Perspectives Article

Raising public awareness: Synthetic biology provides an opportunity to contribute to the conservation of horseshoe crabs*

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The Limulus Amebocyte Lysate (LAL) assay is widely used for purity and sterility testing in the pharmaceutical industry. The assay detects the presence of endotoxins and ensures product safety and quality compliance. A major component of the assay is extracted by bleeding horseshoe crabs. Several synthetic LAL alternatives have been developed utilizing recombinant Factor C (rFC). These animal-free alternatives are available for purchase and commercial use. Despite proven effectiveness, there has been some resistance to adopting rFC in the medical industry. In the following article, the challenges and roadblocks that prevent the reduction of LAL usage and its replacement with synthetic alternatives are explored. For this purpose, stakeholders from a pharmaceutical company, the biotechnology sector as well as a conservation advocacy group were interviewed. Their responses were analyzed and synthesized to identify major issues and propose recommendations. The findings suggest that harmonizing regulatory guidelines across the globe, ensuring that all commercially available synthetic options are of equivalent performance to LAL and affordable, as well as maintaining awareness of the importance of biodiversity to protect horseshoe crabs and achieve a sustainable approach is required to allow the crabs' population to thrive while assuring the safety of medical products continues to be well-controlled and tested.

Keywords: Horseshoe crabs, LAL assay, rFC, conservation, advocacy, animal-free testing, pharmaceutical industry, regulatory health authorities

Horseshoe crabs are marine arthropods, often referred to as a living fossils, with 450 million years of proven evolutionary success. They have been essential for ensuring safety in the production of medicines and medical devices for over half a century (Luck, Luck, Koltstova, Bhattacharjee, and Chauhan, 2023). Horseshoe crabs rely on a copper-based molecule called hemocyanin to carry oxygen, which gives their blood a distinctive blue color. Horseshoe crabs had no need to change over the hundred millions of years of their existence due to their adaptable immune system (Kin and Blazejowski, 2014; Walls et al., 2002). Instead of using specialized immune cells like white blood cells to fight off pathogens, the crabs' blood cells, referred to as amebocytes, produce a substance called coagulogen. Coagulogen detects foreign

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entities and instantly coagulates, initiating an extremely powerful defense against a wide variety of pathogens (Medzhitov & Janeway, 2000; Walls et al., 2002). The coagulogen can detect bacterial components even at concentrations of one per trillion and encapsulates the contaminants in a gel clot for immediate removal from the body (Iwanaga, 2007; Walls et al., 2002).

The amebocytes from horseshoe crabs' blood is a critical ingredient in the Limulus Amebocyte Lysate (LAL) assay. The LAL assays tests for the presence of endotoxins, which are dangerous components of the cell wall of gram-negative bacteria. When introduced intravenously into the bloodstream or spinal fluid, endotoxins cause life-threatening fever or toxic shock. Before medicines and medical devices are made available for purchase, all are tested for bacterial contamination. Such testing is ubiquitous and applied to assess vaccines, syringes, IV drugs, IV lines, hip and knee replacements, and other implants, all drugs injected into human muscle, blood, bone, and skin (Cramer, 2015; Iwanaga, 2007; Walls et al., 2002). If the lines, needles and intravenous or subcutaneous medicines are not sterile and free from toxins, many people may die or develop fatal antibiotic-resistant infections. The LAL assay prevents such danger, and, in effect, the horseshoe crabs save human lives on a daily basis.

Horseshoes crab's significance in pharmaceutical manufacturing

When Bang and Levin (1964; 1968) studied horseshoe crab blood and discovered amebocytes, which coagulate upon contact with bacterial endotoxins, the significance of their research was quickly recognized, and the LAL assay was developed. Before Bang and Levin's discovery, a different animal-derived assay, the rabbit pyrogen test, was used as the primary regulatory-approved method for endotoxin testing (Weary & Wallin, 1973). Comparatively, the LAL was shown to be more robust and sensitive, quicker to complete, required less samples, and was much less expensive. As such, it was quickly adapted, becoming the preferred industry standard. In the 1970s, the LAL test was approved by the Food and Drug Administration (FDA) and included as part of US Pharmacopeia (Tamura et al., 2021; Franco, et al., 2018). Since then, the LAL assay became the official method for detection of endotoxins and a critical release test for the production and sale of pharmaceutical products.

Potential contaminants may be found during various steps in the manufacturing of drugs and medical devices, the packaging of its components, or in the raw materials or equipment used in the pharmaceutical production facilities. Often, endotoxin contamination is introduced through water for injection (WFI) during the drug manufacturing process (Burgenson, 2022). Pharmaceutical companies make a great effort to sterilize and test their products, equipment, and the production process for purity and sterility. And so, as part of compliance, safety, and quality assurance, horseshoe crab-derived LAL assay is widely used to prove that all medical products are safe and free from toxins (U.S. Pharmacopeia-National Formulary, 2017).

As the need for the LAL assay rose, so did the demand for horseshoe crab blood. The LAL clotting agent is valued at $15,000 per quart (Madrigal, 2014). From 2013 to 2017, approximately 525,000 horseshoe crabs were bled annually to produce LAL assay and in 2022, blood from approximately 1 million crabs was harvested for biomedical use (ASMFC, 2011; ASMFC 2023; Smith et al., 2020). The bleeding process involves folding the crabs in half, securing their telson, and sticking a needle in their heart to passively collect approximately 50 mL of blood from a small adult and as much as 400 mL from a large female (Armstrong and Conrad, 2008). See Figure 1 for an example of a bleeding facility and process. Blood collection takes less than 10 minutes to complete, and the crabs are supposedly promptly returned to the sea. Bleeding-induced mortality is estimated to be 15% on average (Smith et al., 2019; Smith et al. 2020). However, there are contradictory studies, indicating a broad range of mortality rates from as low as 4% and as high as 30% (Smith et al., 2019; Smith et al. 2020).

There are few restrictions on biomedical exploitation of horseshoe crabs (Center for

Biological Diversity, 2024). The crabs can be bled any time of the year and even during the spawning season. The females are preferred, as they are larger with a higher blood capacity. Several researchers speculate that blood loss may not be the leading cause of death. Many females lose their ability to spawn after the capture, bleeding, and release procedures (Anderson et al., 2022). The capture, handling and transportation to and from the facility is traumatic (Hurton and Berkson, 2005; Hurton and Berkson 2006; Walls and Berkson, 2003). Horseshoe crabs are collected from the bottom of the sea with dragging trawls and stacked on a boat before being transferred to plastic containers for extended periods. Crabs are often crushed and their shells and telsons are damaged or cracked, which impedes their survival after harvest. Furthermore, the physiological stress of being removed from the sea may be lethal. When taken out from the water, the crabs are unable to effectively remove CO2 from their blood and regulate PCO2, which results in abnormal hemolymph pH levels and causes hypoxia and metabolic acidosis (Allender et al., 2010; Arnold et al., 2021). Clearly, the horseshoe crabs are exposed to various stressors throughout the biomedical harvest and bleeding processes, starting from capture, transportation, bleeding, and release, which together negatively affects their mortality rates.

The manufacturers of the LAL assay believe that they are not hurting the crabs. John Dubczak, director of operations at Charles River Laboratories, a facility in Charleston, S.C. that makes LAL reagents and assays, claims that his company uses delicate procedures so as not to injure the crabs during harvest and bleeding processes and removes only 30% of blood per specimen (Eisner, 2023). The mortality rate at Charles River Laboratories is estimated at only 4% (Chesler, 2016). Due to contradictory information, it is difficult to ascertain the exact impact of the biomedical industry on horseshoe crab populations. It may be significant, but so is the impact of LAL assay on assuring safety and preventing health severe complications and potentially death in patients. Human life is of great value, and proper safety measures must be adhered to. Many stakeholders in the pharmaceutical and biomedical industry are actively engaging and collaborating with the conservation groups to strike a balance between protecting horseshoe crabs and continuing to provide safe and efficacious medications.

Threats to horseshoes crabs

The biomedical exploitation is not the only threat to horseshoe crabs. The crabs are being over-harvested for use in the fishing industry at alarming numbers. They are mainly used as bait for commercial welk and eel fisheries and exported for sale in Asian markets. In 1998, horseshoe crab harvest was estimated at 2.5 million (more than twice as much as that for the biomedical industry) with a mortality rate of 100%. Since then, due to rapid decline in spawning numbers, some fishing harvest quotas have been enforced. Unfortunately, these quotas did not help with the population recovery, and it is estimated

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Figure 1. Bleeding of Horseshoe Crabs. A) Example of bleeding facility and B) process. Photo credit: Ariane Mueller.

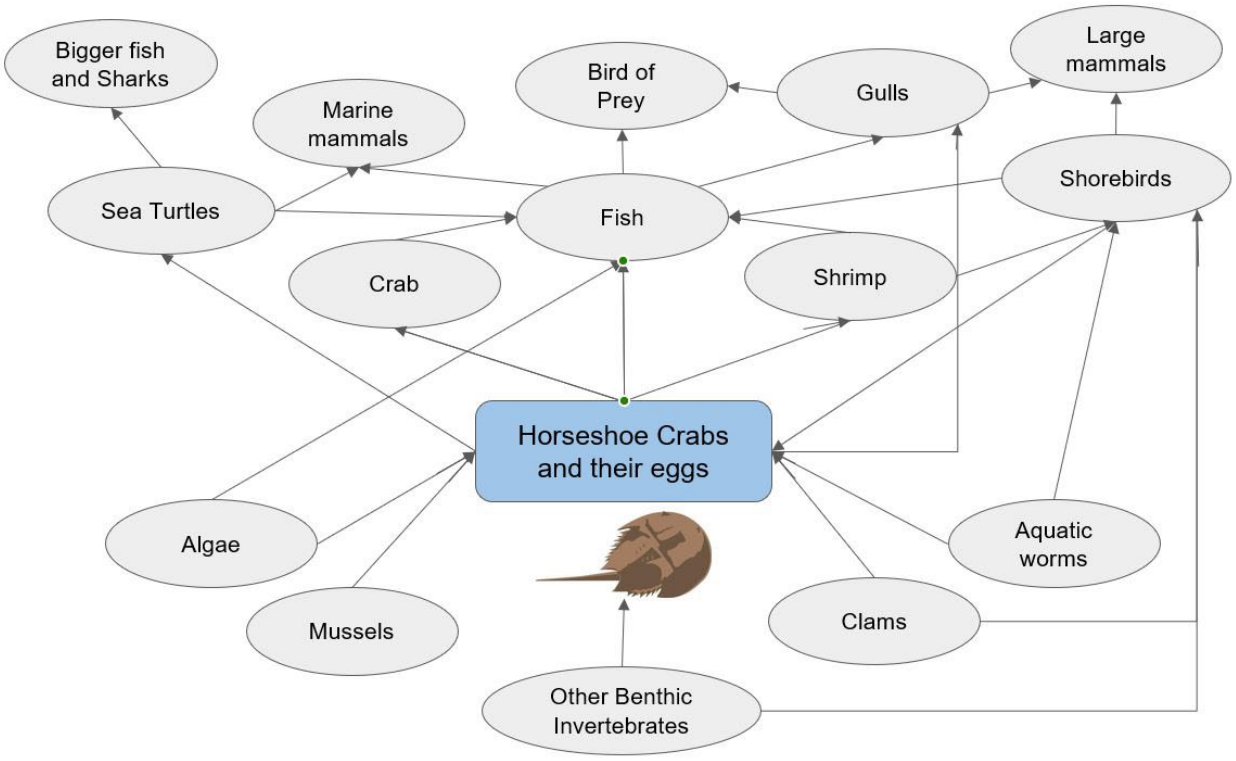

Figure 2. Diagram of Feeding Patterns of the Horseshoe Crabs and Their Ecosystem. Directions of arrows indicate interactions between different groups (Krisfalusi-Gannon et al., 2018); Horseshoe crab image credit: Hopscotch; Figure credit: Alexander and Adrian Luck.

that the horseshoe crab numbers remain at only one third of the population before the 1990's over-harvest tragedy (Horseshoe Crab Recovery Coalition, 2023).

Habitat loss is another threat to horseshoe crabs. Increased human development and subsequent degradation of habitat due to shoreline hardening and sea level rising along the Atlantic coastline have likely contributed to the declining numbers (Horseshoe Crab Recovery Coalition, 2023). Groins and seawalls fortify shorelines and prevent erosion while at the same time stopping natural beach migration, and bulkheads halt access to intertidal spawning areas (ERDG, 2024). The population of horseshoe crabs is largely dependent upon proper spawning habitats. This is challenging as the crabs compete for space with the ever-growing human population that occupies and moves to the same beaches the horseshoe crabs have depended on for many thousands of years. Inevitably, human expansion wins.

Ecosystem impact

Although the issue of what threatens horseshoe crabs and to what extent is contentious, dwindling crab populations have and will have definite impact on the biological community that they are part of (Botton, et al., 2003; Tamura et al., 2021). Figure 2 illustrates the horseshoe crabs' importance in the food web and marine ecosystem. Horseshoe crabs are considered a keystone species and are critical in the marine food web, particularly for shorebirds and sea turtles, some of which are federally listed as threatened species (Krisfalusi-Gannon et al., 2018). Sea turtles depend on horseshoe crabs as their main food source. Maintaining an abundant population of adult horseshoe crabs may be critical for ensuring the long-term survival of loggerhead sea turtles in the Delaware and Chesapeake Bays (Tanacredi et al., 2009; Seney, 2007).

While sea turtles feed mostly on adult crabs, shorebirds feed mostly on crab eggs. As many species of birds migrate south or north depending on the season, horseshoe crabs are essential in replenishing their

energy, aiding in the birds' journey, reproduction process, and survival. In the book *Narrow Edge*, Deborah Cramer (2015) describes how the fate of horseshoe crabs, elusive tiny birds - the red knots, and humans are intertwined. Cramer explains that red knots are not the only birds closely connected with the horseshoe crabs, but they may be particularly vulnerable. To protect horseshoe crabs is to protect red knots, and to safeguard both is to protect their habitat, which are the beaches along a 9,000-mile coastline. Red knots make a challenging journey from the Arctic to the south tip of Argentina and back. They depend on beaches stretching from pole-to-pole for refuge and food. Horseshoe crab eggs are the main calorie-rich food source for red knots. If any beach along their route is destroyed or food sources are threatened, the birds' life cycle is jeopardized.

Many conservation advocates call for restoring horseshoe crab populations so that they can support the ecosystem and the many species that have relied on them for millennia. These efforts have a potential to reverberate beyond just saving shorebird or marine turtles. The crabs' nutritiously rich eggs can restore dwindling diversity and numbers of fish in bay areas, hence supporting local business and communities that support tourism and sport-fishing, which have largely faded away. According to biologist Larry Niles from New Jersey's Wildlife Restoration Partnership, "Walk up to a marina or boat captain today, and they'll tell you there are no fish" (Tangley, 2022). Niles remains optimistic and believes that the current conservation efforts or a potential short-term moratorium on harvesting horseshoe crabs "could bring this ecosystem right back to what it once was" (Tangley, 2022).

Current efforts to protect horseshoe crabs

To ensure a healthy ecosystem and maintain the food chain, an adequate population of crabs must be upheld, and spawning must occur uninterrupted. The impact of biomedical exploitation, fishing industry, and growing human developments must be properly balanced to ensure the survival of all impacted species. Protecting horseshoe crabs is critical, and different efforts have been proposed and implemented. From placing and enforcing quotas on horseshoe crab fishing to forming alliances with national, state and local organizations that help to recover horseshoe crab populations and safeguard key spawning habitats…to motivating community volunteer efforts, such as the Just Flip 'em!™ program, which is popular in Delaware and Maryland and encourages people to flip stranded crabs over during the spawning season, to forging industry and academia collaborations, a strong push to protect and sustainably manage horseshoe crabs has been initiated.

The biomedical community also has been doing its part. In the 1990s, Ling Ding Jeak and Bo How were able to synthesize a recombinant Factor C (rFC), key enzyme in the development of the clotting reaction in the LAL assay. They were the first to clone the rFC DNA and express its rFC protein (Ding et al., 1995; Bolden and Smith, 2017). Their work enabled the development of an animal-free endotoxin detection assay (See Figure 3**)**. There are many benefits to using the non-animal-derived rFC tests. They are comparable in sensitivity and, in some instances, may be more sensitive (LAL assays offer sensitivities in the region of 0.005 EU/mL, while rFC sensitivity can be as

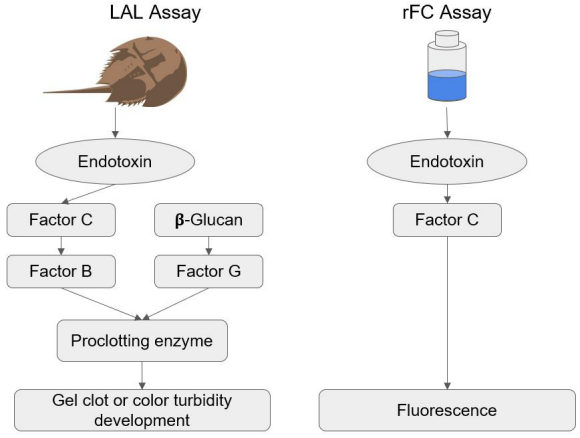

Figure 3. Mechanism of Action of LAL Assay in Comparison to the rFC Alternative (Ding et al., 1995; Bolden and Smith, 2017); Horseshoe crab image credit: Hopscotch; Figure credit: Alexander and Adrian Luck. (Images and Powerpoint in Supporting Files folder.)

high as 0.001 EU/mL) due to the use of fluorescence detection to determine the endotoxin concentration. Also, while the LAL assay may be prone to false positives through the β-glucan pathway, in the rFC assay, the reaction depends only on Factor C activation, thus eliminating the chances of false positives.

There are several commercially available rFC assays. They have been available since 2003. They include PyroGene™ Assay from Lonza, Endosafe® Trillium™ Recombinant Cascade Reagent (rCR) from Charles River Laboratories, Bioendo™ rFC Endotoxin Test Kit from Xiamen Bioendo Technology, and rFC from Creative Biolabs. There is evidence that the efficacy of rFC is equivalent to LAL in the detection of endotoxin (Bolden et al., 2017). In some studies, some rFC-based methods yield better endotoxin recovery rates than the LAL assay and were deemed equivalent or superior and suitable for testing of medicines and medical devices (Piehler et al., 2020). Based on various comparability and validity studies, the US Pharmacopeia (USP) published a Compendia Notice introducing rFC as a synthetic non-animal derived alternative to the reagents derived from horseshoe crabs, and rFC was incorporated as part of the official text of the USP National Formulary. This was a big step to widening acceptable methods and reagents for detection of bacterial endotoxins and provided an opportunity for pharmaceutical companies to potentially switch to the synthetic alternative (USP, 2017). Although there is ample evidence that rFC is sufficient for endotoxin testing and listed as a valid reagent in the USP, adapting this new alternative is slow.

Based on some estimates, switching to rFC would result in a 90% reduction in the demand for LAL. This change would have the potential to save an estimated 100,000 crabs annually, significantly reducing mortality rates in North America. (Maloney et al., 2018). Yet, the industry is reluctant.

Current investigation

In the following paper, several key stakeholders from the biomedical and pharmaceutical industry, as well as from a local biodiversity conservation and advocacy group, were interviewed to better understand some of the challenges associated with widely adapting rFC and helping to protect horseshoe crabs. It is unclear why the pharmaceutical industry is hesitant. High cost of the rFC and push back from the regulators may be the reasons. It seems that the health authorities and global regulators are even slower to accept rFC as a viable alternative to LAL. Being cautious in accepting and integrating new technologies is justifiable. However, there is a strong ecological need to innovate and a lot of evidence that the animal-free alternative is effective. Below we share findings from interviews conducted with several stakeholders who utilize LAL and who are concerned about the welfare of horseshoe crabs. Our findings are below.

Approach and findings

To garner a better understanding of the current processes of endotoxin testing and the challenges with adapting synthetic rFC assay, we interviewed several subject matter experts SMEs) from different branches of the industry to learn about their interdependence and reliance on horseshoe crabs (see Figure 4). Pharmaceutical companies are required to test their products for the presence of endotoxins to assure safety before marketing and selling their products to patients. Biomedical producers of LAL and rFC assays supply endotoxin testing kits and reagents to pharmaceutical companies. Regulatory agencies, like FDA, provide oversight of both the pharmaceutical and biomedical sectors to ensure that released medications are safe, efficacious and meet quality standards and that the testing methods used to ensure safety and quality are accurate and appropriate. On the other hand, conservation and advocacy groups aim to preserve biodiversity in local and regional ecosystems and manage natural resources in a bio-responsible and sustainable way. To protect horseshoe crabs and address the declining population trends, various efforts have been initiated, such as public education, engaging private companies to responsibly harvest and release crabs, and placing pressure on lawmakers to protect spawning beaches and control harvests. All of the

aforementioned parties play a unique role in the life and the future of these ancient creatures. Clearly, much needs to be discussed as these species will not survive the indifference of these key stakeholders and the public.

In the following briefs, the perspectives of a conservation group, Southeastern Massachusetts Pine Barrens Alliance, a manufacturer of LAL and rFC tests and reagents, Lonza; and a pharmaceutical company, Pfizer, that purchases LAL and rFC products for endotoxin testing of their medicines and medical devices are presented. Their responses are provided for general review and synthesized in the Discussion section.

Perspective of conservation group

The first interview was with two representatives from the Southeastern Massachusetts Pine Barrens Alliance (SEMPBA), Sharl Heller and Frank Mand. The organization focuses on the "preservation and enlargement of the 40 natural communities" in the Coastal Pine Barrens, a globally rare ecoregion and one of the only three left in the world (Southeastern Pine Barrens Alliance, 2024). SEMPBA efforts are supported by grants and volunteer work. One of their goals is the conservation of horseshoe crabs along the East Coast.

According to Heller, the main threats to horseshoe crabs are "definitely overfishing and biomedical exploitation." Heller believes that "bay harvest and biomedical carelessness" are leading to a depletion of these ancient arthropods. There are few studies supporting the claim that horseshoe crabs' numbers are declining. However, these studies are contentious. Heller and Mand point out that most surveys tracking population dynamics started after a noticeable decline in the numbers became a cause for concern. In the 1950s and 1960s, "horseshoe crabs' numbers were in the millions" and now they are "in the hundreds of thousands." Heller and Mand describe how the fishing and biomedical industries use this gap in trends of data to their advantage. Without a proper reference point and without data to compare the present numbers to, the private sector claims that horseshoe crab

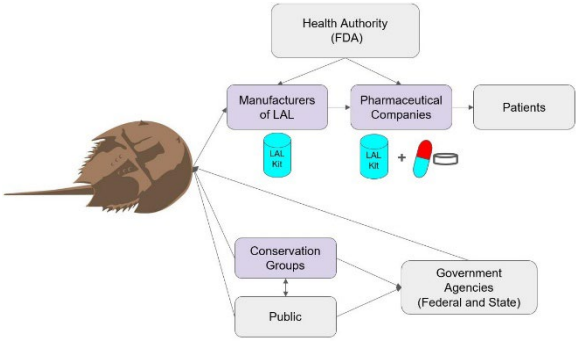

Figure 4. Diagram of Relationships. Arrows indicate direction of impact. Highlighted in purple are interviewed SMEs. Manufacturers of LAL assay market and sell LAL kits to pharmaceutical companies. Pharmaceutical companies use the LAL assay to ensure product safety before selling to patients. Health authorities (e.g. the FDA) regulate the manufacturers of LAL, as well as pharmaceutical companies. Conservation groups work together with the public to preserve horseshoe crab populations and habitats as well as aim to influence government agencies and legislature on both federal and state levels. Horseshoe crab image credit: Hopscotch; Figure credit: Alexander and Adrian Luck.

populations have remained stable, healthy and plentiful, and there is not enough information to warrant concerns or place restrictions on harvesting.

Although Heller and Mand acknowledge that fishing for horseshoe crabs and the accidental capturing of horseshoe crabs via bycatch is just as much of a threat as the bleeding by the biomedical industry, they outline a few dangers specific to pharmaceutical purposes. While fishing and bycatch is harmful to all horseshoe crabs equally, biomedical companies tend to prefer catching sexually mature large females, as they contain more of the precious blue blood. Capturing females alters the ratio of females to males and has negative consequences on spawning. Additionally, the mature females that do survive bleeding avoid returning to the place they were captured, leading to an even bigger discrepancy between male and female dynamics and mating. Heller and Mand note that in some surveyed areas, the ratio of males to females was 1 to 5, while in other places, the ratio was 20 to 1. Most females that do remain in the spawning areas are not sexually mature and so are unable to

reproduce, dwindling the overall population numbers even further.

Heller and Mand emphasize that the effects of exploitation and over-harvesting reach beyond the horseshoe crabs themselves. The crabs are a critical part of the ecosystem's food cycle. Their eggs are a vital food source for "birds migrating from the North to South." Red knots, for example, used to come in millions to feed on horseshoe crab eggs, yet now are on the list of endangered species. Horseshoe crab eggs and juveniles are also an important source of food for different types of fish and turtles, which are now equally at risk. The decline in the horseshoe crab population poses a significant danger to the many species that depend on their survival and uninterrupted life cycle.

To protect horseshoe crabs and preserve their habitats, SEMPBA develops educational materials, produces forums, monitors habitat and species, and collaborates locally and nationally. The organization has successfully obtained local, state, and federal grants, and it shares the majority of those funds with scientists, experts and conservation entities with similar priorities and focus. Most importantly, the SEMPBA engages the public and encourages many citizen science projects, stimulating interest and passion for the environment, its preservation, and ways to transition to sustainability. Heller and Mand strongly believe that empowering citizens is necessary to build effective and long-lasting public partnerships that hold influence and change the legislature accordingly. Figure 5 illustrates the advocacy efforts of SEMPBA. Furthermore, SEMPBA collaborates with other groups and petitions to change regulations and influence the legislature. In one instance, they were hoping to place restrictions on the amount of horseshoe crabs harvested by various industries. The Horseshoe Crab Recovery Coalition, for example, is a group of activists who work with the Alliance and sponsor their work. The lack of data on pre-1990's populations has so far prevented the efforts of listing horseshoe crabs as an endangered species, but they continue with their advocacy and renewed their appeals to government agencies. Their collective efforts are focused on influencing different states; Connecticut already passed laws to ban all horseshoe crab collection, and they are working to encourage other states to follow. In Massachusetts, they petitioned to close 4 Cape Cod beaches from fishing for crabs, but so far have been unsuccessful. In general, they are met with unenthusiastic policymakers who are skeptical of their appeals and favor the perspective of the private sector. However, their efforts do not waver, and they continue to update and resubmit appeals every year.

Overall, SEMPBA and similar groups believe that placing strict limits on horseshoe crab harvest is necessary. They do not doubt that horseshoe crabs are endangered and that different stakeholders, including the private sector, government agencies and the public, must come together to prevent further damage to their population. They make efforts to educate and encourage the public to volunteer and participate in various projects to study endangered ecosystems, restrict their exploitation, and create a sustainable future that is mutually beneficial.

Perspective of manufacturer of LAL and rFC

After the interview with Heller and Mand, the authors acquired an appreciation for the importance of horseshoe crabs in the marine ecosystem and the potential threats the species are facing. To broaden this investigatory study, the authors reached out to one of the most respected biomedical companies, Lonza. Lonza is based in Switzerland with 21 facilities across the US, and it is one of the leading manufacturers of LAL and rFC (PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay, 2024). Lonza's rFC-based assay, called PyroGene®, is advertised as environmentally sustainable and more precise than the animal-derived LAL counterpart. It is widely popular and continues to gain traction (PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay, 2024).

Dr. Allen Burgenson, a Lonza employee who has been involved in the development of both LAL and rFC and was instrumental in moving his company away from the original Rabbit Pyrogen Test (RPT) to the new and improved LAL assay, was interviewed.

Figure 5. Advocacy Efforts of SEMPBA. (a) Horseshoe crab mating at Long Beach in Plymouth, MA. Image credit: Dodie Frank. (b) Female horseshoe crab. Image credit: Barbara Tevelev. (c) Horseshoe crab eggs. Image credit: Joan Pierce. (d) Volunteers surveying horseshoe crab population during spawning season. Image credit: Barbara Tevelev. (e) Public engagement and spreading awareness of the need to protect the horseshoe crab fragile ecosystem. Image credit: Sharl Heller.
(Images and power point in Supporting Files folder.)

Burgenson has a deep understanding of the ecological and regulatory issues, as he sits on the Horseshoe Crab Advisory Panel of the Atlantic States Marine Fisheries Commission (ASMFC) as well as on the Horseshoe Crab Working Group of the International Union for the Conservation of Nature (IUCN). Burgenson also published one of the original papers on validation rFC assay in the Pharmacopeial Forum, which is the official journal of the USP. While he is passionate about the conservation of horseshoe crabs since the age of three, he is also wary of an abrupt transition away from LAL that could potentially harm humans via inadequate safety testing.

Burgenson supports the efforts of conservation groups to manage and study horseshoe crab populations and advocates for the transition from LAL to rFC. Burgenson underscores that the important step in the transition process and convincing regulatory agencies of the switch is conducting and publishing a plethora of studies that prove that rFC is either equivalent or better in performance than LAL. Often the organizations that review scientific publications are staffed by volunteers who

only meet once every six months. At each meeting, they read the paper and suggest edits; after correction, the author must wait another six months before submitting the next version, and so on. Burgenson's own paper, "Comparison of four endotoxin detection reagents in measuring autochthonous endotoxin levels in four representative parenteral products," took five years to publish, delaying the exchange of findings and continued research and development efforts.

Burgenson also highlighted that pharmaceutical companies have to adhere to the strictest testing guidelines set forth by global regulatory agencies via the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) in order to make their products available in markets across the world. While some regulatory agencies have accepted rFC and encourage the use of non-animal testing (i.e. USA-based Federal Drug Administration [FDA] and European Medicines Agency [EMA]), other health authorities maintain the animal-derived tests as the required expectation for release. This continues the utilization of LAL. Burgenson indicates that at Lonza, the manufacturing ratio of LAL to rFC assays stands at approximately 10 to 1, so more work needs to be done to further popularize the assay and validate it as an acceptable alternative to LAL.

Burgenson points out that rFC has some drawbacks that need to be addressed before the assay is integrated into mainstream pharmaceutical practices. Not all synthetic rFC assays are equal in performance and not all work with the same efficiency. So far, there are no regulations placed on the production of rFC tests, therefore anyone can produce and sell them. From the perspective of both the regulatory agencies and the consumers, the lack of quality standards and non-regulated procedures impacts trustworthiness and data credibility. Meanwhile, the production of LAL is regulated by the FDA and endorsed by USP global compendial methods, trusted national and global agencies. Burgenson cautions that even "nonagram amounts of endotoxins in the human blood can be lethal," and so he believes that it is best to avoid rushing the process of switching from LAL to the synthetic alternatives. Robustness and consistent performance needs to be proven before rFC can be used in evaluating human-related products.

In addition, there is the problem of cost. The LAL reagents and assay kits are cheaper to produce than rFC. The production of rFC involves an expensive pharmaceutical-grade process. Burgenson highlights that "clean rooms" that are used to manufacture rFC cost about $1 million each month. On the other hand, the production of LAL assay is 10 times cheaper. Pharmaceutical companies that are dedicated to switching to non-animal tests and that apply for product registration globally are faced with higher costs as they are expected to provide rFC results in markets that demand animal-free tests and LAL in markets that have not adapted animal-free tests yet. It is costly to do the "right thing."

Burgenson admires conservation efforts. He expresses his concern for plankton-sized horseshoe crab babies that are highly susceptible to death, and he talks fondly of programs that capture and protect these juveniles, later releasing them as adults. In contrast to Heller and Mand, Burgenson does not believe horseshoe crabs are an endangered species. "There are 43 million of them," he says, claiming that conservation groups are exaggerating the danger to their populations.

Interestingly, Burgenson mentions that it is also inaccurate to claim that the synthetic alternatives are completely "animal-free," as armyworms and animal-derived supplements are used in the production and purification of rFC. Burgenson explains how the biomedical industry is actually the smallest threat to the horseshoe crab population, as the LAL kits have been optimized to use less of the crab-derived blood. Burgenson shifts attention to the fishing industry as bycatch and bait are the biggest threats (see Figure 6). Because Lonza's operation involves bleeding only one sinus of the crab and bleeding only once a year, Burgenson believes the mortality rate at Lonza may, in reality, be even below 5%. Furthermore, Lonza participates in horseshoe crab replenishing projects, which regenerate the populations. This makes Lonza "a net provider" of horseshoe crabs. Burgenson also

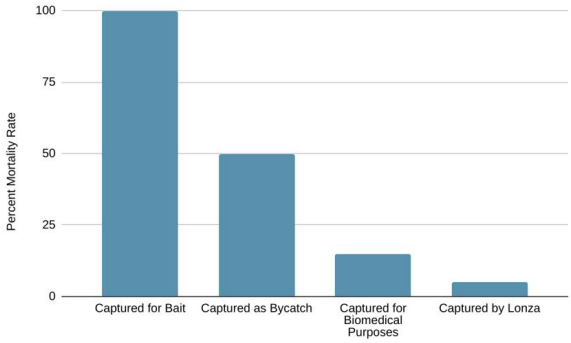

Figure 6. Graph Comparing the Amount of Harm to Horseshoe Crabs by capture type, in percent killed as per Burgenson comments. (Images and Powerpoint in Supporting Files folder.)

warns that increasing horseshoe crab numbers too quickly may endanger other species such as "mussels, starfish, and oysters, which are their main source of food" When asked what actions would best support the local horseshoe population, Burgenson encouraged them to consider participating in volunteer activities that help save horseshoe crabs on the beaches, such as the Just Flip 'em!™ program . On the other hand, he advised against putting too much social pressure on regulatory agencies and pharmaceutical companies to change policies and switch testing reagents too rapidly. rFC assays take longer to make and are more costly. The burden of cost will likely be transferred to customers, raising the cost of vital drugs, and it may take longer to approve medicines. To Burgenson, the movement to regulate horseshoe crab harvesting is not worth the potential risk to human lives and availability and affordability of medical products.

Perspective of pharmaceutical company

For the final interview, the authors met with a representative from a pharmaceutical company that develops and manufactures medicines and uses LAL and rFC assays for endotoxin testing. Thomas J. Schomogy, an employee of Pfizer, agreed to explain the pharmaceutical perspective, the process of manufacturing and safety testing and the challenges associated with transitioning away from animal-derived to animal-free assays.

Schomogy explains that most of Pfizer products are tested with LAL. In line with other interviewees, he describes how testing is done according to the strictest requirements, which currently require animal-derived assays. Products made for human use are the most regulated and therefore must utilize the most stringent testing methods. Schomogy points out that most power lies within the regulatory agencies, which set, enforce and monitor testing requirements and specifications.

Meanwhile, Schomogy speculates that some companies that manufacture only LAL may initially resist the transition to rFC. While there is favorable data that indicates that horseshoe crabs are not in grave danger due to biomedical exploitation, in reality, many crabs do die during transport or harvest, especially the larger females are of big concern. Though a potential transition to producing rFC instead of LAL may lead to initial revenue loss for some of the manufacturers and may also be costly for the pharmaceutical industry due to the higher cost of making rFC, the size and diversification of the product line should provide sufficient traction in the market and buffer the initial loss.

Schomogy admits that regulatory agencies, on the other hand, pose a serious roadblock. For now, many consider LAL the "gold standard" and are hesitant to accept the rFC. What's more, individual regulators who review market registration applications may deny the validity of the synthetic test based on personal bias. Navigating through the regulatory environment is not simple, and applicants are weary of many potential risks.

When considering the efficiency and accuracy of the LAL vs different rFC assays, Schomogy believes there are few truly unbiased comparisons, which aren't trying to prove one better than the other. Another challenge in comparing is the lack of regulations, as the quality of rFC assays may differ from one company to the next. This aligns with some of the commentary expressed by Burgenson. One manufacturer may produce an assay that is comparable to LAL, while the next could significantly underperform, casting doubt on all such types of assays. An accurate and unbiased analysis would require "engineering and time" and willingness from academic circles. This is

possible but requires collaboration and funding. The authors hope that this publication will stimulate such efforts in the near future.

Schomogy echoes Heller's and Mand's sentiments and hopes that the rFC assays will aid in protecting horseshoe crabs and the environment that depends on their survival. Schomogy believes that the rFC assays have the potential to be more precise (eliminating false positives), with a higher throughput and increased convenience. Schomagy also echoes Burgenson, highlighting commitment to patients' safety and underlining the high cost and rigidity of rFC. In general, LAL is cheaper to purchase than rFC. Moreover, while LAL assays utilize many types of data collection (color-based, turbidity, gel clotting), many of which don't require expensive equipment, the fluorescence-based system for most of the rFC assays requires specialized and expensive equipment. Together, all of these factors increase the cost of testing and may affect the affordability of medicines.

To achieve change, Schomogy proposes that multiple companies across multiple countries align in their efforts to integrate rFC and provide sufficient data to convince the regulatory agencies that switching has no impact on the safety and quality of medicinal products. So far, both regulatory agencies and private sector companies from the U.S.A, European Union, and Brazil advocate for switching to rFC. On the other hand, regulatory agencies and companies from Japan and China (another native habitat for horseshoe crabs) are not so accepting. Unlike Burgenson, for Schomogy, an important factor is public pressure on government policies and regulatory agencies. Due to public advocacy groups, regulators in the EU successfully removed the Rabbit Pyrogen Test, previously a staple in the industry for biosafety testing. This test is no longer used in Europe due to public dismay.

Schomogy cautions that putting pressure on health authorities requires being well-informed and educated on the subject and considering the issue from multiple perspectives. Blindly forcing change may not lead to favorable results, and harm another part of the industry or the environment. Schomogy explains that change is often slow and never easy, but achievable when different collaborators come together and form creative solutions. After all, "we are all in this together".

Discussion

Human development, human exploitation of natural resources, and gradual climate changes are impacting ecosystems all over the world, including that of the horseshoe crab. Along the New England coastline, increased real estate and property development and improper structural management of shorelines result in habitat loss and destruction of breeding areas. The mating patterns of horseshoe crabs are disturbed gravely and, as a result, their numbers are dwindling.

Industrial exploitation compounds the problem. Horseshoe crabs are heavily used by the fishing industry as bait for whelks and eels. They are also often caught accidentally as by fishing nets designed to capture other species. Increasing numbers of crabs each year face demise at the hands of the fishing industry. Since fishing is localized to particular areas along the shoreline, fewer crabs return to their host beaches for spawning and fewer eggs are laid and fertilized, explaining much of the 28-year-long decline in the American red knot population, as well as other shorebird and marine species (Cramer, 2015).

Horseshoe crabs are also used by the biomedical industry. Their capture and bleeding causes 4 to 30% mortality. Although not as lethal as the 100% death rate from the fishing industry, the harvesting process is associated with behavioral and physiological changes that pose a risk to horseshoe crab viability and reproduction. Together, all of these threats have brought the future of the horseshoe crab's existence into question.

Undoubtedly, horseshoe crabs save human lives. For over 40 years, they have been bled to make sure medical products are safe and patients do not die from contaminants. Can humans return the favor?

Many environmentalists and members of conservation groups, like Heller and Mand, point to the importance of restoring and maintaining habitats to support healthy

populations of horseshoe crabs so that the food chain and the ecosystem that they are a part of can be continually sustained. Manufacturers of LAL assays and pharmaceutical companies have been actively participating in the conservation efforts. Many donate funds and time to support educational programs and help coastal communities save breeding grounds and foster replenishing attempts. Furthermore, scientists, like Burgenson, are spearheading efforts to validate synthetic alternatives to LAL and collaborate with pharmaceutical companies to promote non-animal testing across the medical industry. Molecular engineers, like Schomogy from Pfizer, are also eager to transition their companies to animal-free testing, as many would prefer to use rFC for global product registration.

It seems that the private sector recognizes that rFC has the potential to replace LAL and provides an opportunity to save horseshoe crabs. However, there are some doubts about rapid change, which may mean more expensive and uncertain methods of testing. The possibility that some rFC assays may not be equivalent in quality to LAL, and thus under-report endotoxin levels, is the most concerning as it impacts patients' safety. More optimization is required to transition away from LAL.

For rFC assays that are equivalent to LAL, the roadblock is the regulatory agencies that oversee the biomedical and pharmaceutical industries, and have the final say on acceptance of diagnostic assays and approval of drugs. Some agencies accept the synthetic alternative, but many do not. For pharmaceutical companies that apply for submission in global markets, harmonization of regulatory requirements is needed, otherwise, the cost of production increases, hindering the affordability of medicines and accessibility in certain markets. In this case, it is beneficial that the public places pressure on the health authorities to recognize non-animal derived testing and increase funding for research to optimize those methods and make them more affordable.

Lastly, the authors encourage public engagement and interest in expanding their knowledge base to better understand pharmaceutical drug manufacturing processes, as well as how human industrial development impacts surrounding ecosystems. As Schomogy underscores, a solid knowledge of all elements involved is essential to create solutions for a sustainable future. Ignorance is not an excuse.

The authors are inspired by Heller's and Mand's assertion that nature has the right to protect itself. They point to the evolution of thought and law as represented by social acceptance and implementation of the Rights of Nature (RoN). RoN is a legal instrument that enables nature (i.e. ecosystems, rivers or species) to have inherent rights and legally to have the same protection as people. As such, biological communities have the legal prerogative to exist, thrive and regenerate. After all, even corporations, not the people who govern them, have the legal ability to sue and to be sued. And so when a river is polluted or the species are exploited, both should have the legal right to be protected. Mand points out that this concept is "not just an abstract idea." Nature is not abstract, but tangible and essential for human survival.

Heller encourages the appreciation of nature and highlights nature's incredible resilience. "Nature will come back, if you allow it." She reminisces about the group's last restoration project: the Tidmarsh Farm. Around a massive cranberry bog operation, dams were removed, and Atlantic white cedars were planted. Just within two years of removing those dams, the herring came back, which had not been there in over 200 years, while new growth and the scent of baby cedars filled the air. This is the power of conservation, human generosity, and activism. The same is possible for horseshoe crabs.

Next steps

To create a more comprehensive review of the topic and to understand the best path forward for both humans and horseshoe crabs, it would be optimal to conduct interviews with different government agencies. First, reaching out to health authorities that regulate the biomedical and pharmaceutical industries, such as FDA, and asking about the regulatory perspective on the acceptance and validation of rFC as an

alternative option to LAL. Second, reaching out to federal and state agencies that regulate the fishing industry and protect wildlife, such as the US Fish and Wildlife Service, would allow to inquire about their stance and strategy for protecting horseshoe crabs and associated species, shaping a complete understanding of the important and intricate task of ensuring the safety of humans and horseshoes crabs alike.

Furthermore, it may be relevant to determine the economic impact of eliminating LAL manufacturing. Discussing the issue with biomedical business analysts and independent economists would help to establish the degree of job loss and whether or not the manufacturing of rFC would replace the void created by abandoning LAL.

Saving horseshoe crabs is a complex issue. Ensuring consistent supply of safe medical products without impacting accessibility and affordability, while maintaining job security are all important considerations and are linked directly with the fate of horseshoe crabs.

Author contributions

A. J. L., A. E. L., and A. K. contributed equally to the literature search and interview process, as well as overall preparation of the paper.

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