



Ethogram of *Limulus polyphemus*

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Abstract – Developing an understanding of how organisms interact with their environment is crucial in creating best management practices in managed-care settings and improving conservation efforts. It similarly is important in understanding and developing research questions surrounding animal behavior and cognition. For many invertebrates, the knowledge surrounding behavior, welfare, cognition, and husbandry practices is greatly limited, posing a multitude of concerns when establishing these best management practices and general awareness for these animals. The American horseshoe crab (*Limulus polyphemus*; horseshoe crab), is often referred to as a living fossil species. However, their behaviors, like many invertebrates, are not well documented in scientific literature. This study presents the first peer-reviewed ethogram of *Limulus polyphemus*, operationally defining 47 behaviors. Horseshoe crabs were observed in both *in-situ* and *ex-situ* settings. The intention of this study was (1) to create a behavioral foundation that can be expanded upon and referenced, (2) to standardize the behavioral language regarding the species, (3) to demonstrate inter-rater reliability of the presented ethogram, (4) to propose potential applications, and (5) to further cultivate specialized research questions about horseshoe crab behavior.

Keywords – Behavior, Welfare, Ethogram, Horseshoe Crab

The American horseshoe crab, *Limulus polyphemus* (horseshoe crab), can be traced back in the geologic record to the Ordovician period, about 445 million years ago (Rudkin et al., 2008). These arthropods are often referred to as “living fossils” as their morphology has remained relatively unchanged from their ancestors (Bicknell & Pates, 2019). While they have persisted through extreme environmental events (i.e., the last ice age and post-Pleistocene climatic fluctuations; Faurby et al., 2010), historical and current anthropogenic pressures (i.e., bait harvest, Shuster & Botton, 1985; Smith, Millard et al., 2009; biomedical harvest, Davis et al., 2006; habitat loss, Botton et al., 1994; Jackson & Nordstrom, 2009; Loveland & Botton, 2015; pollution events, Botton & Itow, 2009; Strobel & Brenowitz, 1981; Venosa et al., 1996; growth in human populations/development, Faurby et al., 2010, and climate change, Loveland & Botton 2015) continue to be a main threat. As of 2016, the IUCN Red List assessment listed the American horseshoe crab as vulnerable with their population trend decreasing (Smith et al., 2016). At a regional level, horseshoe crabs may warrant more concern both in wild and managed-care settings due to variable environmental conditions, population sizes, and regulations across their large geographic range (e.g., Smith et al., 2016, 2017).

Since the early 1900s horseshoe crabs have been heavily utilized for biomedical bleeding in which they are bled to obtain *Limulus* Amoebocyte Lysate (LAL), an important clotting agent used to detect the presence of endotoxins pathogenic to humans in injectable drugs (Berkson & Shuster, 1999). Previous studies have shown that the bleeding process causes reduced activity (ranging from a 33-66% reduction; Krisfalusi-Gannon et al., 2018), reduced spawning activity (Owings et al., 2019; Smith et al., 2017), and decreased expression of circatidal rhythms (Anderson et al., 2013; Owings et al., 2019). Anderson and

colleagues (2013) found that decreased expression of tidal rhythms coincided with reduced activity levels in bled horseshoe crabs. Understanding how an organism interacts with its environment is vital to developing and implementing best management practices in managed-care settings (i.e., research labs and zoos/aquariums) as well as aiding conservation efforts in the field. However, this requires a foundational understanding of a species' behavioral repertoire, which is currently lacking for horseshoe crabs.

Behavior has frequently been used as a good assessment of the health and welfare of animals (Martins et al., 2010; Watters et al., 2021) and as a method to evaluate the effectiveness of husbandry practices (Mendes da Silva et al., 2020). Ethograms are essential tools for such monitoring and assessment of animal behavior. An ethogram is a catalog of mutually exclusive and objective behaviors displayed by an animal, with corresponding definitions of each using detailed descriptions that aim to avoid subjectivity and functional inference of their possible purpose (e.g., Stanton et al., 2015; Howe et al., 2015). The use of an ethogram can quantify species-specific behaviors, enable comparisons between different populations of a given species, and serve as a tool to quantify changes in observed behaviors as a result of anthropogenic or environmental disturbances (Howe et al., 2015; Stanton et al., 2015). While ethograms have been commonly developed to describe and define the behavior of larger vertebrates (e.g., Howe et al., 2015; Seeber et al., 2012; Stanton et al., 2015), they are an underutilized tool for the management of behavior and welfare in invertebrate species. Similar benefits observed from applications of ethograms in larger vertebrate species can benefit invertebrate species. For example, Do Nascimento and colleagues (2019) created a behavioral repertoire of the freshwater crab (*Kingsleya attenboroughi*), a species at risk for extinction, in a laboratory environment in order to better understand their physiological and ecological interactions which helped contribute to the development of conservation strategies. Through analyzing the behavioral activity of this freshwater crab, Do Nascimento and colleagues (2019) were able to make informed inferences on how environmental factors, as simple as time of day, impact when the species may be most vulnerable to stressors.

Monitoring behavior is a common practice used in managed-care environments such as laboratories and zoological/aquaria facilities. Indicators of low welfare and inadequate husbandry are often represented by stereotypies or abnormal behaviors which can develop when an environment lacks stimulation, limits natural movements/tendencies, and/or is not sufficient to allow the expression of natural movements (Mendes da Silva et al., 2020; Smith & Wassmer, 2016). The use of an ethogram in these various managed-care settings can serve to measure changes in behavior and/or activity when new enrichment is provided (e.g., Mendes da Silva et al., 2020; Bashaw et al., 2016), changes in social groupings occur (e.g., Weladji et al., 2019), or when there are alterations to the physical habitat (e.g., Biolatti et al., 2016; Weladji et al., 2019). Using behavior as a main informant for creating best management practices and oversight for horseshoe crab research will likely allow for more effective care, as well as, provide more detailed information on how horseshoe crabs respond and adapt behaviorally in different environments. Specifically, it will allow for management practices and standard operating procedures for the maintenance of horseshoe crabs in managed care settings to be developed through a data-driven approach and include a more complete understanding of the species behavioral repertoire.

The lack of behavior and welfare-based research has contributed to the absence of best management practices within the industry for horseshoe crabs. There are immense opportunities to better utilize behavioral research to develop improved techniques and welfare monitoring methods that promote optimal care given to this species, specifically with their use for biomedical bleeding. More knowledge on horseshoe crab behavior, and the development of a species-specific ethogram, could greatly contribute to future management action, improving biomedical bleeding practices, enhanced welfare assessment tools, and calls attention to improving other laboratory standards/protections for horseshoe crabs and other invertebrates. A best management practices document on the use of horseshoe crabs for biomedical bleeding was established in 2011 by the Horseshoe Crab Biomedical *ad hoc* Working Group (The Horseshoe Crab Biomedical *ad-hoc* Working Group, 2011). While the document is a good starting point, the language used is extremely broad and lacks specific criteria that would enable these best management practices to be implemented and standardized across facilities. Further, this document has yet to be updated since its establishment in 2011.

Additionally, invertebrates being used for research, with the exception of cephalopods and some decapod crustaceans (Harvey-Clark, 2011), are not required to undergo Institutional Animal Care and Use Commission (IACUC) review and approval within the United States. This is due to the immense diversity within the invertebrate genera, a lack of understanding regarding invertebrate welfare, and differing standards of moral concern within the genera (Harvey-Clark, 2011). Currently, many IACUCs approach invertebrate reviews by dividing the genera into ‘advanced’ invertebrates (cephalopods and some decapod crustaceans), meaning the species is worthy of increased moral concern, and ‘not advanced’ (i.e., all other species) (Harvey-Clark, 2011). However, it is argued that this distinction is not appropriate, and all invertebrates should be given proper care that ensures their welfare. Harvey-Clark (2011) further suggests invertebrate protocols should be focused on detailed species-specific requirements. The ethogram presented here is a significant step in that direction.

We created an ethogram of forty-seven observed behaviors by *L. polyphemus* in both *in-* and *ex-situ* settings. The aim of this study was (1) to create a behavioral foundation that can be expanded upon and referenced, (2) to standardize the behavioral language regarding the species, (3) to demonstrate inter-rater reliability of the presented ethogram, (4) to propose potential applications of the ethogram (i.e., monitoring welfare, evaluating husbandry practices, informing the creation of species-specific best management practices, and quantifying changes in behavior) and (5) to further cultivate specialized research questions about horseshoe crab behavior. These observations can inform a better understanding of their behavior and provide direction for the future care and welfare of the American horseshoe crab and other non-charismatic species.

Methods

Ethics Statement

This research was carried out under a Florida Fish and Wildlife Special Activity Permit SAL-22-2398-SR.

Collection and Housing

Fifteen horseshoe crabs were included in this study (Table 1). All horseshoe crabs were collected and released from varying sites in the St. Petersburg/Tampa, Florida, USA, surrounding area under a Florida Fish and Wildlife Special Activity Permit (SAL-22-2398-SR). Individuals were collected at various times from March 1 to May 12, 2022. Only individuals that were not buried and were freely moving in the environment were collected. Those found buried, or males attached to buried females, were not collected to minimize stress on the individuals and to avoid interfering with potential mating events. Upon collection, each individual’s intake weight (g) and prosoma width (cm) were measured. Individuals were weighed by placing them into a felt bag hooked up to a hanging scale. The final intake weight of an individual was then calculated by subtracting the weight of the ‘bag only’ from the weight of the ‘bag and the horseshoe crab.’ Prosoma width was measured from the two farthest points on the individual’s prosoma. Additionally, each individual’s age was estimated based on Smith and colleagues’ (2013) carapace condition index (Table 2). Individuals were noted for being attached or unattached at collection and for any damages/notable features (i.e., barnacle presence, missing limbs, broken telson, compound eye damage, pitting, and carapace damage). Each individual was also given a name to aid in identifying and distinguishing individuals during behavioral observations. Individuals were transported to Galbraith Marine Science Laboratory (GMSL) at Eckerd College for *ex-situ* observations.

Table 1*Horseshoe Crab Intake Information*

Horseshoe Crab	Sex	Intake Weight (g)	Prosoma Width (cm)	Estimated Age Category	
1	Athena	F	1170	20.3	M
2	Zeus	M	395	14.5	M
3	Poseidon	M	325	13.4	M
4	Apollo	M	285	11.6	M
5	Hermes	M	300	11.2	M
6	Selene	F	680	17.4	M
7	Kleio	F	1320	22	M
8	Calypso	F	920	18.5	M
9	Hera	F	940	19.5	M
10	Percy	M	530	16.5	O
11	Dio	M	430	15	O
12	Orion	M	285	11.1	O
13	Aries	M	315	10.8	O
14	Atticus	M	248	12.8	O
15	Olympus	M	510	16	O

Note. Fifteen individual horseshoe crabs were observed ex-situ at Galbraith Marine Science Laboratory from March 1 - May 12, 2022. Intake data included sex (F - female; M - male), intake weight (g), prosoma width (cm), and estimated age (Y - young; M - medium aged; O - old).

Table 2*Horseshoe Crab Age Index*

Age	Carapace Condition
Young (Y)	Light in color with no erosion or pitting and little mucus present.
Medium (M)	Small degree of erosion and pitting and some mucus present.
Old (O)	Dark in color with a great amount of erosion, pitting, and mucus present.

Note. Based on Smith and colleagues (2013) carapace condition index.

Horseshoe crabs were housed in a large outdoor tank (4.572 meters x 16.459 meters) below GMSL (Figure 1). Males and females were separated by a brick wall within the same tank. Specific temperature and salinity day-to-day data were unable to be collected. However, the water in the housing tank was directly pumped in from Boca Ciega Bay, and thus should align with the expected water temperature and salinity levels in the Bay between March 1 to May 12, 2022. The water was aerated with an airstone on both sides of the brick wall. Additionally, a layer of sediment, from Boca Ciega Bay, with varying thickness was added to the tank floor. Individuals were fed shrimp every other day. Shrimp were purchased raw/frozen and defrosted prior to feeding. When an individual did not eat for two consecutive feeding sessions, they were immediately released back into Boca Ciega Bay.

Ethogram Development

The ethogram was assembled based on live and video-recorded observations of horseshoe crabs in both *in-* and *ex-situ* settings. Observations in both of these contexts were essential to creating a comprehensive ethogram. Initial observations were made opportunistically in the St. Petersburg/Tampa Bay area. *Ad libitum* sampling was used during these sessions. Descriptive notes taken during initial observation sessions were used to formulate operational definitions. A literature review was then conducted to refine initial operational definitions where behavioral descriptions found in the literature were similar to those defined in the ethogram (e.g., Brockmann, 1996; Brockmann & Penn, 1992; Cohen & Brockmann, 1983; Vodstatka, 1969).

Figure 1*Housing and Observation Tank at Galbraith Marine Science Laboratory*

Note. Male and female horseshoe crabs were housed on separate sides of the brick wall within the same tank. Water was pumped in directly from the Boca Ciega Bay, USA. A layer sediment, from Boca Ciega Bay, was present on the tank floor.

A second phase of observations was conducted utilizing all-occurrence sampling. Live *ex-situ* observations and video recorded *ex-* and *in-situ* observations were used to further refine operational definitions for behaviors in the ethogram as well as allowed for additional descriptive data used for defining new behaviors. *Ex-situ* observations were conducted with fifteen individuals (10 males, 5 females) at GMSL for varying lengths of time between March 1 to May 12, 2022. The horseshoe crabs were recorded one to two times a day for approximately 45-60 min in the morning (8:00 a.m. - 12:00 p.m.) and/or the afternoon/evening (5:00 p.m. - 9:30 p.m.). A total of 366 hr and 48 min were recorded. Data was recorded on a GoPro10 setup in an aerial view (Figure 1).

In-situ observations were conducted by analyzing video data collected from St. Petersburg/Tampa study sites. Additionally, video data from Sandy Point Island located on the Connecticut-Rhode Island border was provided by John Anderson from Terramar Productions (179 videos). The provided footage was collected within a few days before and after the full moon events of April 28, 2010 and April 17, 2011. All footage was shot in the late afternoon to around dusk. Due to these recordings being short (ranging from 5-44 s) and typically including 1-4 horseshoe crabs, an all-occurrence sampling method was used to observe behaviors. A new behavior was added to the ethogram after it had been noted and described at least five times. These video observations allowed for a wider variety of angles and a closer view of the horseshoe crabs exhibiting their behaviors which were used to establish more detailed and well-rounded operational definitions as well as incorporate new operationally defined behaviors into the ethogram.

Inter-Rater Reliability

Video data for reliability testing of the behavioral observations was coded by six researchers to assess inter-observer reliability to demonstrate the validity of this study's behavioral data and the efficacy

of the ethogram in its applicability to other environments. Criteria for high reliability was greater than 80% agreement on the presence of behaviors. Videos were randomly selected from *ex-situ* footage from both phase one and phase two of the ethogram development and totaled 170 min. The six coders had varying levels of prior experience and knowledge with horseshoe crabs and ethograms. All observers were given a 1 hr training in which they were exposed to the operational definitions and a video representation of each behavior defined on the ethogram. Additionally, all observers completed three practice videos in which observers watched a subset of video recordings where they coded for behaviors and then compared their observations to the lead researcher. Once training was complete, reliability coding commenced. All observers used the same Microsoft Excel database to record all occurrences of behaviors, although each coder was blind to behaviors recorded by all other coders. Each behavior coded was assessed as “Yes” or “No” as to whether the observers agreed on a behavioral event. The level of agreement between all observers was calculated using Pearson’s *r* correlation coefficient, which measures the pairwise correlation between raters, and the mean of the results was used to calculate the average level of agreement for the group of coders.

Results

Forty-seven behaviors were operationally defined (Table 3). Four behaviors were only observed *in-situ*, eight behaviors were only observed *ex-situ*, and 35 behaviors were observed in both settings. However, it is important to note the presence of a specific behavior in only *in-situ* or *ex-situ* settings does not mean that the behavior cannot occur in both environments. Due to the opportunistic nature of our data collection and video observations, opportunities to exhibit behaviors from the ethogram may not have been present at the time of our observations, but these behaviors could still occur in those settings. All behaviors observed were further classified into three categories (social/interactions, movement, and other) and nine subcategories (Figure 2). Of these behaviors, 21 are classified as states, and 24 are classified as events (Table 4).

Table 3

Ethogram of Limulus polyphemus

(A) Behavior	Operational Definition	Setting Observed
Attachment	The male horseshoe crab has clasped onto another female or male for 30 s or more, using their modified claw-like first legs, ending when the male has <i>detached</i> . The overarching term used to describe attachments in a mating context.	<i>In- and ex-situ</i>
Nesting	Individuals who are <i>Buried</i> in a mating context. Can describe the female as well as males, as long as the individual is at least partially buried.	<i>In-situ</i>
Mating Attachment - Direct Attach	The male horseshoe crab is positioned directly posterior to the female and is in line with the females telson. This behavior ends when the male has <i>detached</i> or the male has adjusted to a <i>Mating Attach - Satellite Attach</i> . This behavior can only occur when a male attaches to a female.	<i>In- and ex-situ</i>
Mating Attachment - Satellite Attach	The male horseshoe crab is in an <i>attachment</i> state positioned on the side spines of the female horseshoe crabs' opisthosoma, in line with the females telson. This behavior ends when the male has <i>detached</i> the male has adjusted into the <i>Mating Attach - Direct Position</i> . This behavior can only occur when a male attaches to a female.	<i>In- and ex-situ</i>
Subject to Mating Attachment	A social interaction in which a female horseshoe crab is being <i>attached</i> to or <i>attempted to attached</i> to by a male. A social interaction that can only be used to describe the behavior of a female horseshoe crab.	<i>In- and ex-situ</i>
Primary Nesting Pair	In a mating context, used to describe a male-female pair. The male must be in a <i>Mating Attachment-Direct</i> or <i>-Satellite</i> position to the female.	<i>In-situ</i>
Satellite Attachment	A male horseshoe crab is clasped onto another male horseshoe crab that is directly attached to a female or another male satellite who is indirectly attached to a female and has the criteria for an <i>Attachment</i> behavior (i.e., remaining clasped for 30 s or more). This behavior only occurs in a mating context. Males exhibiting this behavior can be further classified as a <i>Primary Satellite</i> or <i>Secondary Satellite</i> . This behavior ends when the male of interest unclasps from the male subject to the attachment (<i>Detachment</i>).	<i>In- and ex-situ</i>
Primary Satellite	Used to describe a male whose modified claw-like first legs are clasped onto another male who is directly attached to a female.	<i>In- and ex-situ</i>
Secondary Satellite	Used to describe a male whose modified claw-like first legs are clasped onto another male who is clasped onto another male associated with the <i>Primary Nesting Pair</i> .	<i>In- and ex-situ</i>
Subject to Satellite Attachment	A social interaction in which a male horseshoe crab is being <i>attached</i> or <i>attempted to attached</i> to by another male. A social interaction that can only be used to describe the behavior of a male horseshoe crab in a mating context.	<i>In- and ex-situ</i>
Non-Clasping	In a mating context, used to describe a male who is not hooked onto a directly attached male or a satellite (<i>Primary</i> or <i>Secondary</i>) male but is within 10 cm of the nesting female of interest.	<i>In-situ</i>
Solitary	In a mating context, used to describe a male who is non-clasping but greater than 10 cm from the nesting female of interest.	<i>In-situ</i>
Non-Mating Attachment	A male clasped onto another male for 5 s or more, using their modified claw-like first legs to clasp the other males' opisthosoma, or telson. The clasping male may pull the other male backward. These interactions occur in a non-mating context. This behavior ends when the clasping male <i>detaches</i> .	<i>In- and ex-situ</i>
Subject to Non-Mating Attachment	Male horseshoe crab that is being clasped to by another male in a non-mating context.	<i>In- and ex-situ</i>
Adjusting Attachment	An <i>attached</i> male is folding up, extending their back fifth legs, and or using their telson to lift the other horseshoe crabs opisthosoma, sometimes pushing the other horseshoe crab forward. This may also include minor movements of the male and/or regripping of the females opisthosoma. This behavior may lead the male horseshoe crab to transition from a <i>Mating Attachment-Direct Attach</i> to a <i>Mating Attachment-Satellite Attach</i> or vice versa. This behavior can be seen when a male is attached to another male or female in both mating and non-mating contexts.	<i>In- and ex-situ</i>

Approach Swim	A quick, smooth, and direct <i>Gliding</i> movement closing the distance between themselves and another individual, ending with an <i>Attempt to Attach</i> or an <i>Attachment</i> .	<i>In- and ex-situ</i>
Attempt to Attach	The approach of a male horseshoe crab to another horseshoe crab (male or female), hooking their modified claw-like first legs onto another part of the horseshoe crabs body, typically the opisthosoma or telson. The attempting to attach horseshoe crab can be seen maneuvering around the other individual but fails to reach the criteria of a successful <i>Attachment</i> or <i>Non-Mating Attachment</i> . This behavior ends when the male has <i>detached</i> or when the attempting male horseshoe crab has met the criteria of an <i>Attachment</i> or <i>Non-Mating Attachment</i> .	<i>In- and ex-situ</i>
Detachment	Male horseshoe crab releases their modified claw-like first legs, unhooking from the other horseshoe crab (male or female) so that they are no longer in contact.	<i>In- and ex-situ</i>
Head Bump	Prosoma to prosoma contact between two individuals.	<i>In- and ex-situ</i>
Walk Over	One individual move over/on top of another individual.	<i>In- and ex-situ</i>
Climbing Along Wall	<i>Climbing</i> while moving along a wall-like surface. Individual is clearly seen exhibiting leg/body movement as defined by <i>Climbing</i> , with the exception that they are not staying in the same spot along the wall-like surface.	<i>In- and ex-situ</i>
Escape Swim	A very quick, smooth and direct <i>Gliding</i> movement. Typically seen to avoid interaction with another individual, being subject to an attachment, or another social interaction.	<i>In- and ex-situ</i>
Gliding	Synchronous movement of all five walking legs extending towards the rear followed by a quick retraction of the legs forward before extending them back again in a repetitive stroke-like motion, causing the individual to propel in a forward direction. This movement can be quick and smooth, as if the individual is gliding forward or be more lethargic which can be observed by the propelling motion to appear more "choppy."	<i>In- and ex-situ</i>
Stop-Go	Small, jagged movements in which the individual inches forward, with short stoppages in movement occurring. The prosoma may be observed anteriorly bent downwards partially in the sediment. This movement tends to follow an irregular path.	<i>Ex-situ</i>
Swimming	This behavior begins by an individual utilizing a wall-like surface to orient itself vertically. The individual then extends their legs, pushing themselves off the wall-like surface and upward so that they are oriented in the water column or at the water surface with their carapace facing the floor. Once oriented in this position, synchronous and rapid stroke-like movement of all five pairs of legs propels the individual forward. This behavior ends when the individual has sunken to the floor, or has used a wall-like surface to reorient themselves back to their resting orientation.	<i>Ex-situ</i>
Walking	Five pairs of walking legs make contact with the floor in a coordinated movement (one after the other), extending slightly when contact is made causing the individual to move in the forward direction. This behavior can be seen with the individual's legs extended causing their prosoma to be lifted off of the floor or with little extension keeping their prosoma close to or touching the floor in which visual of the legs is hindered. This movement can be quick or lethargic.	<i>In- and ex-situ</i>
Walking into Wall	Body (typically the prosoma) of the individual is oriented perpendicular to and in contact with the wall-like surface. As the individual is <i>Walking</i> or <i>Gliding</i> they maintain contact with the wall-like surface, ending when they no longer have contact with the wall-like surface or when they stop <i>Walking/Gliding/moving</i> along the surface.	<i>In- and ex-situ</i>
Walking on Wall	Body of the individual is oriented parallel to the wall-like surface (perpendicular to the floor), in which their legs are in contact with the wall-like surface as they move (motion as described in <i>Walking</i> behavior) in a forward direction along the wall-like surface, ending when they are no longer oriented parallel to the wall-like surface (aka. have returned to the floor).	<i>Ex-situ</i>

Climbing	Typically sporadic (sometimes coordinated) extension of the walking legs in an upward direction, causing the prosoma to lift up along the obstacle or wall-like surface staying relatively in the same place. The 3rd, 4th, and 5th pair of walking legs may be seen extending off of the floor surface while the 1st, and 2nd (sometimes 3rd) pair of legs are typically observed extending up the wall-like surface. After an initial extension of the legs, they will retract causing the individual to move in an up and down repetitive fashion. This behavior ends when they are no longer in the same place along the wall-like surface (transitions to <i>Climbing Along Wall</i>), or when the movement described above is no longer being exhibited by the individual.	<i>In- and ex-situ</i>
Digging	The prosoma-opisthosoma joint is arched inward, causing the individuals prosoma downward into the substrate (sometimes observed as a direct thrusting of the prosoma ridge into the substrate). The first four pairs of walking legs move either synchronously or independently, disrupting the sediment, and pushing it backward, where the 5th pair of legs will synchronously push the substrate out from under the body, typically through the gaps between the prosoma and opisthosoma. This movement is repeated until the individual is fully or partially beneath the substrate. This behavior ends when the individual is <i>Buried</i> , is no longer visible, is no longer beneath the sediment, or is no longer moving further into the substrate.	<i>In- and ex-situ</i>
Escape Dig	A more rapid and forceful form of <i>Digging</i> ; typically exhibited following a social interaction.	<i>In- and ex-situ</i>
Moving into Surface	Individual repeatedly makes contact with a wall-like surface, while staying relatively in the same spot. Most commonly, the individual hits the front of their prosoma into the wall-like surface. This behavior begins after 10 s of the repeated hitting of their prosoma into the wall-like surface and ends when the described movement ends or when they are no longer staying in the same spot along the wall-like surface.	<i>Ex-situ</i>
Pounce	Quick, jolt-like movement forward on top of a food. Prior to the quick-jolt the prosoma may be slightly bent anteriorly into the substrate. <i>Digging</i> /disruption of the substrate typically follows the jolt-like movement.	<i>Ex-situ</i>
Folding	In a <i>Flipped</i> state, the opisthosoma, and sometimes the telson, move inward towards the legs/underside of the prosoma making at least a 90-degree angle at the underside of the prosoma-opisthosoma joint. After an inward movement, the opisthosoma and sometimes the telson, extend in the opposite direction returning to a flat state or creating about a 190 degree at the underside of the prosoma-opisthosoma joint. The telson may fold in or extend out anywhere from 1-90 degrees, measuring from its attachment telson-opisthosoma joint. This behavior ends when the individual ceases the in and out movement described above or when the individual is no longer <i>Flipped</i> .	<i>In- and ex-situ</i>
Leg Extension	Extension of the legs in an upward or sideways direction or extending outward, upward, and back in towards the center of the body in a circular like movement. This behavior occurs only when the individual is in a stationary state (e.g., <i>Flipped</i> , <i>Inactive</i> , time prior to inactivity), acting as an adjustment movement or isolated movement of the individual's body.	<i>In- and ex-situ</i>
Telson Circle	Movement of the telson in a circular fluid motion.	<i>In- and ex-situ</i>
Telson Lift	Individual lifts their telson upwards at least 20 degrees.	<i>In- and ex-situ</i>
Telson Side to Side	Movement of the telson in a side to side/left to right/right to left direction.	<i>In- and ex-situ</i>
Righting	The behavior begins only if the individual is <i>Flipped</i> and ends when the individual has successfully turned themselves back over.	<i>In- and ex-situ</i>

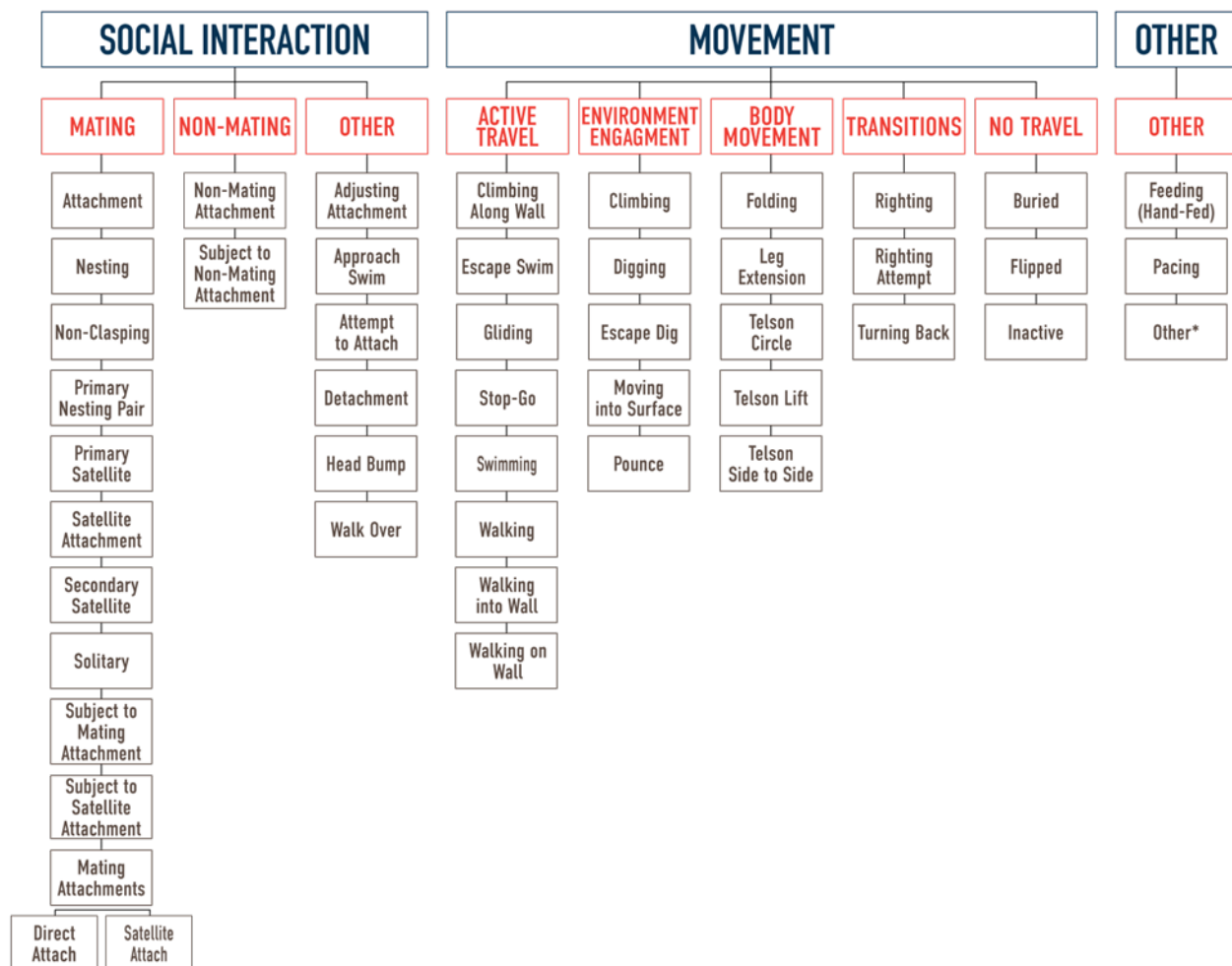
From the flipped position, the individual will fold in their telson and opisthosoma, their legs and gills exhibiting little to no movement. The individual will quickly arch its back, in which their telson is fully extended out and downward, with its tip in contact with the floor, while its legs and gills beat rapidly. This movement is often repeated until they are eventually able to cause their body to roll over on its side, returning to their resting orientation.

Righting Attempt	An individual in a <i>Flipped</i> state who exhibits the movement defined in the <i>Righting</i> behavior, however, the individual fails to turn over and remains flipped. This behavior is typically followed by little to no movement before the individual may attempt again. This behavior ends when little to no movement is exhibited by the individual but remains <i>Flipped</i> .	<i>In- and ex-situ</i>
Turning Back	180 degree change in direction	<i>In- and ex-situ</i>
Buried	This behavior is seen only in sediment and must be preceded by <i>Digging</i> . Individuals may be partially or completely beneath the sediment and exhibit little to no movement (some occasional digging or postural adjustments may occur, digging adjustments lasting longer than 15 s end this behavior). This behavior ends when the individual is no longer beneath the sediment. This behavior cannot occur with an <i>Inactive</i> state as part or all of the body is not visible. This behavior only occurs in non-mating context.	<i>In- and ex-situ</i>
Flipped	The individual is positioned with their carapace in contact with the floor. Can be caused by failing to reorient themselves back to their resting orientation following <i>Swimming</i> , after climbing up on an obstacle, or being turned over by currents or other animals. <i>Righting Attempts</i> are often observed; however, this behavior ends only when the individual has successfully turned themselves back over.	<i>In- and ex-situ</i>
Inactive	Any stationary position, with little to no movement visible, exhibited for at least 2 min (i.e., a quick stop in movement or lack of movement for less than 2 min does not qualify as inactive), ending when the individual exhibits a clear adjustment in position or begins active movement.	<i>Ex-situ</i>
Feeding (Hand-Fed)	The individual has been turned over [by a human]. Food is placed on the top of the individual's mouth. Coordinated small movements of the individual's walking legs help to facilitate their gnathobases to grind up and soften the food. At times the chelicerae, small feeding pinches, will aid in breaking up the food and pushing the food into or deeper into their mouths. Often, spurts of movement of the walking legs, opisthosoma, and/or telson are seen.	<i>Ex-situ</i>
Pacing	A pattern of movement that has been repeated at least 3 times, ending when the pattern has broken or been interrupted by a behavior that was not a part of the initial pattern. Can be observed by the back-and-forth movement between two static points (1 repetition = Point A to Point B; 2 repetitions = Point A to Point B, back to Point A; 3 repetitions = Point A to Point B, back to Point A, Back to Point B). Can also include repeated lap-like patterns or continuous circling. Specific behaviors (i.e., <i>Walking</i> , <i>Gliding</i> , <i>Climbing</i> , etc.) should be taken into account to the pattern.	<i>Ex-situ</i>
Other*	Observation of a behavior that has not yet been defined. *This must be described and explained in the notes section. Five or more observations of the same described new behavior should be operationally defined and added to the current ethogram.	<i>In- and ex-situ</i>

(B) Contextual Term	Definition
Mating Context	A mating context is established when there is a least a male-female pair along the shoreline/in an environment where the female has opportunity to lay eggs and at least one male is attached that could fertilize those eggs.
Non-Mating Context	There is no opportunity for eggs to be laid and fertilized. This could be due to no female presence, no primary nesting pair present, or being in an environment where nesting (laying and fertilization of offspring) does not or could not occur.

Completely Buried	Individual is beneath the substrate up to their median eyes to their entire body is completely beneath the substrate.
Partially Buried	Individual's carapace to their lateral eyes is beneath the substrate with their opisthosoma and telson remain above the substrate.
Wall-Like Surface	Vertical surface or obstacle (i.e., wall, large rock, brick, seawall, etc.).
Resting Orientation	Refers to the typical orientation of a horseshoe crab, in that their carapace is oriented upward.

Note. (A) Behavioral terms and operational definitions of forty-seven behaviors exhibited by *Limulus polyphemus in-situ* and/or *ex-situ*. These behaviors make up the ethogram being presented in this study. Italicized words seen in operational definitions refer to another defined behavioral term found in the ethogram presented. (B) Defines contextual terms used within operational definitions of the main ethogram. The main and mentioned anatomical features of horseshoe crabs can be seen in Supplementary Figure S1.

Figure 2*Ethogram Tree Diagram*

Note. The 47 behaviors defined in the ethogram were divided into three main categories (1) social interaction, (2) movement, and (3) other. The three main categories were further divided into subcategories. *Accounts for a behavior that has not yet been defined. Five or more observations of the same described new behavior should be operationally defined and added to the current ethogram.

Inter-Rater Reliability

Inter-rater reliability was achieved across five of the six coders above criteria (> 80%) agreement for the presence of behaviors (Table 5). Coder “E” achieved reliability with some coders, but not all, while their scores were trending towards reliability. Coder E was then provided an additional training session on new videos not used in the initial reliability coding, before undergoing reliability again. Most notably, coder E’s identification of the behaviors ‘Pacing’ and ‘Climbing Along Wall’ was inconsistent compared to the rest of the group and necessitated additional training to clarify their coding of these particular behaviors. Overall, group reliability was achieved 88% average agreement for presence of the behaviors coded during the initial reliability testing, which indicates strong agreement between coders.

Table 4*Behavior States vs. Events Classification*

States	Events
Attachment	Adjusting Attachment
Buried	Approach Swim
Climbing	Attempt to Attach
Climbing Along Wall	Detachment
Digging	Escape Dig
Flipped	Escape Swim
Gliding	Folding
Inactive	Head Bump
Moving into Surface	Leg Extension
Nesting	Mating Attachment - Direct Attach
Non-Mating Attachment	Mating Attachment - Satellite Attach
Pacing	Non-Clasping
Satellite Attachment	Pounce
Stop-Go	Primary Nesting Pair
Subject to Mating Attachment	Primary Satellite
Subject to Non-Mating Attachment	Righting
Subject to Satellite Attachment	Righting Attempt
Swimming	Secondary Satellite
Walking	Solitary
Walking into Wall	Telson Circle
Walking on Wall	Telson Lift
	Telson Side to Side
	Turning Back
	Walk Over

Note. Two behaviors (other* and feeding-hand fed) were not included in this classification. Feeding-Hand Fed was not included here because the behavior relies on human manipulation of the individual.

Table 5*Inter-Rater Reliability Agreement*

	A	B	C	D	E	F
A	-	0.99	0.90	0.90	0.80	0.96
B	-	-	0.93	0.92	0.75*	0.93
C	-	-	-	0.90	0.74*	0.83
D	-	-	-	-	0.80	0.86
E	-	-	-	-	-	0.82
F	-	-	-	-	-	-

Note. *indicates not achieving the minimum of 80% agreement. Coders are assigned an alphabetical letter indicator. Coders A, B, C, D, and F were all in agreement above criteria upon the first time coding for reliability testing. Coder E, while approaching reliability, did not meet criteria and required additional training.

Ethogram Categorization

The social category was divided into three subcategories, mating, non-mating, and other interactions (Figure 2). The mating subcategory is made up of twelve behaviors that describe attachment interactions between males and females in a mating or reproductive context (defined in Table 3). The non-mating attaches subcategory is made up of two behaviors that describe male-male-specific attachment interactions that do not take place in a mating context. The third subcategory, other, is made up of six behaviors and includes any interactions between at least two individuals and does not fit the criteria of the other two subcategories. We observe that further subcategories of social interactions can be added to this ethogram.

Movement was split into five subcategories, active travel, environmental engagement, transitions, body movement, and no travel (Figure 2). Active travel is made up of eight behaviors that describe high-energy and prolonged movement from one location to another. It should be noted that pacing was not included in active travel due to it potentially being a stereotypic behavior and thus not reflective of typical movement patterns expected *in-situ*. Environmental engagement consists of five behaviors that describe high-energy movement that involves physically manipulating or engaging with the surrounding environment. Body movement consists of five behaviors describing isolated body motions. Transitions are made up of three behaviors that describe a change in direction, position, or orientation of the individual. The final subcategory is no travel, which is made up of three behaviors that describe any time the individual is stationary/not moving from one place to another.

The last category, other, includes three behaviors (Figure 2) that do not fit the criteria of any other categories/subcategories. We recognize further categories, subcategories, and behaviors can be added to this ethogram.

Many observations from the *in-situ* videos were taken of individuals being tagged by researchers for long-term monitoring and tracking. Behavioral observations of individuals and the context in which they were tagged varied too much for specific tagging behavioral states or events to be defined and/or included in the final ethogram. However, leg extensions, folding, telson circle, telson side to side, telson lift, escape swim, and escape dig were commonly observed during and following the tagging process.

Discussion

This study presents the first comprehensive ethogram of horseshoe crab behavior based on horseshoe crabs both *in-situ* and *ex-situ* that illustrates, defines, and classifies horseshoe crab behaviors. This study operationally defines 47 behaviors of *L. polyphemus* forming a comprehensive ethogram that does not currently exist in the literature. This ethogram can help cultivate more specific research questions on *L. polyphemus* behaviors and possible environmental, chemical, or biological cues that may or may not play a role in the initiation and termination of such behaviors. Additionally, this ethogram can be used as a tool to compare different populations and species behaviors more thoroughly and accurately, gain a better understanding of behaviors causative and functional bases, act as a tool to better inform management decisions, and allow for analysis of behavioral impacts due to anthropogenic and other possible disturbances on the species (Howe et al., 2015). Furthermore, this ethogram can and should serve as a tool to be expanded upon with the inclusion of new behaviors as more research is conducted with the American horseshoe crab.

High reliability between observers of the ethogram was achieved, suggesting the ethogram is objective and the behaviors were clearly defined, allowing viewers to be able to recognize and identify the behaviors accurately across multiple individuals. Since observers were student volunteers and had varying levels of experience with horseshoe crabs, as well as ethograms in general, we conclude that this ethogram can be implemented reliably across people in a variety of settings (i.e., labs, managed-care facilities, and in the field). We found that high reliability could be achieved fairly quickly when new observers underwent a training period in which they were exposed to the operational definitions and given practice observations. Through this method of training, new observers, regardless of prior knowledge, gained experience with identifying behaviors as well as with the type of sampling. This approach could be implemented to achieve strong agreement amongst multiple users of this ethogram across various organization types.

Ethograms have been successfully used as a tool in other species for comparing behavioral changes in response to living in a managed-care environment (e.g., Smith & Wassmer, 2016). Due to minimal knowledge regarding the optimal managed-care environment for horseshoe crabs and no current equivalent protections like IACUC for most invertebrates, this ethogram could be used as a tool for monitoring the welfare of horseshoe crabs in such environments. Behavioral patterns are often used as an indicator of the health of the individual and the environment they are inhabiting (e.g., Biolatti et al., 2015; Boissy et al., 2007; Martins et al., 2010). The application of this ethogram can help to inform, develop, and implement proper methods of stimulating horseshoe crab natural behaviors that may enhance a managed-care

environment. Using the ethogram to establish best practices could properly inform the creation of species-specific protections for horseshoe crabs and other invertebrate species.

Implementation of the ethogram established in this study would enable a mechanism for more precise assessments and measurements of horseshoe crab behavior throughout the whole bleeding process (i.e., harvest, transportation, bleeding/time in a laboratory environment, and reintroduction *in-situ*). This necessary information on what and where improvements can be made could greatly reduce the negative behavioral effects currently observed in the bleeding process (e.g., Anderson et al., 2013; Krisfalusi-Gannon et al., 2018; Owings et al., 2019). Most research currently has focused more so on mortality, rather than behavioral effects that could predict mortality, which this ethogram could be used to address. For example, horseshoe crabs that underwent the bleeding process and experienced stressors such as high temperatures and increased air exposure resulted in a significant increase in mortality compared to bled horseshoe crabs under the condition of low stressors (Hurton & Berkson, 2006). While blood loss is a main contributor to the physiological and behavioral effects observed, it is not the only factor that could be contributing to, or be an indication of, mortality. When considering the impacts of stress incurred from collection, reintroduction, transportation, and time spent at the holding facility, it becomes evident that the effects on behavior may be greater than previously realized (Hurton & Berkson, 2006; Krisfalusi-Gannon et al., 2018). Utilizing this ethogram would allow for a thorough assessment of behavior in these environments and under various conditions, providing important insights into how each aspect of the bleeding process affects these animals. Thus, it is important for best management practices to address concerns regarding behavior that can be predictive of mortality or other stressors, and outline specific criteria and standards in every step of the process that is supported by peer-reviewed research.

Another environment where horseshoe crabs are housed is in zoological or aquaria facilities. Monitoring behavior has been highly used in managed-care facilities due to behavioral observations being low in cost, noninvasive, and recognized indicators of welfare (Biolatti et al., 2015; Martins et al., 2010). For many invertebrates, knowledge about behavior, welfare, and husbandry practices has been limited. The ethogram presented in this study provides a well-rounded picture of the behavioral repertoire of horseshoe crabs that can be implemented in managed-care facilities such as zoos and aquariums to evaluate current management practices, habitat design, and enrichment. For example, the addition of sediment to the tank floors in our study enabled the horseshoe crabs to exhibit a larger diversity of behaviors, such as digging, buried, and escape dig that during pilot observations had only been observed *in-situ*. Previous studies conducted with tigers (Biolatti et al., 2015) and farmed fish (Martins et al., 2010) have demonstrated, in combination with species-specific knowledge, that behavioral changes can be associated with good or poor welfare. Use of this ethogram in managed-care facilities can begin to inform which behaviors may be indicators of poor, positive, or enhanced welfare in horseshoe crabs across different housing environments where horseshoe crabs are commonly observed, such as larger aquaria housing or touch tank enclosures.

Conclusion

The ethogram presented provides a behavioral foundation that can be built upon and referenced in order to standardize the behavioral language being used regarding horseshoe crabs. A standardized terminology will allow for more accurate comparability between studies and increase inter-observer reliability (Stanton et al., 2015). Conjointly, consistency in language will likely improve our understanding and lead to more successful conservation, husbandry and welfare practices across facilities.

The current study aimed to develop a comprehensive ethogram that encompassed a broad spectrum of behaviors. A crucial next step is to establish an activity budget for this species in both *in-situ* and *ex-situ* environments. This would enhance our understanding of horseshoe crab behavior and welfare, while also providing a baseline for facilities to compare the welfare of their horseshoe crabs. Furthermore, an activity budget would be a valuable tool for evaluating the potential impact/stressors on horseshoe crabs throughout the biomedical bleeding process. Future studies may also want to further investigate the behavioral categories in more detail such as, feeding/foraging behaviors, social behaviors, etc. More observations should be made regarding females, especially potential female-female interactions as we only observed five

females. While to date, it has been challenging to observe horseshoe crabs underwater when they are not along the shoreline (e.g., Chabot & Watson, 2010), current underwater and tracking technology used in conjunction with this ethogram could open the door to understanding more about their natural behaviors, activity-levels and whereabouts when they are offshore.

This ethogram is by no means the answer to all the questions about horseshoe crab behavior. However, it is a crucial first step to growing our understanding of horseshoe crab behavior by opening the door for deeper behavioral analyses and establishing a means of reliably quantifying how natural, anthropogenic, and laboratory/managed-care factors may be impacting horseshoe crabs.

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Supplementary Material

Figure S1

Diagram Of Horseshoe Crab Main Features as Well as Key Features Linked with their Sensory Structures

