study with horseshoe crabs, we used a hybrid approach that provided data that were similar to the results that we obtained in the current study, but at a lower cost (Owings et al. 2019). An array of VR2s was created that was larger than the one used for this study because they did not have to be close enough together to allow for triangulation of signals. In that study, rather than tracking the sequential positions of the horseshoe crabs to determine where and when they moved, we monitored their activity using acoustic tags that transmitted acceleration and depth data. These data could then be used to determine when animals were active and their approximate location, based on both depth and their proximity to an individual VR2 receiver. As it turned out, these data were sufficient to identify times when animals probably attempted to mate, even though we could not visualize the paths they took or calculate their actual walking speed. We identified mating events as times when animals were active at high tide and moved into shallow water closer to shore (Owings et al. 2019). This same accelerometer approach has also been used to document the biological rhythms horseshoe crabs express in their natural habitat (Watson et al. 2016). In the future, it might be useful to combine this approach with a VPS array so the acceleration data could be used to 'fill in the gaps' when animals could not be detected by 3 VR2s at the same time.

4.2. Locomotion

All the horseshoe crabs in this study walked at a speed of ~80 m h^{-1} when averaged over a 24 h day. However, it should be noted that during a given 24 h period they would also spend time not moving and partially buried (based on diver observations and loss of signals; Watson et al. 2016). For example, in a previous study in a nearby location (Watson & Chabot 2010), horseshoe crabs were not active at each high tide but rather tended to approach beaches more during the daytime high tide. Therefore, they were only consistently moving around for about 4 to 8 h in a day. In the present study, when crabs were obviously walking, as determined by watching playbacks of their tracks with Google Earth, they moved ~220 m h^{-1} (3.6 m min^{-1}), which is similar to the values we calculated in an earlier study using the VRAP system (290 m h^{-1}; Watson & Chabot 2010).

Most of the horseshoe crabs we tracked left the area encompassed by the VPS array within 1 mo, and half of them left within 2 wk, which is not surprising. In the Great Bay Estuary, just prior to mating season in April and early May when water temperatures exceed ~12°C, horseshoe crabs typically migrate about 4–5 km up into the estuary to their preferred spawning areas (Watson et al. 2016). In May and early June, they spawn for about 1.5 mo (Watson et al. 2009, Cheng et al. 2016) and then remain near the vast, widely dispersed intertidal mudflats to feed throughout most of the summer (Watson et al. 2009, Lee 2010, Schaller et al. 2010). In the fall, they move back down the estuary to their deeper overwintering areas (Schaller et al. 2010). Based on the supplemental telemetry data we obtained from colleagues who had VR2s deployed throughout the estuary, some of the horseshoe crabs in the present study expressed this same pattern, but others appeared to remain in the Great Bay portion of the estuary and did not migrate very far in the fall. We are not sure at this

time why some individuals failed to express the more general seasonal migration pattern, but there was no clear correlation of treatment type with this behavior.

4.3. Mating

Bled females attempted to mate about half as often as control females, which is consistent with the data obtained previously in the same location using the slightly different telemetry methods described above (Owings et al. 2019). Bled females expressed apparent mating events 2 times wk^{-1} during the first week after they were released, while control females appeared to try to mate 4 times wk^{-1}. This mating frequency is consistent with data from horseshoe crabs in Delaware Bay that were obtained by Brousseau et al. (2004) using radio telemetry during an 8 d period, as well as tag/recapture spawning surveys conducted along the Florida Gulf Coast by Brockmann & Johnson (2011). The Delaware females spawned 2 to 6 times and many of them did so on consecutive nights, while the Florida females were 'resighted' on beaches an average of 1.5 to 2 times. It was also noted in these studies that many of the females spawned on the beaches where they were originally captured, tagged, and released, which we also observed (Fig. 2).

The tendency for bled females to approach beaches less often than controls was not a function of their overall activity. Moreover, when we plotted distance moved per hour vs. mating attempts, the correlation was very poor for both bled and control horseshoe crabs. Therefore, it appears as if bled females, while healthy enough to move around, may have been allocating their energy to seeking food to improve their nutritional status and restore energy reserves rather than searching for a good site where they could dig a nest and lay eggs. Also, given the fact that females only spawn during about one-third of the high tides possible during the peak of the mating season, it probably even takes healthy females time to recover between mating events, so bled females might require a little more time and thus try to mate less.

It should be noted that we identified mating events as times when females approached beaches around high tide, yet it is possible that they failed to mate during some of these excursions. However, several observations suggest this was not the case. First, shortly after we released the tagged females during a high tide, several of them were observed digging pits to spawn and they already had males attached to them. We were unable to determine, without disturbing them, whether these were control or bled females. However, at least for some of the females, the capture, tagging, and release process did not seem to influence their immediate tendency to spawn and their ability to attract a male. Second, we rarely see single females in the vicinity of mating beaches in the Great Bay Estuary (97% are paired; Cheng et al. 2016), and thus it is unlikely any of the tagged females in this study approached a beach alone and did not attempt to mate. Finally, on one occasion, about 1 wk after they were released, we observed one of the bled females mating, and we were able to correlate this observation with a track of her approaching the beach. Therefore, while we are not certain each movement to a spawning location represented an actual successful mating event, our direct observations support our telemetry data.

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LITERATURE CITED

Anderson RL, Watson WH III, Chabot CC (2013) Sublethal behavioral and physiological effects of the biomedical bleeding process on the American horseshoe crab, *Limulus Polyphemus*. Biol Bull 225:137–151

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67: 1–48

Brockmann JH, Johnson SL (2011) A long-term study of spawning activity in a Florida Gulf Coast population of horseshoe crabs (*Limulus polyphemus*). Estuaries Coasts 34:1049–1067

Brousseau LJ, Sclafani M, Smith DR, Carter DB (2004) Acoustic-tracking and radio-tracking of horseshoe crabs to assess spawning behavior and subtidal habitat use in Delaware Bay. N Am J Fish Manage 24:1376–1384

Chen L, Mozier N (2013) Comparison of *Limulus* amebocyte lysate test methods for endotoxin measurement in protein solutions. J Pharm Biomed Anal 80:180–185

Cheng H, Chabot CC, Watson WH III (2016) Influence of environmental factors on spawning of the American horseshoe crab (*Limulus polyphemus*) in the Great Bay Estuary, USA. Estuaries Coasts 39:1142–1153

Espinoza M, Farrugia TJ, Webber DM, Smith F, Lowe CG (2011) Testing a new acoustic telemetry technique to

quantify long-term, fine-scale movements of aquatic animals. Fish Res 108:364–371

Hurton L, Berkson J (2006) Potential causes of mortality for horseshoe crabs (*Limulus polyphemus*) during the biomedical bleeding process. Fish Bull 104:293–298

Hurton L, Berkson J, Smith S (2005) Estimation of total hemolymph volume in the horseshoe crab *Limulus polyphemus.* Mar Freshw Behav Physiol 38:139–147

James-Pirri MJ (2010) Seasonal movement of the American horseshoe crab *Limulus polyphemus* in a semi-enclosed bay on Cape Cod, Massachusetts (USA) as determined by acoustic telemetry. Curr Zool 56:575–586

James-Pirri MJ, Veillette PA, Leschen AS (2012) Selected hemolymph constituents of captive, biomedically bled, and wild caught adult female American horseshoe crabs (*Limulus polyphemus*). Mar Freshw Behav Physiol 45: 281–289

Krisfalusi-Gannon J, Waleed A, Dellinger K, Robertson L and others (2018) The role of horseshoe crabs in the biomedical industry and recent trends impacting species sustainability. Front Mar Sci 5:185

Kurz W, James-Pirri MJ (2002) The impact of biomedical bleeding on horseshoe crabs, *Limulus polyphemus*, movement patterns on Cape Cod, Massachusetts. Mar Freshw Behav Physiol 35:261–268

Lee WJ (2010) Intensive use of an intertidal mudflat by foraging adult American horseshoe crabs *Limulus polyphemus* in the Great Bay estuary, New Hampshire. Curr Zool 56:611–617

Leschen AS, Correia SJ (2010) Mortality in female horseshoe crabs (*Limulus polyphemus*) from biomedical bleeding and handling: implications for fisheries and management. Mar Freshw Behav Physiol 43:135–147

Meckley TD, Holbrook CM, Wagner CM, Binder TM (2014) An approach for filtering hyperbolically positioned underwater acoustic telemetry data with position precision estimates. Anim Biotelem 2:7–20

Novitsky TJ (2009) Biomedical applications of *Limulus* amebocyte lysate. In: Tancredi JT, Botton ML, Smith DR (eds) Biology and conservation of horseshoe crabs. Springer, New York, NY, p 315–330

Owings M, Chabot CC, Watson WH III (2019) Effects of the biomedical bleeding process on the behavior of the American horseshoe crab, *Limulus polyphemus*, in its natural habitat. Biol Bull 236:207–223

Owings M, Chabot CC, Watson WH III (2020) Effects of the biomedical bleeding process on the behavior and hemocyanin levels of the American horseshoe crab (*Limulus polyphemus*). Fish Bull 118:225–239

R Core Team (2021) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rillahan C, Chambers MD, Howell WH, Watson WH III (2011) The behavior of cod (*Gadus morhua*) in an offshore aquaculture net pen. Aquaculture 310:361–368

Rudloe A (1983) The effect of heavy bleeding on mortality of the horseshoe crab, *Limulus polyphemus*, in the natural environment. J Invertebr Pathol 42:167–176

Schaller SY, Watson WH III, Chabot CC (2010) Seasonal movements of American horseshoe crabs, *Limulus polyphemus*, in the Great Bay Estuary, New Hampshire (USA). Curr Zool 56:587–598

Thompson M (1998) Assessments of the population biology and critical habitat for the horseshoe crab, *Limulus polyphemus*, in the South Atlantic Bight. MS thesis, University of Charleston, Charleston, SC

Walls EA, Berkson JM (2000) Effects of blood extraction on the survival of the horseshoe crab, *Limulus polyphemus.* Va J Sci 51:195–198

Walls EA, Berkson JM (2003) Effects of blood extraction on horseshoe crabs (*Limulus polyphemus*). Fish Bull 101: 457–459

Watson WH III, Chabot CC (2010) High resolution tracking of adult horseshoe crabs (*Limulus polyphemus*) in a New Hampshire estuary using fixed array ultrasonic telemetry. Curr Zool 56:599–610

Watson WH III, Schaller SY, Chabot CC (2009) The relationship between small- and large-scale movements of horseshoe crabs in the Great Bay Estuary and *Limulus* behavior in the laboratory. In: Tanacredi JT, Botton ML, Smith DR (eds) Biology and conservation of horseshoe crabs. Springer, New York, NY, p 131–147

Watson WH III, Johnson SK, Whitworth CD, Chabot CC (2016) Rhythms of locomotion and seasonal changes in activity expressed by horseshoe crabs in their natural habitat. Mar Ecol Prog Ser 542:109–121

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