RESEARCH ARTICLE

Defining Optimal Husbandry conditions for the Atlantic Horseshoe Crab, *Limulus polyphemus*

A comparative study on literature and current culture practices in public aquaria

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ABSTRACT

The Atlantic horseshoe crab, *Limulus polyphemus* L. (Xiphosurida: Lumilidae) plays a vital role in coastal ecosystems and is an important species in physiological studies. Over the past three decades, horseshoe crab populations have rapidly declined due to wild capture for fishing industries and biomedical industries, stressing the necessity for conservation efforts and raising public awareness. Public zoos and aquaria largely contribute to this cause by keeping horseshoe crabs in captivity. However, a complete and detailed set of optimal rearing conditions was still lacking. This study provides first evaluation of current husbandry practises among public aquaria and comparisons with natural and culture conditions suggested in literature. As a result we could determine that optimal water conditions are; 19-21°C, salinity 29ppt, 7.9pH, Dissolved Oxygen (DO) 7.1mg/L, Alkalinity 2.7mEq/L for juveniles and ±18°C, salinity 20-28ppt, ±7.7pH, DO 8.5-9.3mg/L for adults. Flow-through tanks and sufficient filtration including trickling- or sand filters in combination with UV or ozonation should secure a viable habitat for the animals. Toxic substances as ammonia and nitrates should be kept as low as possible, the maximum levels of these, however, are not yet known. Important for good health is to supplement horseshoe crab diet with plant-based food sources and to limit the food quantities to their daily energy and protein requirements.

KEY WORDS: *Limulus polyphemus,* horseshoe crab, husbandry, aquaculture, captive, rearing

INTRODUCTION

Horseshoe crabs, commonly referred to as living fossils, have survived virtually unchanged for over 200 million years and oldest fossils of ancestral species date back even further, to approximately 445 million years ago (Rudkin & Young, 2009; Rudkin, Young, & Nowlan, 2008) As a group of chelicerate, aquatic arthropods, horseshoe crabs 'Leach, 1819' (Xiphosurida; Limulidae) are more closely related to modern-day scorpions and spiders than to true crustaceans. In fact, recent genetic analysis revealed that horseshoe crabs might actually represent a group of aquatic arachnids (Ballesteros & Sharma, 2019).

Limulus polyphemus is the most studied of the four extant horseshoe crab species found worldwide and is endemic to the Atlantic coast of North America ranging from Maine to the Gulf of Mexico. The other 3 species; *Carcinoscorpius rotundicauda, Tachypleus gigas* and *Tachypleus tridentatus*, can be found in coastal areas between India and Japan (Smith & Berkson, 2005; Walls, Berkson, & Smith, 2002).

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The Atlantic horseshoe crab is an important species used as a laboratory model animal in prominent research regarding vision & circadian rhythms, embryology of marine invertebrates and their unique endocrine system (Berkson & Shuster, 1999; Liu & Passaglia, 2009; Rudloe, 1979; Zaldívar-Rae, Sapién-Silva, Rosales-Raya, & Brockmann, 2009). Additionally, *Limulus polyphemus* is a key species in coastal ecosystems and benthic food chains, where the eggs provide an essential food source to supplement the diet of more than a dozen species of migratory shorebirds (Clark, Niles, & Burger, 1993; Gillings et al., 2007; Graham, Botton, Hata, Loveland, & Murphy, 2009).

Since 1980 horseshoe crab populations have been rapidly declining with large numbers of animals captured in the wild for their use in fertilizer, cattle feed and as bait in commercial fishing industries (Berkson & Shuster, 1999; Botton, 1984). It has been estimated that a 2.5 million crabs are harvested annually for eel- and whelk fishing alone, grossing an economical value of approximately $13 - $17 million (Manion, West, & Unsworth, 2008). In recent years *L. polyphemus* has become most notable for the collection of its copper-based blue blood, which contains an important endotoxin indicator 'Limulus Amoebocyte Lysate' (LAL). The underlying mechanism involves a cascade of serine proteases capable of detecting miniscule quantities of pyrogenic endotoxins produced by Gram-negative bacteria (Ding & Ho, 2010). Therefore, horseshoe crab blood has been widely used in testing the sterility of medical devices, implants and injectable drugs over the past three decades (Ding & Ho, 2010; Smith & Berkson, 2005). An estimated 250,000 horseshoe crabs are captured, transported and bled annually. These the animals are put under considerable stress and it has been shown that post-bleeding mortality rates increased with 7.5% for *L. polyphemus* kept in captivity and up to 15% for animals released back into the wild (M. Thompson, 1999; Walls et al., 2002).

In early 2000s, researchers succeeded in mapping the genes underlying the production of Limulus Amoebocyte Lysate and identified the primary component (Factor C) of the coagulation cascade (Ding & Ho, 2001; Ding, Navas, & Ho, 1993). Using recombinant technology and genetic engineering, they have accomplished the production of a recombinant Factor C (rFC), which is now incorporated into the PyroGene kit, an alternative test for LAL (Ding & Ho, 2010). Although the recombinant Factor C has been commercially available for almost 15 years and has been found to be equivalent to the LAL test, its use in pharmaceutical industries worldwide is still lacking. This is presumably due to historical single-source supplier concerns, ongoing validation procedures and compendial standards, whilst the horseshoe crab populations are still being diminished by fishing and blood-harvesting industries (Bolden & Smith, 2017; Williams, 2018).

Due to its ecological value and the importance of *L. polyphemus* in the physiological research and biomedical field, there is high interest in restoring the horseshoe crab populations (Mishra, 2009). Culturing the animals in the lab has been suggested to reduce the necessity for wild-dcaptured horseshoe crabs and ensure a year around source of biological research material (Kropach, 1979). Additionally, horseshoe crabs kept in public zoos and aquaria could largely contribute to education and raising awareness on the conservation status of this species. Carmichael & Brush (2012) have reviewed multiple studies to determine optimal growth and survival conditions for *L. polyphemus* in laboratories, but no literature currently exist on horseshoe crabs kept in public aquaria. This BSc thesis research project aims to provide best practises information for keeping horseshoe crabs in public aquaria by (i) reviewing existing literature on the life history, the physiology and the husbandry conditions for juvenile and adult *L. polyphemus* (ii) evaluating husbandry conditions amongst public aquaria and zoos in Europe. The research questions to be answered are a) what is the life history of *L. polyphemus*, b) what are important environmental conditions for wild horseshoe crabs?, c) how are juvenile and adult horseshoe crabs currently kept in captivity according to literature? and d) what are best practises for horseshoe crab husbandry in public aquaria? In all this information similarities and differences are analysed and possibly explained based on the known traits of wild horseshoe crabs. From this, the best husbandry practises for *Limulus polyphemus* in captivity will be extracted.

METHODS

Data collection

Literature review

For reviewing and comparing literature on horseshoe crab husbandry, information was gathered by searching large databases including Scopus and Wageningen University & Research library for scientific articles on optimal living conditions for *L. polyphemus* in captivity. A number of categories were distinguished for 'husbandry': a) growth & reproduction; b) housing; c) water conditions; d) diet & feeding; f) health issues and management. Search terms are given in Table S1. Useful sources were thoroughly reviewed and the collected information was organised in an excel database following the six categories.

Aquaria survey

Information on husbandry conditions for *L. polyphemus* in aquaria was collected by conducting a survey among public zoos and aquaria in Europe. 20 members of the European Union of Aquarium Curators (EUAC) were contacted, of which 15 responded that they were willing to participate in this study. A questionnaire was made and questions were organized into four main categories: a) enclosure dimensions and animal density; b) diet & feeding; c) water conditions; d) animal health. The questionnaire was sent to the curators of the 15 aquaria involved, out of which 8 responded with valuable data.

Analysis

Determining survival and life stage

Studies on *Limulus polyphemus* in captivity generally used growth (increase prosoma width * $time^{-1}$) or wet weight as an objective measure to evaluate culture success. However, public aquaria were not expected to closely monitor size or weight. Therefore, mean annual survival percentage was calculated using data from literature and by using the population sizes per year reported by public aquaria. Public aquaria were asked to provide i) *PSE* = Population Size at the End of year (x); and ii) number of deaths during that year. New introduced individuals (*NI*) were either marked by the curator or calculated with:

$$NI = \left(PSE\ year\ (x) + \#\ deaths\ year\ (x)\right) - PSE\ year\ (x-1) \qquad (1)$$

PSB, the Population Size at the Beginning of year (x) was determined as the sum of all animals from the previous year and any newly introduced individuals. Percentage Survival (*S*) was then calculated as the total number of animals at the end of the year divided by the total number of animals at the beginning of that same year:

$$S = 100 * \frac{PSE\ (\#\ animals\ end\ of\ year\ (x))}{PSB\ (\#\ animals\ beginning\ of\ year\ (x))} \qquad (2)$$

Summation of the Percentage Survival of every year and subsequent division by the number of years resulted in the Mean Annual Survival Percentage (*MS*).

The life stage of a horseshoe crab is often expressed in instar stage (INST), wet weight (WW) or prosoma width (PW). The metric used varies from study to study. Therefore we made a conversion table to describe the relationships between these variables. Sekiguchi et al. (1988) reported mean sizes of PW for juvenile stages 1-14 and gave predicted values for instar 15, 16, 17 and 18, of which the latter two are adults. The regression equation describing PW-WW relationship (3) a = 2.935, b = 15.55. in wild Atlantic horseshoe crabs (Graham et al., 2009) was then used to calculate wet-weight values for instar stages 1-18 (Table S3.). In the questionnaire, aquaria were asked to report approximate PW which was then used to calculate WW and INST.

$$log_e\ (Wt) = log_e\ (PW) \cdot a + log_e\ (b) \qquad (3)$$

Wt = weight of a horseshoe crab (kg);
PW = prosomal width (mm);
α = slope;
$log_e(b)$ = y-intercept (PROC REG, SAS).

Diet composition and food requirement

Based on field studies on diet composition of wild horseshoe crabs and artificial diets of *L. polyphemus* in culture, common food sources were organized into eight categories: Bivalvia, gastropods, Annelida, Arthropoda, plant-based, fish, squid and commercial fish food. Most literature studies and aquaria reported food type, however quantities and proportions were often lacking. When literature reported food quantity, it was often expressed as % of body weight (BW) or total amount of food per week for all animals. Recent study on energy demand and food digestibility in horseshoe crabs (Tzafrir-Prag, Schreibman, Lupatsch, & Zarnoch, 2010) reported daily energy and protein requirements of 224J and 8.7mg per gram body-weight respectively. To evaluate and compare these data to current feeding protocols in the public aquaria, both qualitatively and quantitatively, aquaria were asked to provide information on the food sources fed to the horseshoe crabs, which were subsequently organized into the eight categories stated earlier, and for each food source the quantity as:

$$food\ quantitiy\ ; \frac{g}{animal * feeding} \qquad (4)$$

Daily energy (kcal) and digestible protein (mg) requirements for each instar stage were calculated by multiplying wet weight with 0.0535373 kcal (224J) and 8,7 mg respectively (Table S3.).

RESULTS

Life history

The Atlantic horseshoe crab spends most of its adult life in waters as deep as 6-20m where they overwinter. They return to the coastal areas to spawn, which is marked by an annual migration of sexually mature horseshoe crabs during the late spring and early summer (J. Cohen & Brockmann, 1983; Shuster & Botton, 1985). Although peak spawning has been found to differ longitudinally presumably due to varying water temperatures, migration often coincides with high tides and new full moons (J. Cohen & Brockmann, 1983; Rudloe, 1979). Spawning is characterized by mating pairs arriving on the beaches, with males attached to the females. These mating pairs are often followed by clutches of single males – so called 'satellites' (J. Cohen & Brockmann, 1983). Female horseshoe crabs dig nests in the sand near the intertidal zone and deposit clutches of green, oval eggs of ±2mm diameter at a depth of 10-20 cm below the sediment surface while the males release their free-swimming sperm to fertilize the eggs (J. Cohen & Brockmann, 1983). Trilobite larvae, also called '1st instar' hatch about 2-4 weeks after fertilization and settle on the sediment of shallow lagoons and mudflats where they moult into the second juvenile instar stage (Botton & Loveland, 2003; Botton, Tankersley, & Loveland, 2010; Rudloe, 1981). After this moult the juveniles take about 9-12 years to reach sexual maturity, following stepwise growth through 16-17 instar stages (Shuster & Botton, 1985). Females take approximately a year longer to mature, moulting at least once more than males and are therefore significantly larger (factor 1.2 x) than males (Shuster, 1955, 1982).

Culture Review and Analysis

Few studies have been conducted on *L. polyphemus* husbandry in relation to optimal growth and survival, therefore information on husbandry conditions is scarce. When studies reported water conditions, temperature and salinity were often provided, while other parameters including alkalinity and ammonia-, dissolved oxygen-, and nitrite concentrations were missing. Precise age classes of the horseshoe crabs in the studies were rarely mentioned, or categorized as 'juvenile' and 'adult'. Data on larger juveniles (instar 10-17) could not be found. Little information on living conditions of wild horseshoe crabs is available. Only one study reported temperature and salinity for wild adult horseshoe crabs, which represented the most extreme values at which animals were found in the field. No information was found on living conditions of wild juvenile horseshoe crabs.

Enclosure and maintenance

Recirculating aquaculture systems and flow-through tanks were the most commonly used enclosure types in both laboratory studies and by the public aquaria. In public aquaria, multiple aquaria (with different species) were often interconnected and water was recirculated between the holding tanks. Additional species besides *L. polyphemus* were present in all enclosures except two. public aquaria, dimensions of the enclosures ranged in size and volume from 1.8-303m^2 and 650-70,000L respectively. Mean animal density was generally high, but no significant correlation was found between animal density and mean annual survival percentage (Pearson correlation coefficient: $r = 0.395$, $P = 0.292$, $n = 8$). Water conditions were checked daily to weekly to maintain appropriate water conditions. Filtration flow (L/h) ranged between 25-111% of the total volume in the enclosures. Trickle filters, sand filters and protein skimmers were the three most commonly used filtration systems both in literature and by the aquaria, often supplemented with UV or ozonation to kill any harmful pathogens in the water. Substrate was present in all aquaria. Sand (Ca-CO3) n=4 and coral sand n=3 were the most common types. Clay was used by one aquarium on the dry area as a digging substrate for two other species in the aquarium: *Uca pugnax* and *Uca tangeri*.

Diet composition

Diet composition of wild horseshoe crabs has been widely studied and was found to change when the animals grow larger (Carmichael et al., 2009). Early instar horseshoe crabs (2-7) almost exclusively rely on a diet of detritus and particulate organic matter (POM) suspended on the seafloor. As the juveniles grow (instar 8-11), they will switch to more animal-based food sources, with a diet consisting of POM, small polychaetes and crustaceans (Carmichael et al., 2009; Gaines, Carmichael, Grady, & Valiela, 2002). Larger juveniles consume the same small prey: polychaetes and small crustaceans like amphipods and isopods (Mark L Botton & Ropes, 1989; Gaines et al., 2002). Adults are important predators of bivalve molluscs (*Mya spp., Gemma spp.*), marine gastropods and marine worms (*Nereis* spp., *Cerabratulus* spp., and *Pectinaria* spp.), which comprise almost 80% of their diet (M. L. Botton, 1984; Shuster, 1950; Tanacredi, Botton, & Smith, 2009; Tzafrir-Prag et al., 2010). The diet of captive horseshoe crabs in literature commonly included fish, squid, small crustaceans, bivalves and brine shrimp, periodically supplemented with high-protein commercial fish pellets (35-40%) (Smith & Berkson, 2005; Tanacredi et al., 2009). Early instar juveniles (instar 2-12) also readily feed on a mix of small invertebrates like brine shrimp nauplii (ca. 450μm), earthworms, rotifers, sea urchin eggs and microalgae, (Brown & Clapper, 1981; Carmichael & Brush, 2012; Smith & Berkson, 2005; Tanacredi et al., 2009). Horseshoe crabs in the public aquaria were fed similar diets of bivalves, arthropods and fish as the primary food sources (Figure 1.). Only one aquarium reported plant-based food. Another aquarium added vitamins to the food twice a week. Two aquaria supplemented the diet with commercial food pellets and one aquarium reported to exclusively feed the horseshoe crabs 'Vitalis Pellets' (50.1% protein, 20.8% moisture, 17.7% inorganic matter, 10.3% fat content, 1.7%. crude fibre). The animals in this aquarium were fed 1g each per feeding, thrice a week and the animals ranged in WW between 147-621g (instar 15-17). Daily energy and protein demands were calculated to range between 7.9-33kcal and 1.3-5.4g respectively. Although current feeding quantities for this aquarium were far below calculated requirements, survival was highest; *MS* = 95%, compared to the other aquaria. Few aquaria reported feeding quantities and in three aquaria tanks were fed as a whole. Due to insufficient data, no correlation analysis between food quantities and survival could be performed.

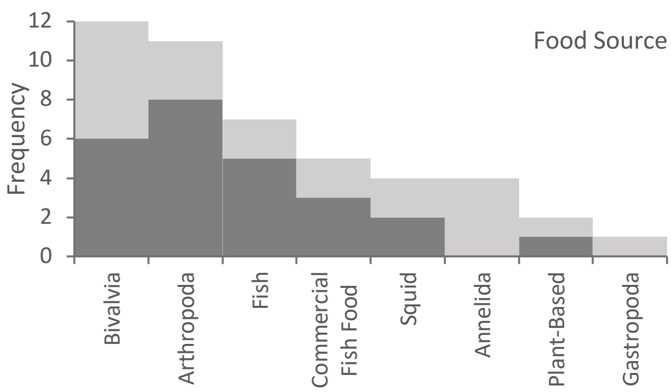


Figure 1. Frequency of food types fed to juvenile and adult horseshoe crabs according to literature studies (light bars; n = 10) and based on aquaria questionniares (dark bars; n = 8 from questionnaire). Columns are stacked, e.g. Bivalvia: *fAq* = 6; *fLi* = 6. (*) indicated food types associated with highest Mean Annual Survival Percentage of horseshoe crabs in captivity. Bivalves included mussels, cockles and chopped clam meat; Arthropods included small crustaceans like mysis, brine shrimp (Artemia spp.), prawn and krill; Fish included European Smelt, Hake, Blue fish, Herring, Cod and Flatfish.

Water conditions in culture

Water temperature and salinity

Temperature and salinity were determined to be the primary factors involved in successful horseshoe crab culture (Carmichael & Brush, 2012; Nolan & Smith, 2009). In literature, maximum growth and survival of juvenile horseshoe crabs was associated with 26°C, while optimal temperatures for culture of (sub)adults was found to be between 15-21°C (Carmichael & Brush, 2012; Nolan & Smith, 2009). Temperatures amongst the aquaria ranged between 19-28°C for juvenile horseshoe crabs and optimal survival; *MS* = 95% was associated with temperatures between 19-21°C (Figure 2.). However, most aquaria maintained temperatures between 23-26°C for juveniles, only one aquarium reported temperatures as high as 28°C. Minimum temperatures at which adult horseshoe crabs were kept in the aquaria were lower, one aquarium reported temperatures as low as 14-18°C; *MS* = 100% for prolonged periods but also kept adult animals at temperatures ranging 22-23°C; *MS* = 92%. Optimal temperature ranges for both juvenile and adult horseshoe crabs in aquaria approximated the optimal temperature ranges suggested in the literature (Carmichael & Brush, 2012; Nolan & Smith, 2009).

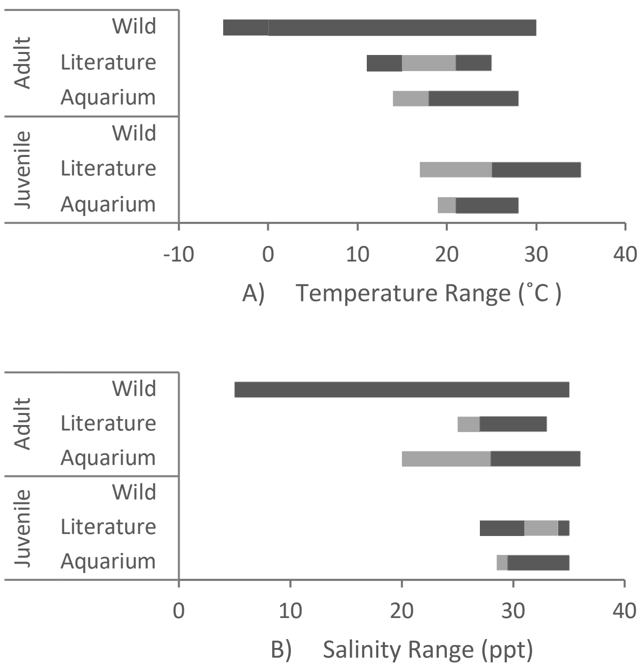


Figure 2. A) Temperature and b) salinity ranges reported in literature and by public aquaria, compared to maximum and minimum values at which *Limulus polyphemus* has been found in the wild. Bars indicated minimum and maximum values reported amongst all sources. Light bars indicated optimal ranges suggested in literature or associated with highest survival amongst the public aquaria, while dark areas represent values that fell outside the optimal range. No bar was drawn when data was absent.

Salinity ranges at which juvenile horseshoe crabs were cultured in literature and by the aquaria were almost identical, however optimal salinity ranges differed strongly (Figure 3.). Lower values, ±29ppt were reported for aquaria in contrast with 31-34ppt suggested in literature (Carmichael et al., 2009). Most aquaria kept salinity levels between 29-34ppt for juveniles. Optimal salinity ranges for adult horseshoe crabs in literature were lower, between 25-27ppt. Only one aquarium reported salinity ranges for adults, which ranged between 20-28ppt and 32,5-36ppt, of which the former yielded highest survival percentage *MS* = 100% in contrast to the 95% survival in the higher salinity range.

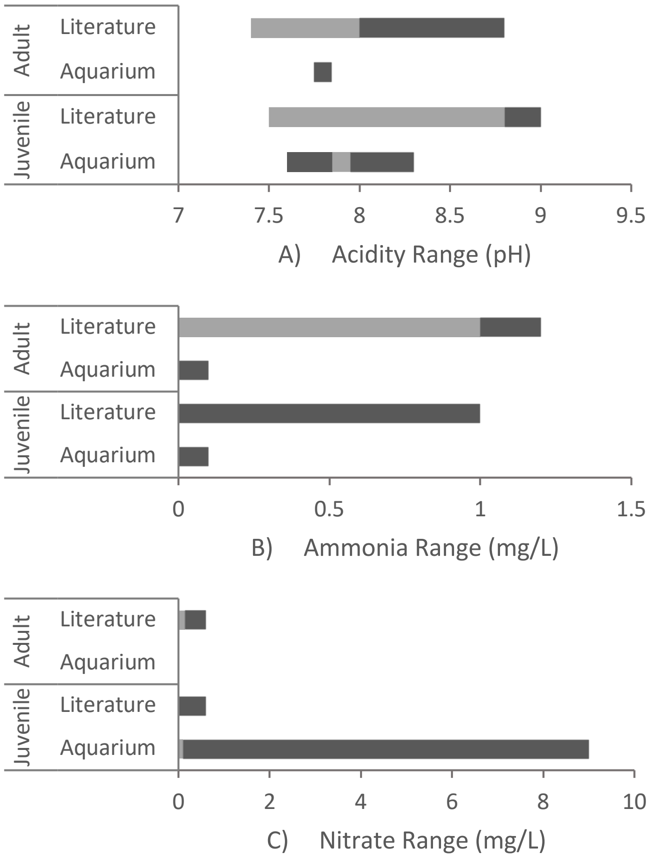
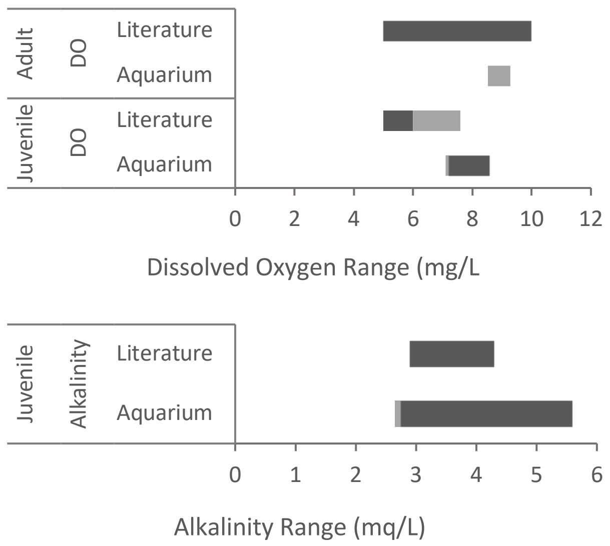


Figure 3. Reported water quality parameters a) acidity (pH), b) ammonia (mg/L) and c) nitrate (mg/L) in literature culture and by public aquaria, no data was found on these variables for horseshoe crabs in the wild. Bars indicated minimum and maximum values reported amongst all sources. Light bars indicated optimal ranges suggested in literature or associated with highest survival amongst the public aquaria, while dark areas represent values that fell outside the optimal range.

Acidity, ammonia, nitrate and dissolved oxygen

Acidity, ammonia and dissolved oxygen (DO) were determined to be important factors involved in the physiology and homeostasis in *Limulus polyphemus* (Hans et al., 2018). In literature, maximum and minimum acidity values for juvenile and adult horseshoe crabs were very similar and ranged between 7.5-9.0pH and 7.4-8.8pH respectively (Figure 3a). Reported acidity ranges amongst aquaria were considerably smaller; 7.6-8.3pH for juveniles and ±7.8pH for adults.

DO levels reported in literature for laboratory cultures were generally lower than reported by the aquaria and lower for juveniles than for adult animals (Figure 4a). Highest survival was associated with DO levels around 7.1mg/L for juveniles and between. Ammonia concentrations were significantly lower (<0.1) amongst the aquaria than suggested as optimal in literature (Figure 3b) (Schreibman & Zarnoch, 2009). Nitrate levels; <0.6mg/L for juveniles and 1-1.2mg/L for adults were suggested in literature contrasting the high values 0-9mg/L reported amongst aquaria (Figure 3c). Highest survival *MS* = 95% was observed when ammonia levels were kept at ±0 mg/L. Three aquaria reported extremely high nitrate values of 25, 50 and 150mg/L, while survival (*MS*) was 78%, 41% and 92% respectively. The curator of the aquarium that reported nitrate concentrations of 150mg/L said that denitrification filters are under construction to lower these values to 50mg/L. Alkalinity ranged between 2.7-5.6mEq/L in aquaria (Figure 4b). Only one source reported alkalinity for juveniles; 2.9-4.3mEq/L, no studies reported alkalinity for adult horseshoe crabs.



Graph 4. A) Dissolved Oxygen (mg/L) and b) alkalinity (mEq/L) ranges reported in literature and by public aquaria, no data was found on these variables for horseshoe crabs in the wild. Bars indicated minimum and maximum values reported amongst all sources. Light bars indicated optimal ranges suggested in literature or associated with highest survival amongst the public aquaria, while dark areas represent values that fell outside the optimal range.

Animal growth and health

Health implication

Out of the eight aquaria, only two reported heath implications with the horseshoe crabs in culture. One aquarium mentioned that four horseshoe crabs ended up stuck between the roots of mangrove trees (above the surface) in the enclosure, which eventually lead to death. White flatworms were encountered on the gills of the animals in the other aquarium. Transferring the infected animals to a freshwater bath for 12 minutes (same temperature as the enclosure) or gently brushing the parasites off proved successful in treating the animals.

Moulting

Moulting of horseshoe crabs was observed in five of the eight aquaria. One aquarium observed moulting throughout the year, one did not specify and horseshoe crabs in the other three aquaria were only found to moult either during February, December or June. The aquarium that observed moulting in June reported that all horseshoe crabs moulted during that month. Implications during moulting were observed in four out of the five aquaria that encountered moulting. Incomplete moulting or inability to exit the old skin resulting in death were the most frequent problems. One aquarium observed interspecific interaction after moulting, another reported loss of appetite (±1 month) in a female horseshoe crab preceding ecdysis, which they treated by moving the individual to a separate tank and elevating temperatures over a span of 5 days. One aquarium found specific behavioural patterns in their horseshoe crabs characterized by prolonged periods of inactivity, alternated with periods of either nocturnal or diurnal activity. This phenomenon was reported not to be correlated with temperature. Overall, aquaria attributed most of the mortalities due to moulting implications or indirect effects of moulting on the health of the animals.

DISCUSSION

Captive rearing of *L. polyphemus* has been widely suggested as a measure to reduce the necessity for wild-captured horseshoe crabs and relieve the pressure on current populations. Although various studies on physiology and diet of wild and captive horseshoe crabs have been conducted, none reported complete or detailed information on the full range of husbandry conditions, as culture was not the main focus. Temperature, salinity and food sources were the most common variables reported along with growth (PW) or survival percentage as a measure of culture success. However, data on important water parameters like DO, acidity and toxic substances (ammonia and nitrates) were rarely provided. Inconsistency in the use of different metrics for horseshoe crab age; PW, WW or INST complicated any comparison between studies or between literature and aquaria. This underscored the necessity for a conversion table with both PW and WW expressed per instar stage. No such table existed before now and few studies have been performed on the relationship between these parameters. We combined reported and predicted PW values per instar stage (Sekiguchi, Seshimo, & Sugita, 2007) with a PW-WW equation developed by Graham et al. (2009). Although the equation was initially based on horseshoe crabs with a PW between 118-329 mm, calculated weight fitted reasonably when compared to studies that reported both PW and WW for smaller individuals (Schreibman & Zarnoch, 2009; Tzafrir-Prag et al., 2010). However, Graham et al. (2009) reported alpha and beta values for female (all stages) and male (all stages) separately, but aquaria did not report the gender of their animals. Therefore the alpha and beta values for the sexes were averaged. A study specifically on the change in PW and WW per instar stage would enable more precise comparisons and greatly benefit the scientific community. With regard to sample sizes, data from the aquaria, particularly regarding food quantities, animal density and survival were insufficient to run any statistical analysis. Larger sample sizes (including data from more aquaria worldwide) and information on other parameters like light-dark regimes could provide more detailed and precise conclusions. Although we provided a simple conversion table for PW, WW and INST, there is need for a study that primarily focusses on precisely linking horseshoe crab age to these parameters. Absence of data on living conditions of wild juvenile horseshoe crabs made it difficult to link data on juveniles in the aquaria to their physiology in the wild. Our results support the inference that optimal husbandry conditions differ between adult and juvenile horseshoe crabs, however, it is yet unclear whether this difference only holds between early instars and adults or gradually decreases when juveniles approach adulthood. This could be of major consideration in defining optimal holding conditions and should therefore be further studied. Nevertheless, it is clear that more data and detailed observations are in demand. Inclusion of all important aspects of horseshoe crab husbandry is imperative to provide sufficient data for comparison and evaluating culture success with rearing conditions in future studies. Reporting water conditions, particularly temperature, salinity, acidity, alkalinity, dissolved oxygen, ammonia and nitrate should be standardized and the use of common metrics for culture success, including PW and survival percentage would greatly benefit the scientific community.

Importance of enclosure maintenance and water conditions

Limulus polyphemus is known to be tolerant of a wide range of environmental conditions (Smith & Berkson, 2005). Adult animals have been found in waters with temperatures from -5°C to 35°C and salinities of 5 ppt to 35 ppt (Carmichael & Brush, 2012; Nolan & Smith, 2009), minimum and maximum values reported in literature were less extreme. Adult horseshoe crabs generally live in deep waters while juveniles primarily inhabit shallow coastal embayments where evaporation and freshwater input result in highly fluctuating conditions. Adaptation to these specific conditions could explain temperature and salinity tolerances for juveniles to be in the higher ranges (Ehlinger & Tankersley, 2009). Conform to literature, public aquaria generally kept juvenile horseshoe crabs at higher temperatures and salinity than adults. However, while most aquaria maintained similar temperatures and salinity relative to each other, survival percentages differed significantly; *MS* = 41%-92%, suggesting that neither temperature nor salinity were the primary factors responsible. Although one aquarium reported temperatures as high as 28°C survival was still high; MS = 78%. However, research has shown that hemocyanin and amoebocytes in the blood decreased with increasing temperatures, eventually leading to inhibited immune responses (Coates, Bradford, Krome, & Nairn, 2012). Higher temperatures also provide more viable habitats for harmful pathogens and e.g. aerobic bacteria, which could result in higher chances of infection and decreased DO concentrations. These findings might be important considerations in defining optimal temperature conditions for husbandry.

Sediment was determined as one of the key variables for horseshoe crabs in the natural habitat (Sekiguchi & Shuster, 2009), serving as a natural rubbing surface to prevent epibiotic growth and providing shelter during moulting (Smith & Berkson, 2005). In culture, substrate in holding tanks was discommended for difficulty in cleaning and potentially harbouring harmful pathogens (Smith & Berkson, 2005). Hypoxia and subsequent death of horseshoe crabs in one of the aquaria was associated with excessive accumulation of microorganisms in the thick layer of substrate. This emphasizes the necessity of proper filtration and aeration of the water to sustain appropriate oxygen levels for respiration. Introducing natural grazers like sea cucumbers, sea urchins and marine snails into the holding tanks of *L. polyphemus* could reduce accumulation of microorganisms. Such eco-minded measures would simultaneously provide additional food sources for the animals and induce natural predatory behaviour. In aquaculture acidity, ammonia and nitrate levels are regarded as key factors. It was however, not possible to define optimal culture conditions for these variables because of absence of sufficient data in literature or reported from the aquaria. Accumulation of faeces, unconsumed food and dead animals can increase turbidity and provide growth medium for harmful bacteria (Beatriz, 2005). Recent studies found that when horseshoe crabs were kept in a fouled pond containing waste material and decaying fish parts, the animals secreted a thick dermal exudate. The exudate likely acted as a barrier to prevent epibiotic growth and pathogenic infections (Braverman, Leibovitz, & Lewbart, 2012; Harrington & Armstrong, 1999). This stresses the importance of sufficient filtration and maintaining low levels of ammonia and nitrate in the water.

Horseshoe crab growth and health

Moulting implications and symptoms described by the aquaria were generally consistent with, but somewhat more frequent than reported for cultured horseshoe crabs in literature. Various external cues have been found to complicate moulting, including temperature extremes, overcrowding and short photoperiods (Aiken, 1969; Bliss & Rouillion Boyer, 1964; Hartnoll, 2001; Mohamedeen & Hartnoll, 1989). Moulting readily occurs in the warm summer months and it was shown that increasing light duration resulted in higher percentage of horseshoe crabs that started moulting (Schreibman & Zarnoch, 2009). However, mortality also increased with light duration, with 10% mortality at 18-24 hours interval versus 0% mortality in the 0-12 hour range. Therefore, a 12/12 light dark-cycle was implemented for optimal moulting conditions. Both temperature and photoperiods are inevitably linked to seasonal changes in the wild and although data on light-dark cycles in aquaria is absent, inconsistency in the months at which moulting was observed suggests that photoperiodic patterns could be involved. Moulting is generally preceded by a quiescent period of a few days, marked by inactivity of the horseshoe crab during which it uses energy storages to produce new shell material (Barthel, 1974). Such periods of inactivity were reported by two aquaria and as ecdysis is highly energy demanding, insufficient feeding might be an underlying cause for incomplete moulting. Further research may provide more detailed information on the relationships between these variables and could lead to better adjusted feeding regimes that correspond with the alternating periods of (in)activity displayed by ready-to-moult individuals.

Moulting itself is a hazardous and energy-depleting process (Smith & Berkson, 2005; Tzafrir-Prag et al., 2010). Particularly in captivity, limited space or overcrowding of horseshoe crabs may result in traumatic injuries from fellow crabs (Smith & Berkson, 2005). Wild horseshoe crabs are known to be cannibalistic and lack of hiding places or burrowing substrate could promote this behaviour, resulting in missing appendages, puncture wounds, fracture or mortality (Sekiguchi & Shuster, 2009; Smith & Berkson, 2005). Although no significant correlation was found between animal density and mean annual survival percentage, interspecific interaction was reported by one aquarium. This could have been the result of high animal density (1.84 animals/m^2) combined with a lack of hiding places and might therefore also add to high mortality after ecdysis. Additionally, insufficient water flow and filtration may impede moulting and increase the chance of infection (Thompson, Ahmeid, Picket, & Neil, 2011). In both spiders and crustaceans the old cuticle and moulting fluid covering the book lungs during ecdysis significantly compromised functionality of the respiratory organ (Krishnakumaran & Schneiderman, 1970). DO concentrations in the aquaria were generally higher than suggested in literature and therefore hypoxia-induced death in the water seems unlikely. However, moulting horseshoe crabs are commonly buried in the substrate where water flow is limited and suffocation due to local hypoxia is plausible. Comparisons on animal health and diseases could not be made due to insufficient data, only one aquarium reported health issues. Despite regular cleaning and filters to kill harmful pathogens, green algae-, and bacterial infections (*Pseudomonas sp.*) were commonly encountered in culture (Carmichael et al., 2009; Nolan & Smith, 2009). Although such pathogens rarely cause any mass mortalities in the wild (Sommerset, Krossøy, Biering, & Frost, 2005), horseshoe crabs in culture are prone to infection (Smith & Berkson, 2005; Thompson et al., 2011). Pathogens are continuously introduced through feed and high densities of animals in the enclosure increase the risk of infection (Thompson et al., 2011). Therefore, monitoring health by regularly checking the animals for parasites and discolorations of the exoskeleton is recommended.

Importance of diet composition and enclosure maintenance

Besides temperature and salinity ranges, diet composition was most often reported in literature and determined as a key factor in captive rearing (Carmichael & Brush, 2012; Schreibman & Zarnoch, 2009; Smith & Berkson, 2005). The most important aspect of horseshoe crab diet appeared to be change in food preference. Juveniles primarily feed on particulate organic matter, subsequently moving up the food-web as they grow towards a more animal-based diet in adults (Carmichael et al., 2009; Gaines et al., 2002). These findings indicate that in addition to diet composition, prey size is an important aspect in horseshoe crab feeding regimes. Similar diets for horseshoe crabs were reported in literature and by the aquaria, but more data is needed to link feeding quantities to survival. However, daily energy and protein demands have been calculated per INST and should be used to ensure sufficient food is provided to the horseshoe crabs. Simultaneously, low levels of nitrate should be maintained, e.g. by minimizing excess food. Supplementing horseshoe crab diet with commercially prepared fish food showed successful (Carmichael et al., 2009; Schreibman & Zarnoch, 2009). Artificial food sources are rich in protein, vitamins and minerals that might be lacking in current feeding regimes in horseshoe crab culture. Horseshoe crabs maintained for prolonged periods (>6 months) can develop hypoproteinaemia as a result of insufficient food quantities and inadequate diet formulations (Smith & Berkson, 2005). Studies also found high-protein, animal-based diets to be poorly assimilated and suggested that additional plant-based food sources are required for optimal growth and survival (Carmichael et al., 2009; Schreibman & Zarnoch, 2009). Vascular plant material was abundant in the digestive systems of wild horseshoe crabs and other marine arthropods (blue crabs and Atlantic rock crabs) (Botton, 1984). Many organic molecules, including some amino-acids crucial in chitin production and the antioxidant astaxanthin can only be derived from plant detritus, POM and marina algae (Carmichael et al., 2009). Supplementing horseshoe crab diets with algae increased growth and survival compared to high animal-protein (>70% protein) feeds (Carmichael et al., 2009). Another study showed that algae are a vital dietary component in culture of the lobster species *Homarus americanus* (Botton, 1984; Darnell, 1958). These findings highlight the importance of algae-enriched diets for *L. polyphemus* in culture.

CONCLUSIONS

Presentation of horseshoe crabs by public aquaria is of upmost importance in raising awareness and initiating conservation efforts for the rapidly declining horseshoe crab populations in the wild. This study provides first ever evaluation of current husbandry practises among public aquaria and comparisons with culture conditions suggested in literature. Flow-through tanks with high water circulation, sufficient aeration and multiple filtration systems; trickling- or sand-filters in combination with UV or ozonation should provide a viable habitat for captive horseshoe crabs. Substrate, particularly sand or crushed coral, could benefit animal health by providing burrowing places and rubbing surfaces, but accumulation of pathogens and risk of hypoxia increase with increasing layers of substrate. Water conditions should be kept at; 19-21°C, 29ppt, 7.9pH, Dissolved Oxygen (DO) 7.1mg/L, Alkalinity 2.7mEq/L for juveniles and ±18°C, 20-28ppt, ±7.7pH, DO 8.5-9.3mg/L for adults. Although temperature and salinity were most often reported in literature and by the aquaria, more attention should be given to the other important water parameters, most notably nitrate and ammonia concentrations as these can have detrimental effects on animal health. In addition, more emphasis is demanded on feeding regimes, particularly with regard to supplementing horseshoe crab diet with plant-based food sources and adapting food quantities to their daily energy and protein requirements. Age, size and weight of the animals should be considered in when adapting food quantities. These findings indicate that even though aquaria generally provided viable conditions for horseshoe crab culture, improvement can be made. In conclusion, although more consistent data would contribute to more precise conclusions, the culture conditions provided in this study could already be of great benefit in improving or adapting current culture conditions for horseshoe crabs in laboratories and public aquaria.

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REFERENCES

Aiken, D. E. (1969). Photoperiod, Endocrinology and the Crustacean Molt Cycle. *Science*, *164*(3876), 149 LP-155. https://doi.org/10.1126/science.164.3876.149

Ballesteros, J. A., & Sharma, P. P. (2019). A Critical Appraisal of the Placement of Xiphosura (Chelicerata) with Account of Known Sources of Phylogenetic Error. *Systematic Biology*. https://doi.org/10.1093/sysbio/syz011

Barthel, K. W. (1974). Limulus: a living fossil. Horseshoe crabs aid interpretation of an Upper Jurassic environment (Solnhofen). *Naturwissenschaften*, *61*, 248–433.

Beatriz, T. B. (2005). *Organic matter decomposition in simulated aquaculture ponds*. publisher not identified. Retrieved from https://library.wur.nl/WebQuery/wda/1757025

Berkson, J., & Shuster, C. N. (1999). The Horseshoe Crab: The Battle for a True Multiple-use Resource. *Fisheries*, *24*(11), 6–10. https://doi.org/10.1577/1548-8446(1999)024<0006:THCTBF>2.0.CO;2

Bliss, D. E., & Rouillion Boyer, J. (1964). Environmental regulation of growth in the decapod crustacean Gecarcinus lateralis. *General and Comparative Endocrinology*, *4*, 15–41. https://doi.org/10.1016/0016-6480(64)90034-6

Bolden, J., & Smith, K. (2017). Application of Recombinant Factor C Reagent for the Detection of Bacterial Endotoxins in Pharmaceutical Products. *PDA Journal of Pharmaceutical Science and Technology*, *71*(5), 405–412. https://doi.org/10.5731/pdajpst.2017.007849

Botton, M. L. (1984). Diet and food preferences of the adult horseshoe crab Limulus polyphemus in Delaware Bay, New Jersey, USA. *Marine Biology*, *81*(2), 199–207. https://doi.org/10.1007/BF00393118

Botton, M. L. (1984). Diet and food preferences of the adult horseshoe crab Limuluspolyphemusin Delaware Bay, New Jersey, USA. *Marine Biology*, *81*(2), 199–207. https://doi.org/10.1007/BF00393118

Botton, M. L., & Loveland, R. E. (2003). Abundance and dispersal potential of horseshoe crab (Limulus polyphemus) larvae in the Delaware estuary. *Estuaries*, *26*(6), 1472–1479. https://doi.org/10.1007/BF02803655

Botton, M. L., & Ropes, J. W. (1989). Feeding Ecology of Horseshoe Crabs on the Continental Shelf, New Jersey to North Carolina. *Atlantic*, *45*(3), 637–647.

Botton, M. L., Tankersley, R. A., & Loveland, R. E. (2010). Developmental ecology of the American horseshoe crab Limulus polyphemus. *Current Zoology*, *56*(5), 550–562.

Braverman, H., Leibovitz, L., & Lewbart, G. A. (2012). Green algal infection of American horseshoe crab (Limulus polyphemus) exoskeletal structures. *Journal of Invertebrate Pathology*, *111*(1), 90–93. https://doi.org/10.1016/J.JIP.2012.06.002

Brown, G. ., & Clapper, D. . (1981). Procedures for Maintaining adults, collecting gametes, and culturing embryos and juveniles of the horseshoe crab, Limulus polyphemus. In committee on M. Invertebrates (Ed.), *Laboratory animal management: Marine invertebrates* (pp. 268–290). Washington, D.C.: National Academy Press.

Carmichael, R. H., & Brush, E. (2012). Three decades of horseshoe crab rearing: A review of conditions for captive growth and survival. *Reviews in Aquaculture*, *4*(1), 32–43. https://doi.org/10.1111/j.1753-5131.2012.01059.x

Carmichael, R. H., Gaines, E., Sheller, Z., Tong, A., Clapp, A., & Valiela, I. (2009). Diet Composition of Juvenile Horseshoe Crabs: Implications for Growth and Survival of Natural and Cultured Stocks. In *Biology and Conservation of Horseshoe Crabs* (pp. 521–534).

Clark, K. E., Niles, L. J., & Burger, J. (1993). Abundance and Distribution of Migrant Shorebirds in Delaware Bay. *The Condor*, *95*(3), 694–705. https://doi.org/10.2307/1369612

Coates, C. J., Bradford, E. L., Krome, C. A., & Nairn, J. (2012). Effect of temperature on biochemical and cellular properties of captive Limulus polyphemus. *Aquaculture*, *334–337*, 30–38. https://doi.org/10.1016/j.aquaculture.2011.12.029

Cohen, J., & Brockmann, H. (1983). Breeding activity and mate selection in the horseshoe crab, Limulus polyphemus. *Bulletin of Marine Science*, *33*(2), 274–281.

Darnell, R. M. (1958). Food habits of fishes and larger invertebrates of Lake Ponchartrain, Louisiana, an estuarine community. *Contributions in Marine Science*, *5*, 353–416.

Ding, J., & Ho, B. (2010). Endotoxin Detection - from Limulus Amebocyte Lysate to Recombinant Factor C. In X. Wang & P. J. Quin (Eds.), *Endotoxins: Structure, Function and Recognition* (53rd ed., pp. 187–208). Springer. https://doi.org/10.1007/978-90-481-9078-2

Ding, J. L., & Ho, B. (2001). A new era in pyrogen testing. *Trends in Biotechnology*, *19*(8), 277–281. https://doi.org/10.1016/S0167-7799(01)01694-8

Ding, J. L., Navas, M. A. A., & Ho, B. (1993). Two forms of Factor C from the amoebocytes of Carcinoscorpius rotundicauda: purification and characterisation. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, *1202*(1), 149–156. https://doi.org/10.1016/0167-4838(93)90076-4

Ehlinger, G. S., & Tankersley, R. A. (2009). Ecology of Horseshoe Crabs in Microtidal Lagoons. In *Biology and Conservation of Horseshoe Crabs* (pp. 149–162). Boston, MA: Springer US. https://doi.org/10.1007/978-0-387-89959-6_9

Gaines, E., Carmichael, R. H., Grady, S. P., & Valiela, I. (2002). Stable isotope evidence for changing nutritional sources of juvenile horseshoe crabs. *Biological Bulletin*, *203*, 228–230.

Gillings, S., Atkinson, P. W., Bardsley, S. L., Clark, N. A., Love, S. E., Robinson, R. A., … Weber, R. G. (2007). Shorebird predation of horseshoe crab eggs in Delaware Bay: species contrasts and availability constraints. *Journal of Animal Ecology*, *76*(3), 503–514. https://doi.org/10.1111/j.1365-2656.2007.01229.x

Graham, L. J., Botton, M. L., Hata, D., Loveland, R. E., & Murphy, B. R. (2009). Prosomal-width-to-weight relationships in american Horseshoe crabs (Limulus polyphemus): Examining conversion factors used to estimate landings. *Fishery Bulletin*, *107*(2), 235–243.

Harrington, J. M., & Armstrong, P. B. (1999). A Cuticular Secretion of the Horseshoe Crab, Limulus polyphemus: A Potential Anti-fouling Agent. *The Biological Bulletin*, *197*(2), 274–275. https://doi.org/10.2307/1542647

Hartnoll, R. G. (2001). Growth in Crustacea – twenty years on. *Hydrobiologia*, *449*(1/3), 111–122. https://doi.org/10.1023/A:1017597104367

Krishnakumaran, A., & Schneiderman, H. A. (1970). Control of Molting in Mandibulate and Chelicerate Arthropods by Ecdysones C. *The Biological Bulletin*, *139*(3), 520–538. https://doi.org/10.2307/1540371

Kropach, C. (1979). Observation on the potential of amoebocyte lysate. In E. Cohen, J. Levin, & F. B. Bang (Eds.), *Progress in Clinical and Biological Research* (pp. 103–106). Alan, R. Liss, NY.

Liu, J. S., & Passaglia, C. L. (2009). Using the horseshoe crab, Limulus Polyphemus, in vision research. *Journal of Visualized Experiments : JoVE*, (29). https://doi.org/10.3791/1384

Manion, M., West, R., & Unsworth, R. (2008). *Economic Assessment of Mid-Atlantic Horseshoe Crab and Dependent Fisheries including a Qualitative Discussion of the Potential Effects of Addendum IV*. Retrieved from http://www.asmfc.org/files/Science/hscEconomoicAssmt02_08_08.pdf

Mishra, J. K. (2009). Horseshoe crabs, their eco-biological status along the northeast coast of India and the necessity for ecological conservation. In *Biology and Conservation of Horseshoe Crabs* (pp. 89–96).

Mohamedeen, H., & Hartnoll, R. G. (1989). Larval and postlarval growth of individually reared specimens of the common shore crab Carcinus maenas (L.). *Journal of Experimental Marine Biology and Ecology*, *134*(1), 1–24. https://doi.org/10.1016/0022-0981(90)90053-F

Nolan, M. W., & Smith, S. A. (2009). Clinical Evaluation, Common Diseases, and Veterinary Care of the Horseshoe Crab, Limulus polyphemus. In *Biology and Conservation of Horseshoe Crabs* (pp. 479–499). https://doi.org/10.1007/978-0-387-89959-6

Rudkin, D. M., & Young, G. A. (2009). Horseshoe Crabs – An Ancient Ancestry Revealed. In *Biology and Conservation of Horseshoe Crabs* (pp. 25–44). Boston, MA: Springer US. https://doi.org/10.1007/978-0-387-89959-6_2

Rudkin, D. M., Young, G. A., & Nowlan, G. S. (2008). The oldest horseshoe crab: A new xiphosurid from late ordovician konservat-lagerstätten deposits, Manitoba, Canada. *Palaeontology*, *51*(1), 1–9. https://doi.org/10.1111/j.1475-4983.2007.00746.x

Rudloe, A. (1979). Locomotor and Light Responses of Larvae of the Horseshoe Crab, Limulus polyphemus (L.). *The Biological Bulletin*, *157*(3), 494–505. https://doi.org/10.2307/1541033

Rudloe, A. (1981). Aspects of the Biology of Juvenile Horseshoe Crabs, Limulus Polyphemus. *Bulletin of Marine Science*, *31*(1), 125–133. Retrieved from https://www.ingentaconnect.com/content/umrsmas/bullmar/1981/00000031/00000001/art00009

Schreibman, M. P., & Zarnoch, C. B. (2009). Aquaculture Methods and Early Growth of Juvenile Horseshoe Crabs (Limulus polyphemus). In *Biology and Conservation of Horseshoe Crabs* (pp. 501–511).

Sekiguchi, K., Seshimo, H., & Sugita, H. (2007). Post-Embryonic Development of the Horseshoe Crab. *The Biological Bulletin*, *174*(3), 337–345. https://doi.org/10.2307/1541959

Sekiguchi, K., & Shuster, C. N. (2009). Limits on the Global Distribution of Horseshoe Crabs (Limulacea): Lessons Learned from Two Lifetimes of Observations: Asia and America. In *Biology and Conservation of Horseshoe Crabs* (pp. 5–24). Boston, MA: Springer US. https://doi.org/10.1007/978-0-387-89959-6_1

Shuster, C. N. (1950). Observations on the Natural History of the American Horseshoe Crab, Limulus polyphemus. *Woods Holes Oceanography Institute Contribution*, *564*, 18–23.

Shuster, C. N. (1955). *On Morphometric and Serologic Relationships Within the Limulidae, with Particular Reference to Limulus polyphemus (L.)*. New York University.

Shuster, C. N. (1982). A pictorial review of the natural history and ecology of the horseshoe crab, Limulus polyphemus, with reference to other Limulidae. In B. J, B. C, & T. S (Eds.), *Physiology and Biology of Horseshoe Crabs* (pp. 1–52). Alan R. Liss Inc., New York.

Shuster, C. N., & Botton, M. L. (1985). A Contribution to the Population Biology of Horseshoe Crabs, Limulus polyphemus (L.), in Delaware Bay. *Estuaries*, *8*(4), 363. https://doi.org/10.2307/1351874

Smith, S. A., & Berkson, J. (2005). Laboratory culture and maintenance of the horseshoe crab (Limulus polyphemus). *Lab Animal*, *34*(7), 27. https://doi.org/10.1038/laban0705-27

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*(1), 89–101. https://doi.org/10.1586/14760584.4.1.89

Tanacredi, J. T., Botton, M. L., & Smith, D. R. (David R. (2009). *Biology and conservation of horseshoe crabs* (Vol. 84). Springer. Retrieved from http://ir.obihiro.ac.jp/dspace/handle/10322/3933

Thompson, J., Ahmeid, A. ., Picket, C., & Neil, D. (2011). *Identification of cost-effective measures to improve holding conditions and husbandry practices for the horseshoe crab Limulus polyphemus A Scientific Report by Ms Asma A . Ben Ahmeid*.

Thompson, M. (1999). *Assessments of the Population Biology and Critical Habitat for the Horseshoe Crab, Limulus polyphemus, in South Carolina*. Medical University of South Carolina.

Tzafrir-Prag, T., Schreibman, M. P., Lupatsch, I., & Zarnoch, C. B. (2010). Preliminary studies of energy and protein requirements of atlantic horseshoe crabs, Limulus polyphemus, grown in captivity. *Journal of the World Aquaculture Society*, *41*(6), 874–883. https://doi.org/10.1111/j.1749-7345.2010.00430.x

Walls, E. A., Berkson, J., & Smith, S. A. (2002). *The Horseshoe Crab, Limulus polyphemus: 200 Million Years of Existence, 100 Years of Study*. *Reviews in Fisheries Science* (Vol. 10). Retrieved from http://nsmn1.uh.edu/dgraur/popbio/horseshoe crab, Limulus polyphemus 200 million years of existence, 100 years of study.pdf

Williams, K. L. (2018). Specificity in the recombinant factor C test for endotoxin. *Pharmaceutical Review*, *2*.

Zaldívar-Rae, J., Sapién-Silva, R. E., Rosales-Raya, M., & Brockmann, H. J. (2009). American Horseshoe Crabs, Limulus polyphemus, in Mexico: Open Possibilities. In *Biology and Conservation of Horseshoe Crabs* (pp. 97–113). Boston, MA: Springer US. https://doi.org/10.1007/978-0-387-89959-6_6