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Effects of the biomedical bleeding process on the behavior and physiology of the American horseshoe crab, Limulus polyphemus

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EFFECTS OF THE BIOMEDICAL BLEEDING PROCESS ON THE BEHAVIOR AND PHYSIOLOGY OF THE AMERICAN HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

BY

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ABSTRACT

EFFECTS OF THE BIOMEDICAL BLEEDING PROCESS ON THE BEHAVIOR AND PHYSIOLOGY OF THE AMERICAN HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

by

Meghan Owings

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Horseshoe crabs are harvested by the biomedical industry in order to create Limulus Amebocyte Lysate (LAL) to test medical devices, vaccines and pharmaceutical drugs for pathogenic gram-negative bacteria. Previous studies of the impacts of the biomedical bleeding process on horseshoe crabs have primarily focused on mortality rates and sublethal impacts, using animals held in the laboratory. Therefore, the first goal of this project was to determine the effects of the bleeding process on horseshoe crab behavior once they are released back into their natural environment. In addition, previous studies have typically only investigated the impacts of the full bleeding procedure, or just hemolymph extraction, on horseshoe crab mortality and behavior. Therefore, my second objective was to determine the relative impacts of the three main stressors (aerial exposure, increased temperatures, and blood loss) on the locomotor activity and hemocyanin levels of horseshoe crabs. Finally, previous studies have demonstrated that horseshoe crabs held in captivity, even if they are not bled, experience a decline in hemocyanin levels. Therefore, the third objective of this study was to test a food supplement that might reduce these sustained decreases in hemocyanin levels, along with the associated behavioral impacts.

We found that once horseshoe crabs were released back into their natural habitat there were some immediate differences between bled animals and controls. Bled animals appeared to

mate significantly less than control animals within the first week post-release, with the largest differences between bled and control females. However, the only other significant difference we observed between 14 bled and 14 control animals was a tendency for bled animals to remain significantly deeper during the two-year study. Our laboratory studies revealed that the full bleeding procedure typically used commercially (i.e., all three stressors) had the largest impact on mortality, hemocyanin levels, overall activity, and expression of biological rhythms, followed by bleeding along with at least one of the other stressors. We also saw a seasonal trend in hemocyanin levels and a strong, significant relationship between hemocyanin levels and overall activity. Our data also revealed that animals with starting hemocyanin levels of 0.13 mg/mL or less were more likely to die or be impaired by the bleeding process. Therefore, it appears that an awareness of the overall health and hemocyanin levels in animals captured for bleeding might help reduce mortalities and other impacts if companies would avoid using these borderline animals. Another approach might be to use a food supplement to prevent sustained reductions in hemocyanin levels. Our last study showed that animals that were fed had an increase in their hemocyanin levels and overall activity, and maintained the same biological rhythms they had prior to being bled. Therefore, providing dietary supplements to horseshoe crabs either before or after bleeding them might be a logistically realistic way to improve physiological status and maintain a healthy population of this important species.

In summary, we saw negative impacts from the biomedical bleeding procedure on the behavior and mortality of horseshoe crabs both in the laboratory in the field. Our data should provide insight into ways that biomedical facilities can modify the process in order to alleviate these issues. Moreover, these data indicate that one approach would be to develop a food supplement that would help animals maintain healthy hemocyanin levels. Given the importance

of horseshoe crabs to coastal and estuarine ecosystems, and human health, it is crucial to determine a sustainable practice for bleeding these animals to create LAL.

INTRODUCTION

OVERVIEW

The American horseshoe crab, *Limulus polyphemus*, is an ecologically and economically important species found in estuarine ecosystems along the Atlantic coast in North America, including the Great Bay Estuary, New Hampshire. Ecologically speaking, horseshoe crabs are a keystone species in their natural environment by serving as bioturbators as they forage for food and, consequently, oxygenating the marine sediment (Botton *et al*., 2003), and their eggs are also a vital food source for the 425,000 to 1,000,000 shorebirds that migrate up the Atlantic coast each year (Walls *et al*., 2002; Botton *et al*., 2010). Economically speaking, horseshoe crabs were harvested for livestock feed and fertilizer from the 1870s-1960s (Walls *et al*., 2002). However, due to a decline in the population of horseshoe crabs as a result of this fishery, as well as public complaints of odor, and competition with other fertilizers, horseshoe crabs ceased to be harvested for animal feed and fertilizer in the 1960s (Berkson and Shuster, 1999). Currently, horseshoe crabs are being harvested as bait for the eel and whelk fisheries (ASMFC, 1998; ASMFC, 2012), and their blood is used to create Limulus Amebocyte Lysate (LAL; Novitsky, 1984). LAL is used in the biomedical industry to test medical devices, vaccines, and pharmaceutical drugs for pathogenic gram-negative bacteria (Novitsky, 1991; Chen and Mozier, 2013). Currently, while quotas and regulations have been placed on the bait fishery (ASMFC, 2012), the biomedical fishery remains fairly unrestricted and harvest rates continue to steadily increase in certain areas (ASMFC, 2013).

With horseshoe crabs continuing to remain a valuable marine resource and with fewer restrictions for crabs collected for bleeding than for bait, it is critical to understand the consequences of the biomedical bleeding process on horseshoe crabs' fitness and population

dynamics. The capture process in and of itself includes multiple stressors, such as increased air exposure (time on docks and boats and trucks), and increased temperatures (remaining in direct sunlight or insufficient temperature-controlled facilities and transport). The process of extracting blood can also compromise the health of the horseshoe crab. Approximately, 30% of their total hemolymph volume is extracted and, as a result, reports of mortalities range from 5-30% (Rudloe, 1983; Thompson, 1998; Walls and Berkson, 2000; Kurz and James-Pirri, 2002; Walls and Berkson, 2003; Hurton and Berkson, 2006; Leschen and Correia, 2010; Anderson *et al.*, 2013) Moreover, Anderson *et al.* (2013) demonstrated that the bleeding process leads to several sublethal impacts, such as decreased overall activity and lower hemocyanin levels. Given these findings, it is imperative to further investigate the impacts of all aspects of the bleeding process on the behavior and physiology of horseshoe crabs, both in the laboratory and after bled animals are released into their natural environment. Specifically, the objectives of my thesis research were to: 1) use acoustic telemetry to assess the survival and behavior of bled horseshoe crabs released back into their natural habitat; and 2) determine which of three major stressors associated with bleeding (air exposure, increased temperatures, or blood loss) has/have the greatest impact on their behavior and hemocyanin levels.

HORSESHOE CRAB LIFE HISTORY

Commonly referred to as 'living fossils', horseshoe crabs have existed for over 445 million years, making them one of the oldest marine arthropods (Sekiguchi, 1988; Rudkin and Young, 2009). The American horseshoe crab is one of four extant species, but alone makes up the genus *Limulus* and inhabits the Atlantic coast ranging from the Yucatan Peninsula, Mexico to Maine, USA (Shuster, 1982a). The remaining three species are found along the coast of southern

Asia (Walls *et al.,* 2002). Horseshoe crabs can live up to 20 years, with a long maturation period of 9-10 years (Shuster, 1958, Sekiguchi *et al.*, 1988). Before reaching sexual maturity, horseshoe crabs shed their exoskeleton at least 16-17 times (Shuster, 1950). But, because they have a terminal molt, it is difficult to determine the exact age of individuals (Walls *et al.*, 2002).

Horseshoe crabs use a variety of different habitats during different seasons and at different life stages. Their movements to and from these habitats are influenced by a variety of environmental variables including tides, water depth, temperature, and daylight. During the late fall, horseshoe crabs move to deeper waters and even as far out as the continental shelf where they estivate for the winter months (Shuster, 1982b; Moore and Perrin, 2007; Schaller *et al.,* 2010; Watson and Chabot, 2010). As temperatures rise in the early spring, they start to move inland towards estuarine and coastal environments (Barlow *et al.,* 1986; Watson *et al.,* 2016), to mate (Schaller *et al.,* 2010) and forage for food.

While water temperature is probably an important cue they use to guide their large-scale movements (Cheng *et al.*, 2015), beach slope and tides are the major environmental cues used to locate spawning beaches (Botton and Loveland, 1987). During high tides in May-June, thousands of horseshoe crabs move to the high water mark and spawn. Adults can spawn several times each season, but females usually only spawn during high tides within one week and do not return until the next year (Brockmann and Penn, 1992). Males use both vision (Barlow *et al.,* 1982) and pheromones (Saunders *et al*., 2010) to find females on these beaches. Typically, larger females are surrounded by several smaller 'satellite' males, in addition to one that is attached to her abdomen (amplexus), leading to a skewed male-biased sex-ratio on spawning beaches (Loveland and Botton, 1992). Female horseshoe crabs lay about 4-5 clutches of eggs at each high tide and deposit around 3,650 eggs into each nest (Shuster and Botton, 1985). Each nest is a

shallow, burrowed pit about 5-30 cm deep (Brockmann, 1990). Females can develop up to 88,000 mature eggs and, thus, lay up to 20 egg clusters each year (Shuster, 1982a). However, it is not clear exactly how many clutches a given female deposits each year. After the female deposits her eggs, both the male clasped onto her, and the satellite males in the vicinity, release their sperm over the nests to compete for fertilization of the eggs (Brockmann, 1990).

Once the eggs are fertilized, the trilobite larvae usually hatch after incubating for 2-4 weeks or the following spring (Botton *et al.,* 1992). Larvae dig out of their egg cluster and, with the help of wave action, are released into the water where they remain pelagic for a few days before settling to the bottom as they mature and molt (Shuster, 1982b). Juveniles will spend their first and second summer on these intertidal flats feeding before the daytime low tide and burrowing in the mud for the rest of the day. They move several kilometers from their natal spawning beaches as they mature, and then they move into deeper waters of the estuaries or towards the continental shelf when they reach adulthood (Shuster, 1982b; Carmichael *et al.,* 2003). After molting about 16 times, in ~10 years, they become sexually mature and they return to spawning beaches to mate (Sekiguchi, 1988).

HORSESHOE CRAB BEHAVIOR AND BIOLOGICAL RHYTHMS

Horseshoe crabs possess both circadian and circatidal clocks. The circadian clock modulates their visual sensitivity, allowing them to see almost as well during the day and night (Powers and Barlow, 1985; Barlow *et al.,* 1986; Herzog *et al.,* 1996; Barlow, 2001; Barlow *et al.,* 2001). It has been proposed that this enables them to forage and find mates during any high tide, whether it occurs during the day or night (Chabot and Watson, 2010). Horseshoe crabs are typically most active at high tide (Watson *et al.*, 2016), and this is due, in part, to the fact that

they have a circatidal clock that influences their activity, so that even in the laboratory when they are under constant conditions, they will express activity peaks every 12.4 hours (Chabot *et al.,* 2007; Chabot and Watson, 2010). It has been recently argued that this circatidal rhythm of locomotion is controlled by two circalunidian clocks, each driving one of the two bouts of activity and each with a 24.8 h period (Chabot and Watson, 2010; Chabot *et al.,* 2016). Given the robust nature of these animals' tidal rhythms and their importance to both mating and foraging activities, any changes to their rhythms can have deleterious effects on their survival. Therefore, one major goal of my thesis research was to determine if bleeding adult *Limulus* has an impact on the expression of their biological rhythms in their natural habitat.

HORSESHOE CRAB DIET AND FEEDING

Gut content analyses have revealed that the adult *Limulus* diet consists of several species of bivalve mollusks (*Ensis* sp., *Macoma* sp., *Gemma gemma*, *Spisula solidissima*, *Mytilus edulis*, *Tellina* sp., *Siliqua costata*, *Mya arenaria*, and *Mulina lateralis*), sediment, vascular plants, and worms (*Nereis* sp. and *Cerebratulus* sp.; Shuster, 1950; Smith and Chin, 1979; Shuster, 1982a; Botton, 1984a, b; Botton and Haskin, 1984). Immature and mature crabs of both sexes have similarities in both the quantity and quality of infaunal and epifaunal invertebrates in their diet, despite the larger size of females (Botton 1984a; Botton and Ropes, 1989). However, food consumption was higher in crabs that were collected later in the spawning season than those collected during peak spawning activity (Botton, 1984a; Botton and Ropes, 1989). Gut contents of two Asian species of juvenile horseshoe crabs, *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda,* revealed similarities in their diets; these crabs were also selective benthic feeders

with a diet made up of polychaetes and thin-shelled bivalves, but they had a stronger preference for insect larvae (specifically, *Chironomous* sp.; Zhou and Morton, 2004).

Carmichael *et al.* (2012) determined sources of nitrogen and carbon in estuarine food webs in order to attain the diet composition and foraging ranges of horseshoe crabs using stable isotope data. As a highly motile invertebrate benthic predator, N and C isotopes in horseshoe crab feces revealed a mixed diet of a relatively opportunistic consumer that had low assimilation efficiency. This was due to the overall ingestion of low quality organic matter, and it was postulated that horseshoe crabs require habitats with sufficient particulate organic matter, higher water quality, and a diversity of prey, indicative of the estuaries where they are found. Since horseshoe crabs eat a mixed diet, primarily composed of molluscs and bivalves, it is important to understand the feeding behavior of horseshoe crabs that are used in the biomedical bleeding process. Especially, since molluscs and bivalves contain hemocyanin in their circulatory systems, this could become a key development in maintaining healthy hemocyanin levels in bled horseshoe crabs and regulating sustainable practices in the future of this fishery.

COMMERCIAL VALUE AND EXPLOITATION

Ecological Value for Migrating Birds

Every spring, at least 11 species of migrating birds stop at Delaware and New Jersey beaches to feed on horseshoe crab eggs (Myers, 1986). Most of these birds use these spawning beaches as a stopover during their migration from South American wintering grounds to their breeding grounds in the Arctic (Botton *et al.,* 1994). The birds must reach their final destination in the Arctic to lay their eggs before the annual insect hatch, because the insects are the primary food source for shorebird hatchlings (Clark, 1996). The timing of their migration north has

evolved to match with the timing of horseshoe crab spawning every year, because they depend on the nutrition they obtain from the eggs in order to provide the fuel for the rest of their journey north.

As 425,000 to 1,000,000 shorebirds assemble on Delaware and New Jersey beaches to feed (Myers, 1981; Shuster, 1982a; Myers 1986; Myers *et al.*, 1987; Clark *et al.*, 1993), thousands of bird-watching enthusiasts trail behind to witness this event. The birders-watchers contribute greatly to the local economies, as they purchase goods and services related to this event (food, equipment, lodging, etc.; Walls *et al.*, 2002). In fact, the annual social welfare value for this ecotourism industry has been estimated to be between $3-4 million, while providing 120-180 jobs (Manion *et al.*, 2000).

Horseshoe Crabs as Bait

L. polyphemus is also used as bait in the American eel (*Anguilla rostrata*), whelk (*Buscyon* sp.), and some catfish (*Ictaluridae* sp.) fisheries (Walls *et al.*, 2002). Horseshoe crabs are caught by trawl, dredge, by hand on spawning beaches, and/or gillnets (HCTC, 1988). Female horseshoe crabs are preferred for the American eel pot fishery, because eels show a strong preference for chemical odors released by egg-laden females (HCTC, 1998). However, the whelk pot fishery relies more heavily on horseshoe crabs (males and females) than the eel pot fishery, which generates a substantial income for local regions where the whelk fishery is dominant. According to Manion *et al.* (2000), the whelk fishery has an annual value of $11-15 million and creates 270-370 jobs, whereas the eel fishery only contributes $2 million to local economies and creates 70 jobs in these areas. Because of the species dominance in the mid-Atlantic region, fishery effort is concentrated in the mid-Atlantic coastal waters and surrounding

federal waters, but a significant fishery has started to grow in the New England area (ASMFC, 1998; HCTC, 1998).

Limulus Amebocyte Lysate (LAL)

Probably, the most valuable use of horseshoe crabs is to create Limulus Amebocyte Lysate (LAL). The United States Food and Drug Administration (FDA) demands that all biomedical facilities use LAL assays to test for sterility and detection of bacterial endotoxins in pharmaceutical instruments before they are sold to the market and released for use in hospitals (Walls *et al.*, 2002). There have been several new alternatives for LAL recently created and introduced, but biomedical companies are reluctant to use them and most of them do not have the same level of sensitivity for bacterial contamination (LAL is capable of detecting one millionth of a billionth of a gram of endotoxin; Mikkelsen, 1988). As a result, this biomedical fishery generates the largest income out of the three major industries that use horseshoe crabs for commercial purposes. Even though only four companies are currently producing LAL, this industry contributes $73-96 million in annual output to local regions, creates 440-540 jobs, and has an estimated value of over 200 million dollars (Manion *et al.*, 2000; ASMFC, 2012).

LIMULUS *BLOOD: OVERVIEW AND LAL*

The fluid or 'blood' of horseshoe crabs is actually a mixture of blood and lymph, scientifically referred to as hemolymph. The two main constituents of hemolymph in horseshoe crabs are the fluid plasma (where the cell-free respiratory pigment, hemocyanin, is found at a concentration of around 50 g per liter of hemolymph) and amebocytes. In contrast to the iron-protein complex in hemoglobin in humans and other vertebrates, hemocyanin contains copper, and thus their blood turns a dark blue color when it absorbs oxygen. The granular amebocyte is

the only cell type found in *Limulus* hemolymph, whereas most arthropods have multiple cell types. The secretory and adhesive functions of the amebocytes makes them a key part of the immune system and this is also what makes them so good at recognizing pathogens for pharmaceutical applications.

The biochemical components and functions of the amebocytes and their clotting factors has evolved to protect horseshoe crabs from the vast amount of endotoxin present in their natural habitat and to ward off infection (Armstrong and Conrad, 2008). Endotoxins, or bacterial "pyrogens," are a structural component of the cell wall of a large group of bacteria called gram-negative bacteria (Novitsky, 1984). Most marine bacteria are of the gram-negative type, and seawater has been shown to have over 1 million gram-negative bacteria per milliliter and 1 billion bacteria can be found per gram of sand on spawning beaches (Novitsky, 1984). Thus, the protection from gram-negative bacterial infections has been conjectured to allow horseshoe crabs to survive throughout the millennia.

In 1885, W.H. Howell first described the clotting ability of *Limulus* blood, but it was not until the 1950s when Frederik Bang discovered the clotting agent in *Limulus* blood, creating Limulus Amebocyte Lysate (LAL; Novitsky, 1984). In Asia, there is a similar assay called TAL, named after the Asian species, *Tachypleus tridentatus.* Before LAL was discovered and used to test for, and immobilize endotoxins, living animals or living parts of animals were used to test for toxins in different compounds, thus making LAL the preferential new method (Mikkelsen, 1988).

To further understand the advantages of LAL, and its adopted use by pharmaceutical companies, it is useful to review how it is obtained and used. After blood is withdrawn from male and female horseshoe crabs, the amebocytes are separated from the plasma in the

hemolymph. The amebocytes are then lysed to release the biochemical components that constitute the active ingredients in LAL. However, each of the four biomedical companies use slightly different techniques in these steps throughout manufacturing LAL, as well as slightly different methods for reading the assay. The widespread LAL assay biochemistry is shown in Fig. 1 delineating the clotting enzyme cascade (Munford, 2016).

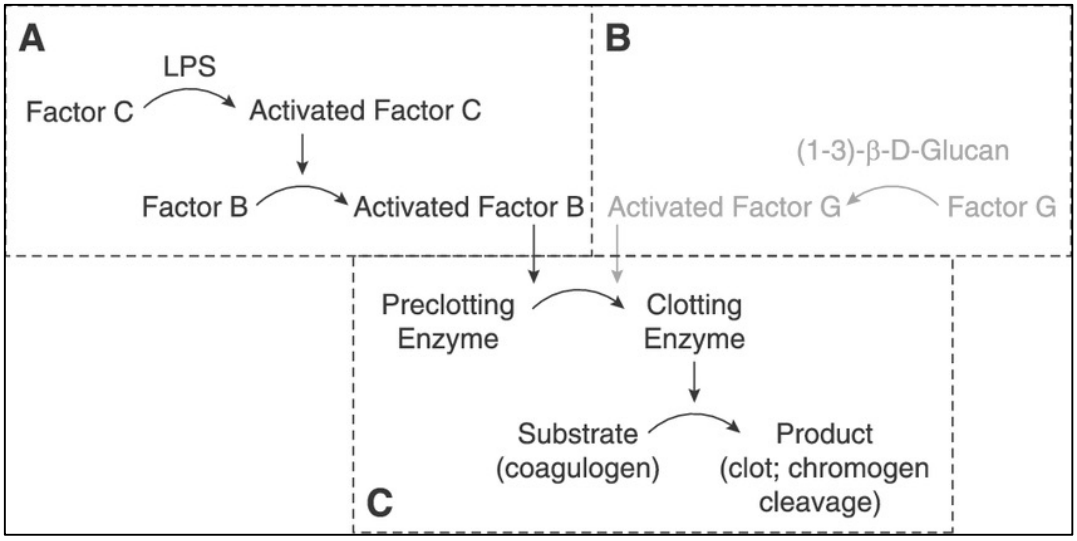

Figure 1. The clotting enzyme cascade in *Limulus* amebocytes in the LAL assay. The cascade is initiated by lipopolysaccharide (LPS) binding factor C (A) or the (1-3) β-D-glucan binding factor G (B). These pathways cause the clotting enzyme to form by cleaving the preclotting enzyme (C; Munford, 2016).

LAL is used to detect pyrogens on a wide range of different substances. For example, purified water, intravenous drugs, recombinant drugs, stored blood, and medical devices are all monitored for endotoxins using LAL. It is also used to test for endotoxins in fresh water, seawater and marine sediments, air quality, and, most astonishingly, in space (Charles River Laboratories, Inc., 2007; Novitsky, 2009). Furthermore, the biochemical components in horseshoe crab blood have been discovered to recognize fungi (specifically, those that contain β-D-1,3-glucan; Morita *et al.,* 1981), and LAL, consequently, has been cleared by the FDA to help diagnose invasive fungal infections (including *Aspergillus and Candida*). Thus, this substance

that originally evolved to aid in the survival of horseshoe crabs by preventing infections, is saving many human lives as well.

Hemocyanin

As previously mentioned, the most abundant protein in the blood of *Limulus* is hemocyanin. Hemocyanin performs the respiratory functions of the blood and is present at a very high concentration, averaging about 50 g per liter of blood (Schuster *et al.*, 2003). Most invertebrates' hemocyanins are made up of large aggregates of smaller protein subunits, but horseshoe crab hemocyanin has a huge protein complex made up of 48 polypeptide subunits (Brouwer *et al*., 1982). The multiple forms of hemocyanin in invertebrates have multiple functionalities. Primarily, hemocyanin plays a crucial role in transporting oxygen to respiring tissues (specifically book gills in *Limulus*). As compared to hemoglobin in vertebrate blood that turns red as the hemoglobin is oxygenated due to iron in the heme cofactor, chelicerate blood turns blue when the hemocyanin is oxygenated as it changes from a Cu I state to a Cu II state (Coates and Nairn, 2014). Besides oxygen transportation, hemocyanins are involved in other physiological processes that include hormone transport (Jaenicke *et al*., 1999), molting (Adachi *et al*., 2005a,b; Kuballa and Elizur, 2008; Kuballa *et al*., 2011; Glazer *et al*., 2013), and protein storage and osmoregulation (Paul and Pirrow, 1998).

There are several factors that affect these functions of hemocyanin in horseshoe crabs. One major factor is temperature. The rate of oxygen uptake depends heavily upon temperature; since horseshoe crabs have a Q_{10} of 1.84 between 15 and 25°C, oxygen uptake rate almost doubles with a 10°C change in temperature (Johansen and Petersen, 1975). With a rise in temperatures, the affinity of *Limulus* hemocyanin for oxygen decreases (reverse Bohr effect; Burnett *et al*., 1988). A decrease in blood pH helps to compensate for the effect of increasing

temperatures on hemocyanin. As the pH decrease increases oxygen affinity, the function of hemocyanin at different temperatures becomes stabilized. An example of this process in the course of a horseshoe crabs' daily life is that as they migrate onto spawning beaches that are at higher temperatures than nearby waters, this adaptation helps them to conquer these physiological demands and adequately spawn to lay eggs or release sperm. In addition, Coates *et al.* (2012) examined the effects of temperature on the biochemical properties of *Limulus,* and they found that an increase in temperature caused a decrease in hemocyanin concentration. Thus, it is important for horseshoe crabs to have a physiological mechanism to maintain homeostasis to function properly at challenging and variable environmental conditions.

Another challenge in the natural habitats of horseshoe crabs is dealing with limited oxygen in hypoxic environments. When horseshoe crabs are exposed to low oxygen conditions, they produce lactate. This lactate becomes metabolized into carbon dioxide (Gaede *et al.*, 1986). Along with lactate buildup is reduced blood pH (a result of metabolic acidosis). As dissolved oxygen passes over the gills, some of the oxygen binds to hemocyanin in the horseshoe crabs (Shuster *et al*., 2003). Related to temperature effects on oxygen affinity of hemocyanin, the ionic environment exerts a strong influence on its oxygen-carrying capacity, as well. Magnum *et al*., (1976) examined the effects of different levels of oxygenated waters on oxygen transport of hemocyanin in horseshoe crabs. They discovered that moderately oxygenated waters (those more similar to the waters in estuaries) would cause the oxygen delivery to be more efficient as compared to well-oxygenated waters where they suggested that hemocyanin would be fully oxygenated and not transfer enough of its bound oxygen to the tissues. Therefore, the increased oxygen affinity helps to remove oxygen from the environment proficiently.

Furthermore, this relates to the response of air exposure in horseshoe crabs during emersion, such as during the mating season. Since the gills are not able to release respiratory carbon dioxide as efficiently when they are in air, this leads to an increase in carbon dioxide in the blood and protons. Similarly to hypoxia, respiratory acidosis decreases hemolymph pH and causes an increased oxygen affinity. Interestingly, as a result, horseshoe crabs exposed to air can maintain oxygen uptake up to 36% of their level of uptake when immersed in water where 90% of the oxygen is transported by hemocyanin (Mangum *et al*., 1975; Burnett, 1988).

As horseshoe crabs show the reverse Bohr effect and cooperative oxygen binding, the carbon monoxide binding properties are very different. Bonaventura *et al*. (1974) revealed that *Limulus* hemocyanin binds carbon monoxide non-cooperatively. The carbon monoxide affinity is not affected by ionic conditions or pH as compared to the effects of these on oxygen affinity.

As a whole, there are many changes to the environmental conditions in the habitats of horseshoe crabs. Considering that these changes can alter hemocyanin levels and functions, it is vital that these organisms have different physiological adaptations to overcome these challenging circumstances.

BIOMEDICAL BLEEDING INDUSTRY

The Bleeding Process

Relatively speaking, the LAL extraction process is simple. Armstrong and Conrad (2008) developed a generally universal method that involves puncturing the cardiac sinus via the arthrodial membrane in adult horseshoe crabs to extract the hemolymph, centrifuging the blood to separate hemolymph from the amebocytes, and freezing the resulting supernatant (cell-free hemolymph) until the actual sterility test is designated to be performed. During this process, up to 30% of the estimated blood volume of individual horseshoe crabs is extracted, or until free-

flow stops and the blood starts to clot (Novitsky, 1984; James-Pirri *et al.*, 2012). Currently, there are four biomedical companies that manufacture LAL in the United States: Lonza (Walksersville, MD; formerly Cambrex Bioscience), Charles River Endosafe, Inc. (Charleston, South Carolina), Associates of Cape Cod (Falmouth, Massachusetts), and Wako Chemicals (Richmond, Virginia). Also, Limuli Labs (House, New Jersey) bleeds horseshoe crabs but does not produce LAL. Out of these companies, Lonza is the largest producer of LAL (Walls and Berkson, 2003), and, altogether, these companies perform 70 million LAL tests each year.

Each company has slightly different collection, handling, and bleeding practices, but an example of a complete bleeding process involves: 1) collecting horseshoe crabs through trawling, or hand-harvest on spawning beaches; 2) holding them on the deck of a boat or in 30-gal Rubbermaid containers for several hours; 3) transporting them to a bleeding facility in trucks (with or without air-conditioning); 4) holding the animals in a cold room (16-18°C) at the bleeding facility for a few hours or overnight; 5) removing ~30% of their blood volume, and then; 6) returning the animals to their original capture location to be released, often after holding them overnight (Hurton and Berkson, 2006). Female horseshoe crabs are preferentially selected for this process due to their larger size and subsequently greater blood volume (James-Pirri *et al.*, 2012). Estimated blood volume via exsanguination has revealed discrepancies in data due to the fact that horseshoe crabs have an open circulatory system, allowing a significant amount of blood to remain in the body in tissue spaces, muscle, and sinuses (Shuster 1982a; Hurton *et al.*, 2005). Regardless, horseshoe crabs are subjected to significant blood loss, as well as other stressors (heat and air exposure) throughout this procedure, which raises concerns about the sustainability of this fishery.

Harvest Regulations and Ecological Implications

Horseshoe crabs are managed by the National Marine Fisheries Service (NMFS) in federal waters (>3 nautical miles offshore) and by the Atlantic States Marine Fisheries Commission (ASMFC) in non-federal waters. In order to successfully manage and provide regulations for any fishery, it is imperative to have scientific information on the status of the population and on population dynamics. However, insufficient data are collected for horseshoe crabs, even they have been harvested for more than 100 years. In the past, horseshoe crabs were considered a "trash-fish" that was not deemed worthy of allocating agency resources and providing proper management to this fishery (Berkson and Shuster, 1999). However, with recent increased scrutiny and concern over the management of this species, spawning surveys and assessments were established in 1990. However, in 1998, an independent Peer Review Panel deemed the methods of these endeavors statistically unsound, lacking standardized methodology, and limited sampling (ASMFC, 1998). This has led to an apparent need for more effective management and efforts to gather more accurate data to informatively manage this fishery, and, as a result, several positive management actions have taken place since 1998.

For the bait fishery, a quota system and several complete closures of coastal waters have been implemented (ASMFC, 1998; ASMFC, 2012). This has caused a significant decrease in harvest levels of horseshoe crabs, from around two million crabs per year in 2000 to 600,000-700,000 crabs per year in 2014 (ASMFC, 2013). In contrast, overall harvest levels for the biomedical industry continue to increase, with levels climbing from 340,000 crabs in 2004 to 610,000 crabs per year in 2012 (ASMFC, 2013). As a whole, the 2013 stock assessment update revealed horseshoe crab abundance continues to decrease in the New York and New England regions, increase in the Southeast (North Carolina down to Florida), and remain stable in the

Delaware Bay region (New Jersey, Delaware, Maryland, and Virginia). The stable population in the Delaware Bay region could be attributed to the harvest restriction of a male to female ratio of 2:1 crabs landed in Virginia (ASMFC, 2012) and prohibition of landing female horseshoe crabs in 2013 in MD (Butowksi and Morin, 2016). For the 2016-17 fishing seasons, the Horseshoe Crab Management Board from the ASMFC set harvest levels in the Delaware Bay region to a limit of 500,000 *male* horseshoe crabs only. This will hopefully continue to preserve the stabilization of the Delaware Bay region horseshoe crab population and influence other areas to adopt these protocols.

There are also several specific regulations concerning the harvest of horseshoe crabs for biomedical purposes. In order to harvest *Limulus* for LAL, biomedical companies must have a collection permit and are required to submit monthly reports on harvest numbers and mortalities before they are released (including mortalities during collection, shipping, handling, traveling, and bleeding; Walls *et al.*, 2002). During collection and up until blood extraction, horseshoe crabs must be inspected to cull out any moribund or damaged animals (ASMFC, 2013). All of the biomedical companies comply with the ASMFC Best Management Practices (BMP) document, but the specifics of those practices vary from company to company. For example, in the state of Massachusetts (MA), Associates of Cape Cod (ACC) is the only biomedical bleeding facility that manufactures LAL. In MA, their letter of authorization (LOA) states that ACC cannot accept more than 1,000 horseshoe crabs from any licensed harvester in a 24-hour period, must return the crabs in good condition to site of harvest, and mark the crabs to prevent the same animal from being re-bled (Perry, 2016). Furthermore, ACC is required to transport collected animals in a temperature controlled truck set at 10-16°C, the temperature cannot exceed 21°C in their laboratories, animals must be kept moist during transport and in the laboratories, and the

storage barrels should remain less than 2/3 full to prevent compression of crabs on the bottom (Perry, 2016). However, not all of the companies follow this exact protocol, and an important observation is that not all companies provide public access to, or communication about, their procedure. As a matter of fact, 100% of the companies do not make horseshoe crab collection and mortality data accessible to the public. It must be cited from papers, personal communication, or other sources. Thus, it remains elusive as to what the precise BMP's are being carried out in all of the facilities.

Besides the specifics of their BMP procedure, along with obtaining permits and pre-screening for injured crabs, the facilities maintain several other universal regulations. They hold the animals in an aseptic environment, use sterile needles and return crabs within 24-72 hours as mandated by the United States Food and Drug Administration (ASMFC, 1998). Since 2001, rather than return them to their natural habitat, these companies can sell bled crabs to the bait market (ASMFC, 2001), but only one company (Associates of Cape Cod) actually implements this routine. Up until 2009, there was no system in place for marking bled crabs to prevent individuals from being bled more than once in a single season (Leschen and Correia, 2010), and currently, only a few of the biomedical companies are tagging their crabs before release. Further, in 1998, ASMFC instructed that all companies manufacturing LAL must assess mortalities from the bleeding process (Schrading *et al*., 1998). From these estimated mortalities, there was a crab cap employed in 2007 and a bled and release mortality threshold of 57,500 crabs was put into place, assuming that number was 15% of the total biomedical harvest (Gauvry, 2015). Since 2007, the coastwide biomedical harvest has increased above this designated threshold (Table 1), suggesting the need for improvements to the present regulations. Thus, with the consistent overages of the mortality threshold, the ASMFC Plan Review Team suggested that the ASMFC

Management Board take actions to evaluate the biomedical mortality and use of horseshoe crabs in the next Adaptive Resource Management review (Butowski and Morin, 2016).

Table 1. Number of horseshoe crabs harvested for LAL and the estimated mortalities (assuming 15% mortality) associated with the bleeding process from 2005 to 2012 and 2015. The numbers in bold represent the years after the cap bled and release mortality threshold was set to 57,500 (assuming 15% mortality), clearly showing that is has surpassed this threshold every year since the institution of that threshold (ASMFC, 2013; Gauvry, 2015).

	2005	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2015**
Harvested for LAL	323,149	367,914	500,251	511,478	512,552	482,704	628,476	611,827	559,903
Total Mortality	44,830	49,182	**63,432**	**63,285**	**60,642**	**75,428**	**80,827**	**79,786**	**70,223**

Several other population assessments of horseshoe crabs continue to raise concerns. One problem is that in several annual reports of horseshoe crab landings to the ASMFC, companies report 'unclassified crabs' (meaning they did not sex them). For example, in Massachusetts, the bait harvest in 2016 was 22,226 females, 19,210 males, and 56,205 unclassified crabs (Perry, 2016). This could lead to inexplicable issues for population dynamics, especially if there are significantly more female horseshoe crabs being captured. Another major concern is that the related TAL fishery in Asia is poorly regulated and unmonitored with a possible declining population (Gauvry, 2015). The underlying major problem is that if the *Tachypleus* population cannot sustain TAL production for the pharmaceutical industries in Asia, these companies will become dependent on the already limited supply of LAL from the USA. Clearly harvesting of horseshoe crabs for TAL is not sustainable. The mortality rate is high, in large part because bled animals are sold to secondary markets for chitin production and human consumption (Gauvry, 2015). Therefore, with the impending growing demand for LAL/TAL as our global population expands and medical advancements improve and/or prolong life, it is debatable as to whether we

will be able to meet the demands for LAL and TAL, especially with the Asian horseshoe crab species being decimated.

Alternatives to using horseshoe crabs for LAL and as bait have been investigated and several replacements have been found, but industries are reluctant to transition away from traditional methods and undergo further regulatory testing from the FDA. These methods include the Recombinant Factor C (rFC) derived from horseshoe crab DNA and the Monocyte Activation Test (MAT) that uses human blood instead of horseshoe crab blood. *In vitro* techniques to maintain viable horseshoe crab amebocytes are currently being attempted, as well as optimizing aquaculture conditions for this species (Coates *et al.*, 2012). More recently, the Horseshoe Crab Ranch and Blood Institute, a project designed by Kepley BioSystems Inc. (Greensboro, NC), is promoting the harvest of horseshoe crab blood from controlled aquaculture pens in a protected estuary where horseshoe crabs would be monitored, fed, and carefully bled. They are looking to establish optimal protocols for "ranching" horseshoe crabs and aiming to reduce environmental and physiological stress on these animals, as compared to traditional practices of wild capture and harvest. They would also estimate ameboycte production, a scientific area that is currently lacking in research. But, even given these advances, most companies continue to use LAL for measuring endotoxins.

In regards to replacements to horseshoe crabs as bait, several alternative measures have been introduced to the bait fishery, but not necessarily exploited. Since it takes over a week for eels and whelks to be habituated to new bait, some fishermen are reluctant to switch to a different bait (Walls *et al.*, 2002). Waste from fisheries, green crabs, surf clams, cownose rays, and other substances have been examined as potential baits, but none have been as attractive as horseshoe crabs to eels and whelks. This might be because there appears to be a chemical cue

found in horseshoe crab eggs' and blood that is thought to attract eels and conchs (Walls *et al*., 2002). Studies are currently exploring inclusion of *Limulus* blood as a bait ingredient (M. Owings, unpublished data), especially since *Limulus* hemolyph would be easily accessible as it is a by-product from the bleeding process.

PREVIOUS WORK ON BLEEDING IMPACTS ON LIMULUS POLYPHEMUS

Mortality

Prior to recent harvest implementations and sporadic stock assessments, it is astounding that, although horseshoe crabs have been used since the 1970s for LAL, no studies were completed to understand the impacts of blood extraction on horseshoe crabs until 1983. Most studies determined mortality rates from the bleeding process (Table 2). Overall, there was a greater increase in mortalities in bled animals (8-30%) as compared to unbled animals (0-2.6%). One study by Leschen and Correia (2010) continued to examine the mortality rates in bled horseshoe crabs, but expanded their study to identify the implications that the estimated mortality rate would have on the current management of the biomedical fishery. Since harvesting occurs primarily directly after the spawning season, females are preferentially selected for due to their size, and are likely to be more physiologically stressed due to the energy expended during egg production (Leschen and Correia, 2010; James-Pirri, 2012), they looked specifically at mortality rates in bled female horseshoe crabs versus unbled female horseshoe crabs. The mortality rate was significantly lower in controls than the bled females (controls: 3%, bled: 22-29.8%). Mortality rates in this study were double those that are used to make regulations and current management procedures in the biomedical industry as described above (5-15%; ASMFC, 2013). Since the biomedical fishery is considered a low mortality "catch and release" fishery (Berkson

and Shuster, 1999), the bait fishery and biomedical fishery are managed differently. Therefore, since mortality seems to be higher than what is currently presumed, management agencies should consider revising their harvest protocols and regulations.

Table 2. Studies of mortality rates in *Limulus polyphemus* due to the biomedical bleeding process. Bled (bold **B**) mortality rates are higher in all studies than control (C) rates.

Author/Year	Location	Mortality		Notes (Type of Study, Experimental Design, etc.)
		Duration of Study	Experimental Groups	
Rudloe, 1983	St. Joseph's Bay, FL	First-year post-release	C: 0% **B: 10%**	Tag-recovery; hemolymph extraction only
		Second-year post-release	C: 0% **B: 11%**	
Thompson, 1988	Bull's Bay, SC	One-week post-bleeding	C: 0% **B: 15%**	Full bleeding process at commercial bleeding facility
Walls & Berkson, 2000	Chincoteague, VA	Two-week post-bleeding	C: 0% **B: 15%**	Full bleeding process at commercial bleeding facility
Kurz and James-Pirri, 2002	Cape Cod, MA	26-day monitoring period	C: 0% **B: 20%**	Telemetry study; hemolymph extraction only
Walls and Berkson, 2003	Chincoteague, VA and Ocean City, MD	Two-week post-bleeding	C: 0.5% **B: 8%**	Full-bleeding process at commercial bleeding facility
Hurton and Berkson, 2006	Ocean City, MD	10-30% hemolymph extracted; one-week post-bleeding	C: 2.6% **B: 8.3%**	Held in laboratory; full bleeding process but different levels of hemolymph extracted
		40% hemolymph extracted; one-week post-bleeding	C: 2.6% **B: 29.4%**	
Leschen and Correia, 2010	Woods Hole, MA	One-week post-bleeding*	C: 3% **B: 22-29.8%**	*Females only; full bleeding process at commercial bleeding facility
Anderson *et al*., 2013	Great Bay Estuary, NH	Two-week post-bleeding	C: 0% **B: 18%**	*Held in laboratory; full bleeding process

Sublethal Impact of Bleeding

Other than the tag/recapture study by Rudloe (1983), all mortality data have been collected from animals that have yet to be released into their natural habitat. Moreover, even if they do not die from the procedure, the process might have sublethal behavioral and physiological effects that could lead to long-term decreases in the population. The first two studies of such impacts were published by James-Pirri *et al.* (2012) and Anderson *et al.* (2013). James-Pirri *et al.* (2012) analyzed selected hemolymph constituents from captive, biomedically bled, and wild caught adult horseshoe crabs handled according to BMP at Woods Hole, Massachusetts. They discovered that captive bled animals in the BMP treatment group had lower protein values signifying that biomedical bleeding may have prolonged impacts on horseshoe crab physiology. This result was similar to the discovery by Novitsky (1984), where it was found that it takes 3-7 days for a bled horseshoe crab to regain its total blood volume, but it takes up to 4 months for amebocyte cell counts to return to baseline levels they were at before bleeding. Interestingly, James-Pirri *et al.*, (2012) also reported that bled crabs released into their natural habitat exhibited a random direction of movement compared to the pattern of movement seen in the control animals, suggesting that the orientation in the bled group was more greatly affected. Anderson *et al.*, (2013) investigated the impacts of bleeding on locomotion and hemocyanin levels in horseshoe crabs collected from the Great Bay Estuary, New Hampshire. They found changes in the bled animals' activity levels and expression of circatidal behavioral rhythms, a reduced ability to orient in the laboratory, significant decreases in their linear and angular velocity, and chronic reductions in hemocyanin levels.

In summary, the bleeding process leads to high mortality rates, and has detrimental sublethal behavioral and physiological effects that have negative implications for the long-term

survival and fitness of horseshoe crabs, especially females. Moreover, as indicated by the study by Hurton and Berkson (2006), the stressors associated with the whole bleeding process, not just the loss of blood, probably play a role in causing both mortalities and sublethal effects. Clearly, this literature review highlights the need for more research to determine which stressors are most deleterious to horseshoe crabs, the impacts of bleeding on both horseshoe crab physiology and behavior, and the extent to which the bleeding process impacts animals after they are released into their natural habitat.

OBJECTIVES

The overall objective of this project was to determine whether the biomedical bleeding process has sublethal behavioral and physiological impacts on *Limulus polyphemus* in the laboratory and in their natural environment. My specific objectives were:

1. Determine if bleeding alters the behavior of horseshoe crabs once they are released back into their natural habitat.
    a. Will they express normal seasonal migrations?
    b. Will they approach mating beaches?
    c. Will they express normal tidal and daily rhythms?
2. Determine which of three stressors associated with the bleeding (high temperature, air exposure, or blood loss) process has the greatest impact on the behavior and physiology of bled horseshoe crabs in the laboratory.
3. Test a food supplement that might be able to reduce the sustained decreases in hemocyanin levels seen in animals that are held in captivity and as a result of the bleeding process, as well as the associated behavioral impacts.

In Chapter One, I present data concerning the impacts of bleeding on the behavior of horseshoe crabs in their natural environment, specifically in the Great Bay Estuary (GBE), New Hampshire. A total of 14 horseshoe crabs were exposed to the bleeding practices followed by biomedical facilities, and then released back into the GBE after fitting them with acoustic transmitters. Another 14 animals, that were not bled, were also released with transmitters as controls. The movement of these animals, as well as times when they were active and their preferred depths, were monitored for ~ 2 years. These data were used to discern if there are differences in spawning activity, expression of rhythms, distances traveled, migratory patterns, and overall activity between bled and unbled animals.

In Chapter Two, I present data concerning the effects of three different stressors (air exposure, increased temperature, and blood loss) on horseshoe crab behavior and hemocyanin levels. In addition, I present data about the seasonal changes in the hemocyanin levels in animals collected each month from the estuary. These data demonstrate that bleeding alone does not have as much of a negative impact on their behavior as bleeding combined with the associated stressors. In addition, I show that hemocyanin levels in animals in their natural habitat varies considerably between May and October. The results from these experiments can be used to provide information to management and regulatory agencies on more sustainable practices for the bleeding industry.

In Chapter Three, I present data evaluating the effects of a food supplement on the hmeocyanin and locomotor activity in bled animals. In addition, I present data regarding hemocyanin levels in bled animals that are held in their natural environment that have access to marine sediment to forage. These data demonstrate that our food supplement that contains green crab extract *and* spent *Limulus* hemolymph have a beneficial impact on reducing the effect of

bleeding on hemocyanin levels and behavior in horseshoe crabs. Using these findings, hopefully a pelleted feed can be created to be distributed to biomedical facilities to be given to bled horseshoe crabs before they are released into their natural habitat.

CHAPTER 1: IMPACT OF BIOMEDICAL BLEEDING ON HORSESHOE CRAB (*LIMULUS POLYPHEMUS*) BEHAVIOR IN THEIR NATURAL HABITAT

Abstract

Horseshoe crabs are harvested by the biomedical industry in order to create Limulus Amebocyte Lysate (LAL) to test medical devices, vaccines and pharmaceutical drugs for pathogenic gram-negative bacteria. Previous studies of the impacts of the biomedical bleeding process on horseshoe crabs have primarily focused on mortality rates and sublethal impacts in the laboratory and two limited field studies. In this study, we focused on the effects of the bleeding process on horseshoe crab behavior once they are released back into their natural environment. A total of 28 horseshoe crabs (14 control and 14 bled) were fitted with acoustic transmitters and released into the Great Bay Estuary, New Hampshire during the spring of 2016. The acoustic tags transmitted information about each animals' activity (acceleration) and depth, and these data were logged by an array of VR2W receivers. These data, along with their locations, were collected from May-December of 2016 and from March-October 2017. There were several immediate impacts of the bleeding process on mortality rates and mating behaviors between the bled and control treatments. Overall, bled animals appeared to mate significantly less than control animals within the first week post-release (bled: 61%, controls: 80%), with larger differences between bled and control females (bled: 38%, controls: 85%) during this time. There were also long-term impacts on horseshoe crab movements, rhythms, and mating events across seasons and years. For example, bled animals remained significantly deeper during the spawning season than control animals; bled animals changed from daily rhythms in June 2016 to arrhythmic behaviors in June 2017; and bled animals did not mate as often in 2017. These negative impacts on horseshoe crab behavior likely have significant impacts on foraging and mating, as well as maintaining sustainable populations of horseshoe crabs in areas where they are harvested for the biomedical industry.

Introduction

The American horseshoe crab, *Limulus polyphemus,* is an ecologically and economically important species found in bays and estuaries along the Atlantic coast in North America, including the Great Bay Estuary, New Hampshire (GBE). They play an important ecological role as bioturbators, as a result of foraging for food (Krauter and Fegley, 1994; Lee, 2010), and their eggs are a vital food source for up to 425,000-1,000,000 migratory shorebirds (Walls *et al.*, 2002; Botton *et al.*, 2010). They are also harvested for use as bait for the eel and whelk fisheries (ASMFC, 1998; ASMFC, 2012), and for their blood, which is used to create Limulus Amebocyte Lysate (LAL; Novitsky, 1984). LAL is used in the biomedical industry to test medical devices,

vaccines, and pharmaceutical drugs for pathogenic gram-negative bacteria (Novitsky, 1991; Chen and Mozier, 2013).

Currently, while quotas and regulations have been placed on the bait fishery (ASMFC, 2012), the biomedical fishery remains fairly unrestricted and harvest rates continue to increase in certain areas (ASMFC, 2013), which could have deleterious effects on populations of this valuable marine resource. A quota system and several complete closures of coastal waters have been implemented for harvesting horseshoe crabs for the eel and whelk fisheries (ASMFC, 1998; ASMFC, 2012), which has caused a significant decrease in the commercial harvest levels of horseshoe crabs, from around two million crabs in 2000 to 600,000-700,000 crabs in 2014 (ASMFC, 2013). In contrast, overall harvest levels for the biomedical industry continue to increase, with levels climbing from 340,000 crabs in 2004 to 610,000 crabs in 2012 (ASMFC, 2013). However, since 2007, the coastwide biomedical harvest has increased above this designated threshold, suggesting the need for improvements to the present regulations.

With the growing demand for LAL as our global population expands, medical advancements improve, and medical needs increase, it is critical to understand the consequences of the biomedical bleeding industry on horseshoe crabs' fitness and population dynamics. The capture process includes multiple stressors, such as increased air exposure (time on docks and boats and trucks) and increased temperatures (remaining in direct sunlight or poorly temperature-controlled facilities and transport vehicles). In addition, the blood extraction process can also compromise the health of the horseshoe crab, as ~30% or more of the estimated blood volume of individual horseshoe crabs is harvested (Novitsky, 1984; James-Pirri *et al.*, 2012). Female horseshoe crabs are also preferentially chosen for this process due to their larger size and subsequently greater blood volume (Rutecki *et al.*, 2004; James-Pirri *et al.*, 2012), which has led

to skewed sex ratios (Leschen and Correia, 2010). Each of the four major biomedical companies that bleed horseshoe crabs has slightly different collection, handling, and bleeding processes, but, nonetheless, horseshoe crabs are subjected to significant blood loss, as well as heat and air exposure stressors. This raises concerns about the sustainability of the fishery and the long-term impacts on horseshoe crab populations.

Although most biomedical companies employ variations of the ASMFC Best Management Practices (BMP), mortalities and sublethal effects on horseshoe crabs have been reported. Mortality rates associated with the bleeding process range from 5-30% (Rudloe, 1983; Thompson, 1998; Walls and Berkson, 2000; Kurz and James-Pirri, 2002; Walls and Berkson, 2003; Hurton and Berkson, 2006; Leschen and Correia, 2010; Anderson *et al.*, 2013) with a differential mortality rate between sexes (15% mortality in males and up to 29% in females; Leschen and Correia, 2010; James-Pirri, 2012). Sublethal impacts include delayed blood volume recovery, reduced blood protein levels, and behavioral deficits. Specifically, Novitsky (1984) found that in aquarium studies that it takes 3-7 days for a bled horseshoe crab to regain its total blood volume and up to 4 months for amebocytes to return to baseline levels. Captive bled animals exhibit significantly lower blood protein values, signifying that biomedical bleeding may have prolonged impacts on horseshoe crab physiology, and bled crabs released back into their natural environment displayed a significant random direction of movement compared to the movement patterns in control animals (Kurz and James-Pirri, 2002; James-Pirri *et al.,* 2012). Finally, and most recently, Anderson *et al.* (2013) examined the impacts of bleeding on locomotion and hemocyanin levels in horseshoe crabs in the laboratory and found changes in the bled animals' activity levels, expression of circatidal rhythms, significant decreases in their linear and angular velocity, and chronic reductions in hemocyanin levels.

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

With the exception of a study by Rudloe (1983) on the mortality rates of bled animals, and the study by James-Pirri *et al.* (2012) regarding bleeding impacts on horseshoe crab orientation, all relevant studies regarding biomedical bleeding effects on horseshoe crabs have been collected from animals in the laboratory. Therefore, the major goal of this project was to determine the behavioral and physiological effects that the bleeding process has on horseshoe crabs that are released back into their natural environment. The animals in this study were collected from, and released back into, the Great Bay Estuary (GBE), because this population of horseshoe crabs has not been previously harvested for biomedical bleeding (ASMFC, 2012) and previous studies have described the behavior of horseshoe crabs in this estuary. This is advantageous since we have a background knowledge of their seasonal movements and

behaviors, as well as it allows us to attribute our findings as results of the bleeding process since these animals have not been previously bled. For example, Schaller *et al.* (2010) and Watson *et al.* (2016) found that horseshoe crabs remained in GBE year-round, but changed depths and locations in the estuary as temperatures changed throughout the year. In the spring (March-April), when water temperatures exceeded 10-11°C, animals traveled to shallower areas and moved to spawning beaches at high tides. After spending the summer and early fall scouring the mudflats for food, they moved down the estuary into deeper waters in the late fall to overwinter. We can use this background knowledge in the literature to compare our findings to these expected behaviors and discern if there are differences due to the bleeding process.

In this study, before being released into GBE, designated animals underwent the bleeding process and then all animals, both bled and controls, were fitted with acoustic transmitters to monitor their movements, the times when they were active, and their preferred depths. These data were collected for ~ 2 years and used to discern if the bleeding process had an impact on spawning activity, expression of daily and tidal rhythms, overall activity and distances traveled, and seasonal migrations.

Methods

ANIMAL COLLECTION AND TAGGING

A total of 28 healthy adult horseshoe crabs (14 male, 14 female) were hand-collected during high tide from a spawning beach on Adams Point, Durham, New Hampshire (Fig. 1.1) in May 2016. All captured crabs were brought back to the University of New Hampshire Jackson Estuarine Laboratory (JEL) to be held in flow-through estuarine-water tanks until they underwent their designated treatment. Half of the animals (7 males: IO width- 8.21 cm ± 0.61, 7

females: IO width- 11.56 cm ± 0.52) were used as controls, while the remaining half (7 males: IO width- 8.31 cm ± 0.47, 7 females: IO width- 11.42 cm ± 0.63) were bled according to the industry-standard procedures followed by the biomedical bleeding facilities, as outlined below.

After treatment, all horseshoe crabs were fitted with VEMCO V13AP ultrasonic transmitters (69 kHz, 147 dB low power output, 13 mm diameter, 48 mm long, 6.5 g in water, estimated battery life ~ 530 days, VEMCO, Bedford, Nova Scotia, Canada). The V13AP transmitters were programmed to transmit acceleration and depth data at random intervals approximately every 3 minutes. They were also programmed to turn off in December 2016 and then turn back on in March 2017. Transmitters were attached to the dorsal carapace of each individual using the following method. First, they were superglued into a piece of Tygon tubing that had two cable ties attached to it. The cable ties were then affixed to the carapace using small screws. Finally, duct tape was super-glued over the entire harness to ensure that the fixture would not become caught on underwater obstructions (Fig. 1.2). In addition, male claspers were super-glued shut to eliminate their ability to attach to females. This ensured that data from males represented their activities and not those of a female to which they were attached.

After the transmitters were attached, the animals were released into the Great Bay Estuary at the same place they were previously collected.

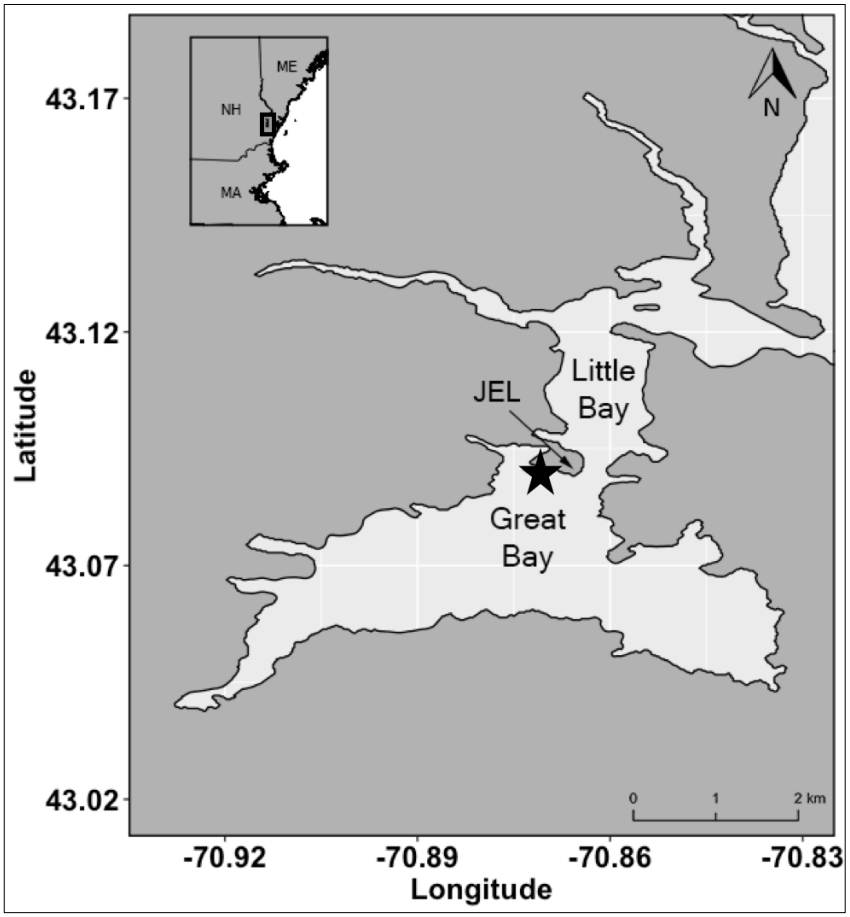

Figure 1.1. Map of study location in the Great Bay Estuary, NH, USA. Black star represents collection/release site at Adam's Point, Durham, NH. The black arrow indicates the location of the Jackson Estuarine Laboratory (JEL).

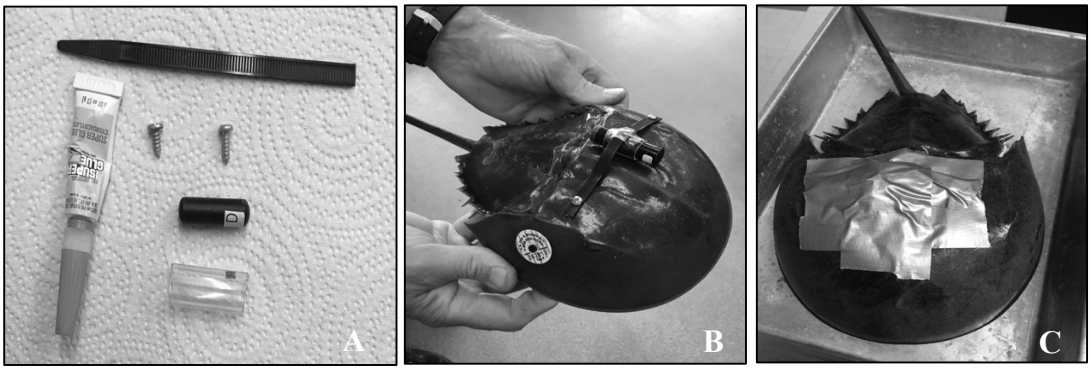

Figure 1.2. Transmitter attachment on dorsal carapace. The materials needed for attaching the transmitter include superglue, Tygon tubing, small screws, and a large zip tie (A). The transmitter was affixed towards the rear of the prosoma and slightly off-centered (B). Duct tape was used to cover the attachment to ensure that the horseshoe crab did not get caught on underwater debris (C).

BLEEDING PROCEDURE

Pre-bleeding Treatment

The bleeding process, replicating industry-standard procedures, took a total of three days (13 to 15 May 2016). Half of the 28 horseshoe crabs (n=14; 7 males, 7 females) were randomly selected to undergo the bleeding procedure. The animals in this treatment group were evenly distributed between two 50-gallon plastic barrels. HOBO temperature loggers were placed in each of the barrels to record temperature. The control animals (7 males, 7 females) remained in the flow-through tanks at JEL (14.1 ± 1.4 °C; mean ± SD) until transmitters were attached and they were released.

The 60-gal barrels with the treatment animals were placed outside of JEL in direct sunlight for 4 h or next to a space heater in the JEL greenhouse (depending upon the temperature and ambient sunlight during the selected day), to replicate the duration of time spent on the deck of boat or dock prior to transport to biomedical facilities. The average temperature they experienced during this time was 32.6 ± 2.7 °C. After the first 4 h, the barrels were placed in the back of a car and driven around for an additional 4 h to simulate time spent in a truck traveling to a bleeding facility (23.2 ± 1.7 °C). After these 4 h, the barrels were placed indoors for 16 h at JEL to simulate time spent overnight at a bleeding facility (20.7 ± 0.6 °C). Finally, after 16 h, hemolymph was extracted as described below.

Hemolymph Extraction

Hemolymph was extracted following the procedure of Armstrong and Conrad (2008), with modifications from Anderson *et al*. (2013). The arthrodial membrane between the prosoma and opisthosoma of each horseshoe crab was exposed, and the hinge joint was sterilized with 70% ethanol. An 18-ga syringe needle was inserted into the membrane and hemolymph was

collected in pre-chilled 50 mL conical tubes until 30% of total hemolymph volume had been reached or until the blood flow stopped. The collected hemolymph was then placed on ice until further examination. Total hemolymph volume for each individual was calculated using the following equation from Hurton *et al*. (2005):

$$H = 25.7\, e^{0.1928(IO)}$$ *[H= hemolymph volume (mL); IO= inter-ocular width (cm)].*

Hemocyanin concentrations were measured using the procedure of Coates *et al.* (2012). Hemolymph samples were centrifuged for 10 minutes at 3000 g and 4°C. Then, an aliquot of hemolymph was diluted 1:100 in 0.1 M Tris-HCl buffer (pH 7.5) in a quartz cuvette with a path-length of 1 cm. The absorbance was measured at 280 nm on a Ultrospec 3100 pro UV/Visible spectrophotometer (Artisan Technology Group, Champaign, IL). Values were standardized using a pre-determined value of 1.39 mg/mL hemocyanin solution. This value was verified using a Pierce Modified Lowry Protein Assay Kit (ThermoFisher Scientific Inc, Waltham, MA).

Post-bleeding Treatment

To minimize the influence of handling on the horseshoe crabs' behavior, immediately after the treated animals were bled, transmitters were attached and the animals returned to their respective barrel. We did not attach transmitters to animals that appeared to be unhealthy (2 animals) or died (2 animals) during the procedure before release. We only used 28 animals that we anticipated we would produce at least one week's worth of data from to avoid the possible loss of a transmitter. For the control animals, transmitters were attached at the same time, but they were returned to the flow-through tanks at JEL. The bled animals remained in their barrels overnight for another 24 h to replicate a second night at a bleeding facility (20.5 ± 1.1 °C). Then, the barrels were placed back into a car for 4 h (21.9 ± 0.9 °C) to simulate transportation back to the dock, where they would be loaded on vessels and returned to their capture location. Finally,

all 28 horseshoe crabs were returned to their collection site at Adam's Point and released into the estuary.

ACOUSTIC TELEMETRY/TRACKING

An array of VR2W acoustic monitoring receivers (n=11; 69 kHz; VEMCO, Bedford, Nova Scotia, Canada) was set up throughout GBE ranging from Fox Point to the Great Bay Discovery Center. The receivers were deployed ~ 0.5 km apart, either attached to a mooring line, suspended ~ 5-10 m from the bottom, or placed in an empty lobster trap, depending on the depth of the listening station (some areas were only ~2 m deep at low tide). Each receiver was capable of detecting a horseshoe crab with a transmitter attached within a 200-500 m range of the receiver due to range tests. The variability in the range is due to currents, turbidity, topography, weather events and high winds potentially affecting the detection range. The transmitters were programmed to take depth (m) and triaxial (*x, y, z*) (acceleration (m s^{-2}) readings at 5 Hz within a 25 s period. Then, every 70-140 s the acceleration data (root mean square of the three axes, see formula below) obtained during the most recent 25 s period (T), or the depth at that time, would be transmitted (depth and acceleration would alternate).

$$\text{m s}^{-2} = \sqrt{x^2 + y^2 + z^2} \text{ averaged over T.}$$

Based on specifications for these transmitters, the proportion of time for which acceleration was measured was only 12% of every 2 to 5 min. This range is dependent on environmental conditions, such as wind speed, fouling on the hydrophone, etc. Each time a transmission was received, the receiver would record the date and time, acceleration, and depth of the animal.

Receivers were downloaded in VUE Software 2.3.0 (VEMCO, Bedford, Nova Scotia, Canada) every 1-2 weeks in the spring and summer, ~once every month in the fall, and removed from the water in the winter after the date the transmitters were programmed to shut off. After each download, if the receiver had multiple detections of different animals, we would keep the receiver in the same location. If the receiver did not contain a viable amount of detections, we would move the receiver to a more suitable location. On several of the receiver mooring lines, HOBO temperature data loggers (Onset Computer Corporation, Bourne, MA) were attached to record water temperature throughout the duration of the project. The temperature data loggers were placed at a receiver near the release site, JEL, and in the middle of Little Bay. Towards the bottom of Great Bay, temperature data was collected by a buoy in Great Bay that was provided by the NOAA National Estuarine Research Reserve System (NERRS). This buoy was located on the outskirts of the deep channel, with the temperature logger ~1m below the surface of the water.

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

DATA ANALYSES

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

For analyses, we chose to use only data from animals that were detected for more than seven days in a row in a given month. Three-way ANOVAs were used to test for effects of treatment group (bled and control), years (2016 and 2017), and sex (males and females) on days at large, and, also, on ranges in the estuary. Days at large was calculated as the first day in a season an animal was detected until the last day an animal was detected. Ranges were measured as the distance from the animal's furthest up-estuary position to furthest down estuary position. Two-way ANOVAs were used to test for effects of treatment groups and sex on mating and, also, on depth changes. A MANOVA was used to look at the impacts of sex, treatment groups, and months on percentage of activity and depth in different animals. In all cases, the percentage of activity, movements, and rhythms of control animals were compared to the experimental animals. Tukey's HSD *post hoc* analyses (with a level of significant difference set at $p < 0.05$) were used to examine differences between means of treatment groups, monthly depths, rhythms expressed at different depths, and sexes. Correlational and single linear regression analyses were

used to determine relationships between temperature and years, as well as activity and depth across months. Unpaired Student's t-tests were used to compare depth or activity in one between treatment groups.

To determine if horseshoe crabs were approaching spawning beaches, changes in animals' depths and tide heights were examined together. Water depth/tide height data was obtained from the Squamscott River Monitoring Station (data provided by the National Estuarine Research Reserve Centralized Data Management Office) and the Great Bay Buoy (data provided through NERACOOS, COOA Great Bay, University of New Hampshire). Horseshoe crabs were considered 'spawning' if they showed high activity levels (> 0.1 m s^{-2}; Watson *et al.,* 2016) around the times of high tides commensurate with a more shallow location, during the spawning period of May-July 2016. The first two-weeks post-release were used for this analysis.

Results

Days-at-Large and Days Detected

A total of 28 horseshoe crabs were successfully tracked in the GBE, from 15 May to 6 December in 2016 (205 days), and data were also obtained from 23 of these between 14 April and 4 October (191 days) in 2017. In 2016, all animals were at large for an average of 158 ± 59 days (max = 205, min = 7) and were detected for an average of 84.3 ± 50 days (max = 180, min = 8; Fig. 1.3). In 2017, all animals were at large for an average of 91 ± 56 days (max = 172, min = 1) and were detected for an average of 32.4 ± 41 days (max = 171, min = 0).

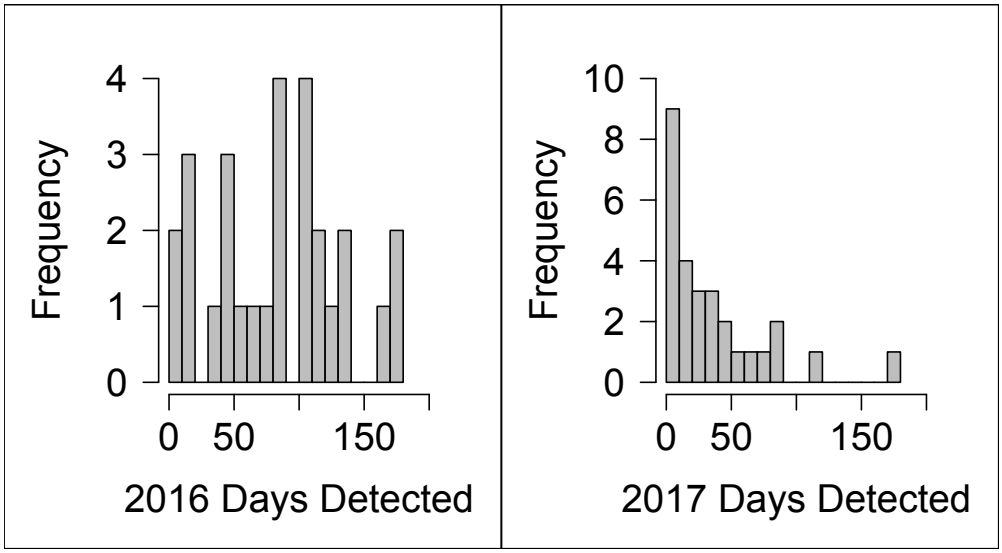

Figure 1.3. Days detected for horseshoe crabs in 2016 (left) and 2017 (right). Animals were detected less in 2017.

Mating Behaviors

Through the New Hampshire *Limulus* Spawning Surveys, it was determined that the mating season for 2016 started on May 9 when temperatures reached 11.2°C, peaked around May 21-26, and ended around June 10. The mating season for 2017 started on May 16 when temperatures reached 11.3°C, peaked around June 10-16, and ended around June 20.

Animals were considered to have appeared to mate during a high tide event during the mating seasons listed above if they had high activity during high tide, were found at shallow depths (moved inshore), and were heard by receivers near spawning beaches (see Fig. 1.4 for an example).

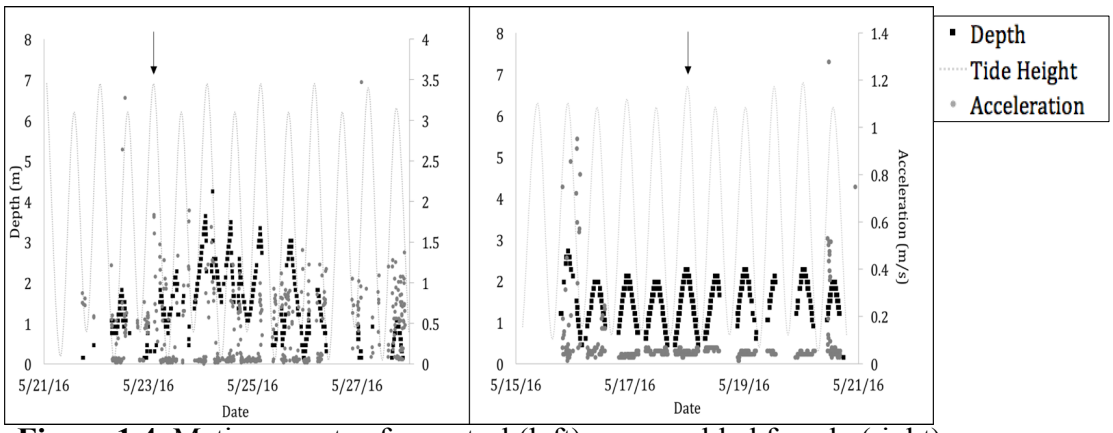

Figure 1.4. Mating events of a control (left) versus a bled female (right). The control female is more active (gray circles), but also moves shallower (black squares) at the start of high tide (dotted gray lines; especially during the latter high tides). It appears that the animal is moving with the tides, but considering the acceleration is 0 m/s and water pressure increases with tides, the animal is not moving and the increased pressure of the tides is detecting by the transmitter as increasing depth. Black arrows represent a good

Likely Mating Events Between Treatments

We determined how often animals likely mated, or not, during the first two weeks after they were released in 2016. Out of all the times animals were detected during each high tide within the first week, females appeared to mate less than males (females: 61%, males: 80%), bled animals appeared to mate less than control animals (bled: 61%, control: 80%), and, especially, bled females appeared to mate less than control females (bled females: 38%, control females: 85%; Table 1.1, Figs. 1.5). Or, stated another way, control females appeared to spawn 4.8 ± 2.5 times during these 7 days in the first week while bled females only spawned 2.0 ± 0.7 times in the first (Table 1.1, Fig. 1.4). Out of the animals that were detected for at least two weeks, there was no difference in likely mating activity between the first week and second week post-release (Student's paired t-test; $p = 0.16$; data not shown). These analyses were not performed in 2017 because there were not enough animals present and they did not appear to mate for a long enough time period.

Table 1.1. Two-way ANOVA results from likely mating events for animals tracked in 2016 comparing different sexes (males and females) and treatments (bled and controls). Bold represents significant difference ($p<.05$).

Likely Mating Events

Source	df	MS	F	p
Sex	1	2568.405	6.019	**0.024**
Treatment	1	3800.770	8.906	**0.008**
Sex*Treatment	1	2449.484	5.740	**0.027**
Error	19	426.746		
Total	23			

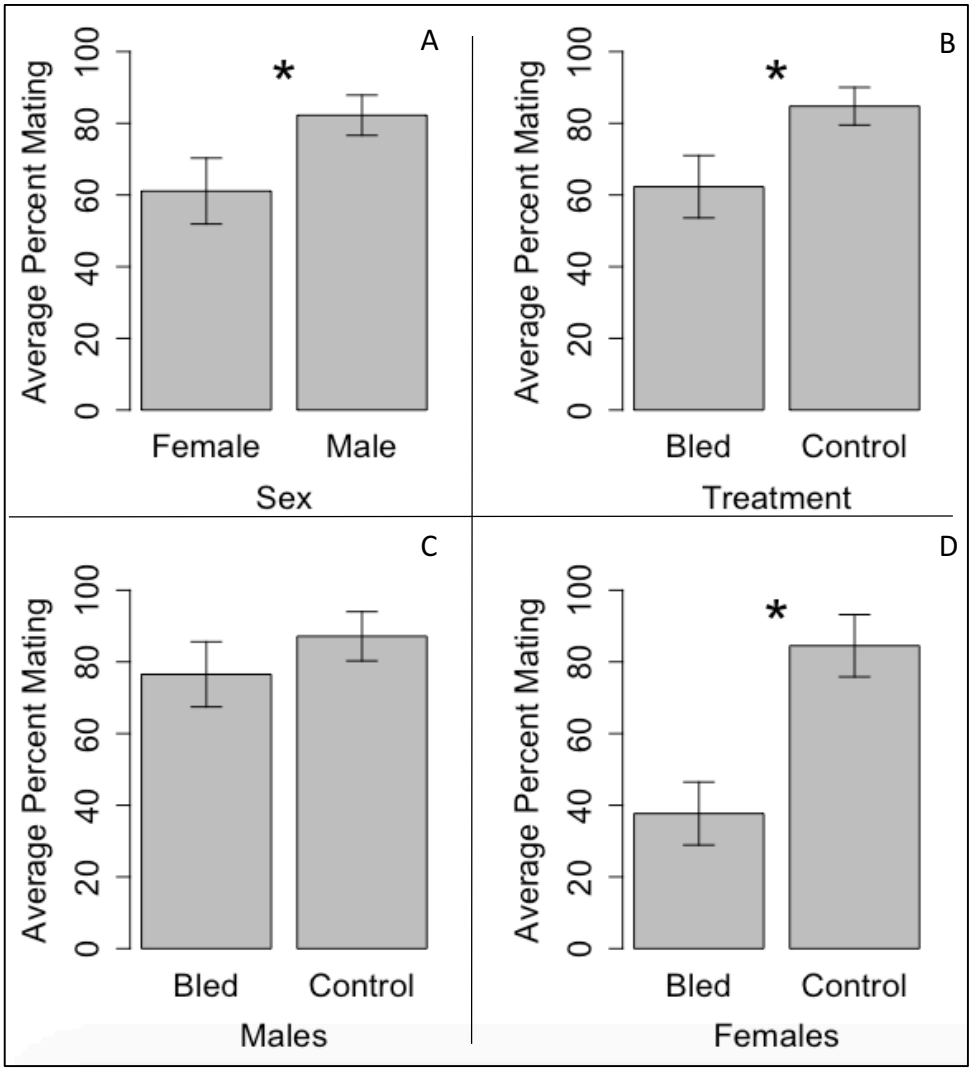

Figure 1.5. Average percent likely to be mating (± SE) for each group within the first week post-release. Females (n=10) appeared to mate less than males (n=13) out of the times they were detected (A). Bled animals (n=12) appeared to mate less than control animals (n=11; B). Males did not differ in likely mating events between control animals (n=6) or bled animals (n=7; C). Bled females (n=5) appeared to mate less than control females (n=5).

2016 Biological Rhythms of Activity

Horseshoe crabs that provided sufficient data for biological rhythm analyses (i.e., at least 7 days of continuous data) exhibited mostly tidal and daily rhythms. In June, all animals expressed tidal rhythms (n = 12; τ = 12.4 h; Table 1.2). This corresponds to the mating season in 2016 in GBE that took place from 9 May to 15 June. In the following months, there was a mix

between arrhythmic, daily, and tidal rhythms. However, there were more control animals detected, but the majority of both treatment groups expressed clear daily or tidal rhythms from June-October 2016 (control: 30/32 animals; bled: 15/16). All animals that expressed daily rhythms (τ = 24 h, Fig. 1.6) were more active during the day than at night, except for Animal 75 that was more active during the night during July and September. There was no clear relationship between depths and expression of rhythms. There was no significant difference between depths for months that animals displayed daily rhythms (4.4 ± 1.4 m) versus tidal rhythms (3.3 ± 1.3 m; unpaired Student's t-test, p = 0.5). This was also true for depths and rhythms in animals that switched rhythms. There was also no clear relationship between sexes and expression of rhythms (Table 1.2, Fig. 1.7). Finally, four animals also expressed a switch in rhythms (from tidal to daily), and these switches took place in July, directly after mating season (Table 1.2, Fig. 1.8).

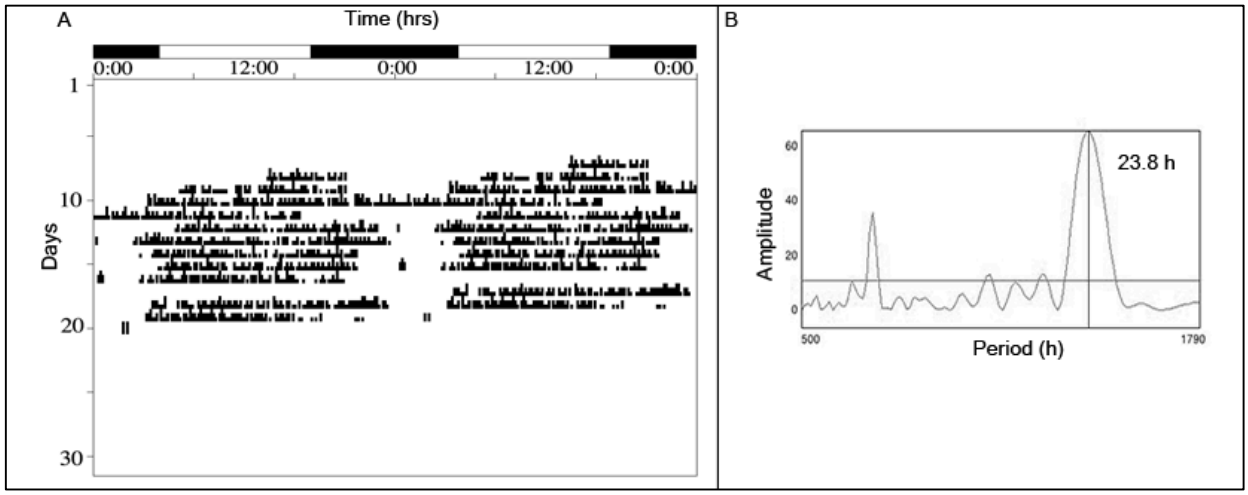

Figure 1.6. Representative example of a control female that expressed a daily rhythm and was more active during the day (A). Each small black tick mark represents the amount of activity per minute. The x-axis is double-plotted to make rhythms appear more evident, showing a span of 48 h for each line. The yellow/dark horizontal bars on top represent the Light/Dark periods of the day. The y-axis represents the 30 days in August 2016. (B) Periodogram showing that this animal had a significant rhythm ($p < 0.05$) at 23.8 h.

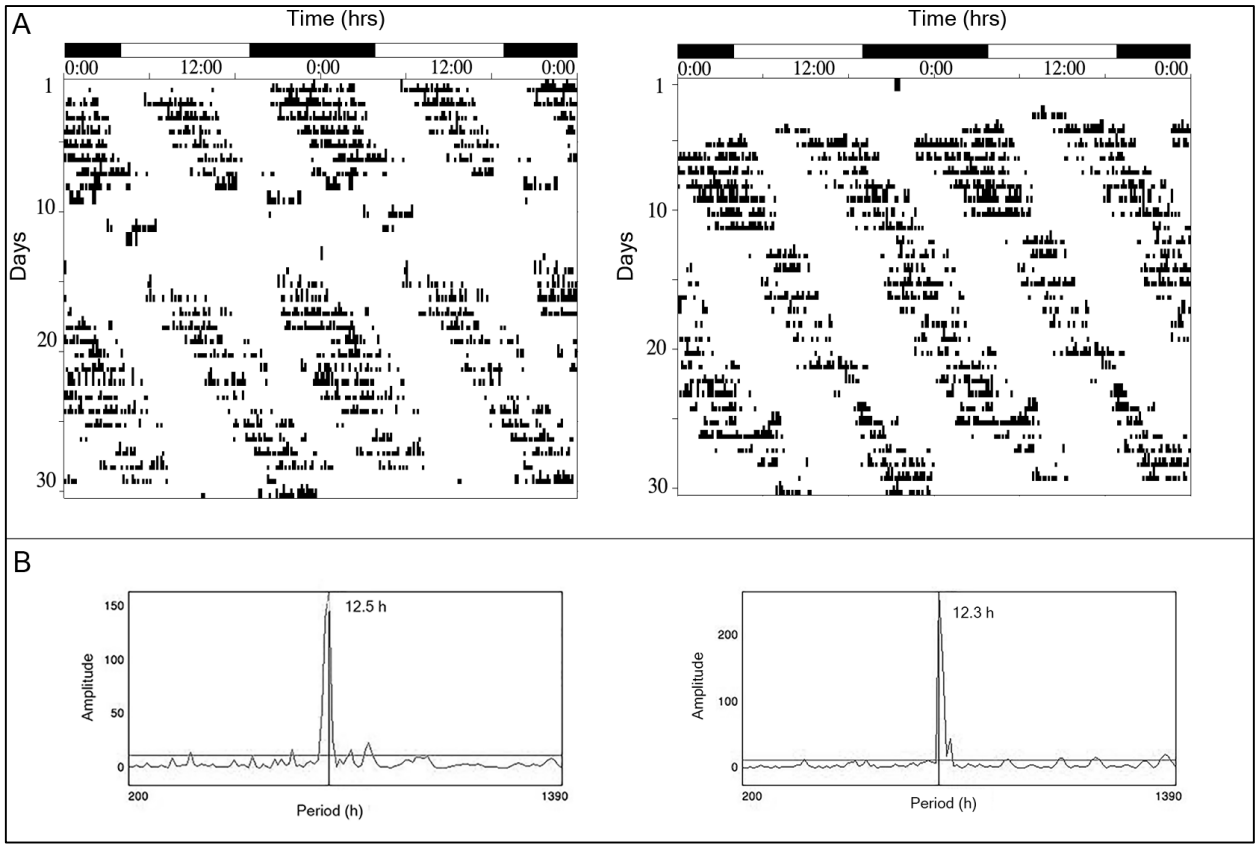

Figure 1.7. (A) Actograms of a control female (left) and bled female (right) in the month of June. The lack of activity around days 11-14 in the animal on the left may represent a span of time when the animal was not within distance of a receiver, and not actual lack of activity. (B) Periodogram displaying significant period of activity ($p<0.05$) for each animal for the amount of time shown in the actograms. The control female on the left and the bled female on the right had similarly strong tidal rhythms ($\tau = 12.5$ h; $\tau = 12.3$ h). See Figure 1.6 for more details about how this figure was produced.

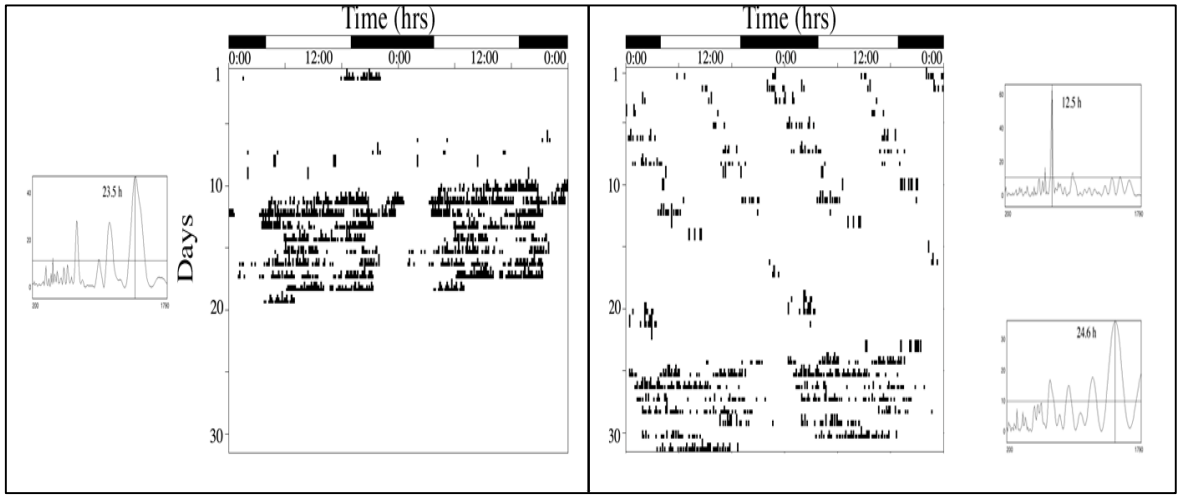

Figure 1.8. (A) Example of an animal that expressed a tidal and daily rhythm in July 2016. (A) Actogram showing a tidal rhythm during the first 25 days, and then a daily rhythm during the last 8 days. (B) Periodogram showing that this animal had a significant rhythm ($p < 0.05$) at 12.4 h and 24.3 h. See Figure 1.6 for more details about how this figure was produced.

2017 Biological Rhythms of Activity

In 2017, these same animals continued to express the same variety of rhythms (Table 1.2). Since different animals were detected at different times in 2017, we kept years separate for following analyses, there was a lower sample size, and a greater distribution in rhythms, we chose to discuss the two years separately. In April, before the start of the mating season, they exhibited tidal rhythms or were arrhythmic. Interestingly, all bled animals (n=4) were arrhythmic. After April, there was no clear difference between the types of rhythms exhibited by both groups. Although in June, there were fewer tidal rhythms than those expressed in 2016 (2016: n=13, 2017: n=1) due to fewer total animals. As in 2016, all animals that displayed daily rhythms were more active during the day than at night. In contrast to 2016, there was a relationship between depths and expression of rhythms. Animals that displayed arrhythmic rhythms were deeper than animals that expressed tidal or daily rhythms (ANOVA; $F_{(2,21)} = 8.22$, $p = 0.002$, Fig. 1.9).

Table 1.2. Rhythms expressed by individual animals for each month in 2016. Thick black bar separates 2016 (left) from 2017 (right). Light gray = tidal rhythm, dark gray = daily rhythm, striped= bimodal rhythm, black = arrhythmic, white = not sufficient data.

Animal #	Treat-ment	Sex	June	July	Aug	Sept	Oct	April	May	June	July	Aug	Sept
69	B	F											
70	B	F											
71	B	F											
39	B	M											
73	B	M											
74	B	M											
75	B	M											
85	B	M											
87	B	M											
37	C	F											
38	C	F											
76	C	F											
77	C	F											
80	C	F											
37	C	F											
38	C	F											
68	C	M											
36	C	M											

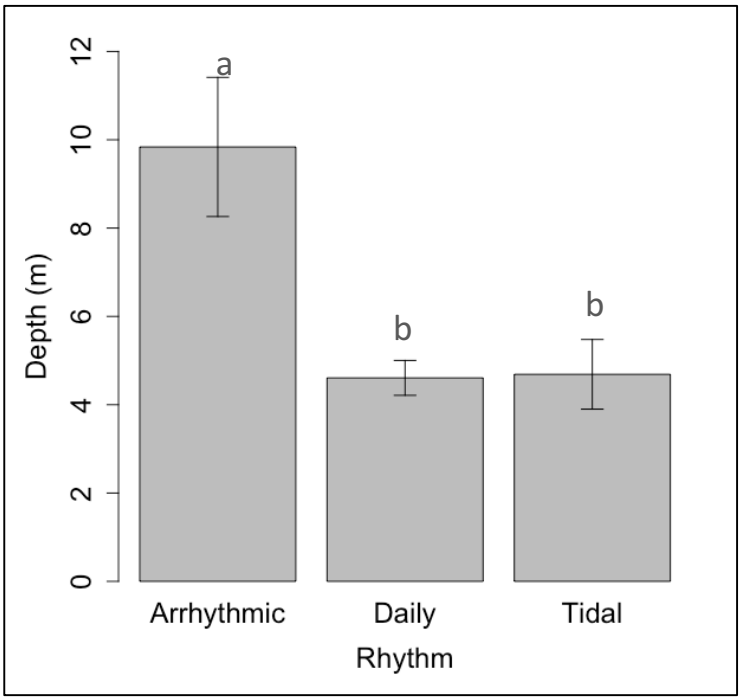

Figure 1.9. Average depth (± SE) for animals showing each type of rhythm in 2017. Arrhythmic rhythms were expressed by animals in deeper waters than daily or tidal rhythms. Different letters represent significant difference (Tukey's HSD test; $p < 0.05$).

Ranges

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

In 2017, 5 out of the 23 detected animals returned to their release site (Fig. 1.10). Four of these animals returned during the mating season in May-June. All four were males, and three of them were control animals and one was a bled animal. The fifth animal was a control female that

returned to the release site in late July. Since this is generally 2-3 weeks after the last recorded spawning in the GBE (Schaller *et al*., 2010; Watson *et al.*, 2016), it probably was not spawning at that time.

Table 1.3. Three-way ANOVA results comparing the ranges of animals tracked in 2016 and 2017. Comparisons included different years (2016 and 2017), males and females, and bled vs control animals.

Range

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

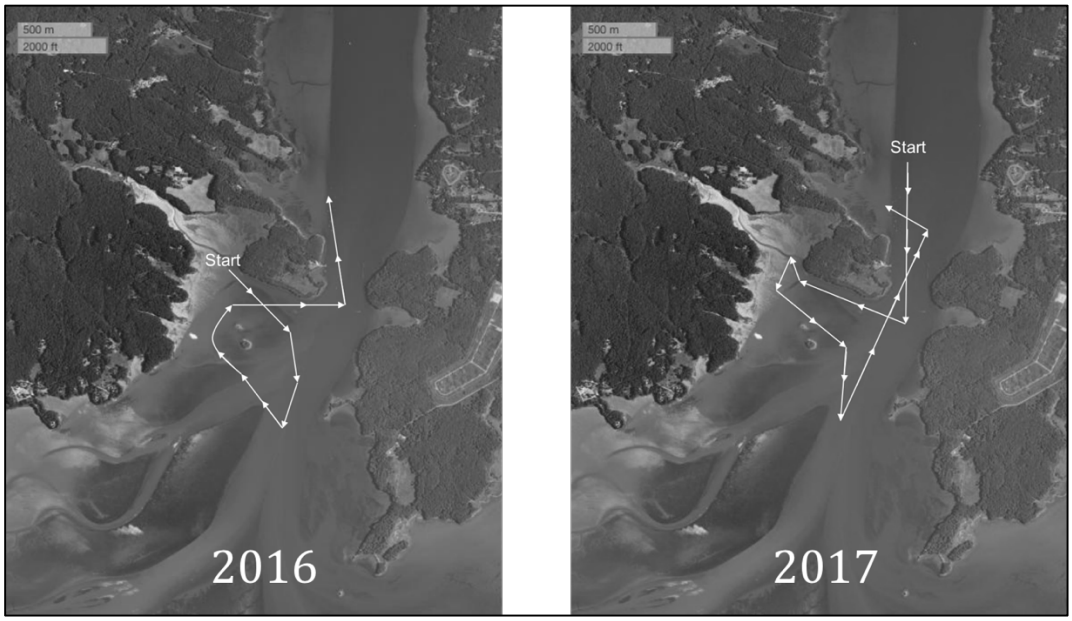

Figure 1.10. Example of a control animal that returned to the release site (indicated by the Start text on the left in 2016) again in 2017 (right). Arrowheads indicate direction of the path traveled by the individual in each year.

Overall Seasonal Changes

The water temperature in the GBE ranged from 9.5°C to 25.4°C during the time when we were tracking animals, and there was no difference in the mean water temperature (2016: 18.9 ± 3.6; 2017: 18.12 ± 3.2) between 2016 and 2017 ($r^2 = 0.85$, $p < 0.0001$; Fig. 1.11). The majority (21/23) of the animals that were detected in 2017 were first detected near the location where they were last detected in 2016 (the 2 animals that were not detected at their exact location from the previous year were still in Little Bay, but ± 2.3 km up or down estuary from their last location). Although transmitters were turned off during the winter season, the animals appeared to remain in the same location the following year before the transmitters turned off, allowing for a near continuous record of the activity, movements, and depth preferences of horseshoe crabs from that two-year span.

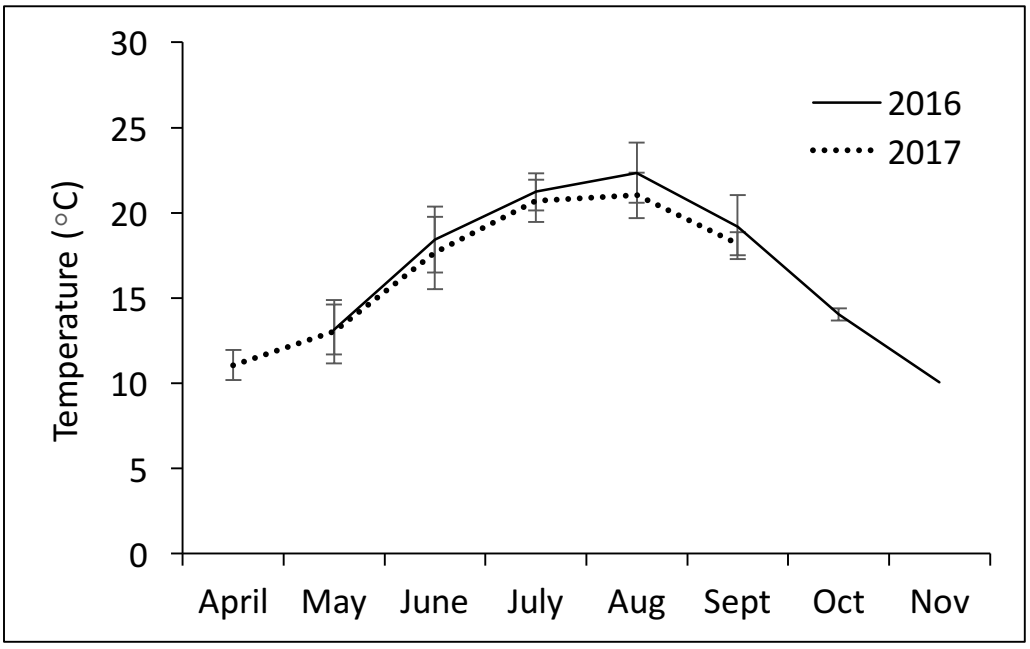

Figure 1.11. Average water temperature (°C ± SD) over a two-year timeframe in the GBE. The average water temperatures for 2017 (dotted line) were slightly lower than average temperatures in 2016 (solid line). Data are shown for months when transmitters were turned on for those years (2016: May-November; 2017: April-September). Water temperature peaked in mid-August in both years.

Overall Seasonal Changes in Depth and Activity

Only animals that were detected and active for at least seven days in a given month were used for depth and activity analyses (2016: n=21, 2017: n=11). Two animals (Animal 35 and 82) were only detected for 3-4 days within the first week and were not detected after that, thus, they were excluded them completely from all analyses. In 2016, there was a significant correlation between activity and depth (r^2 = -0.35, p = 0.01), where animals exhibited a higher percentage of activity at shallower depths (Fig. 1.12).

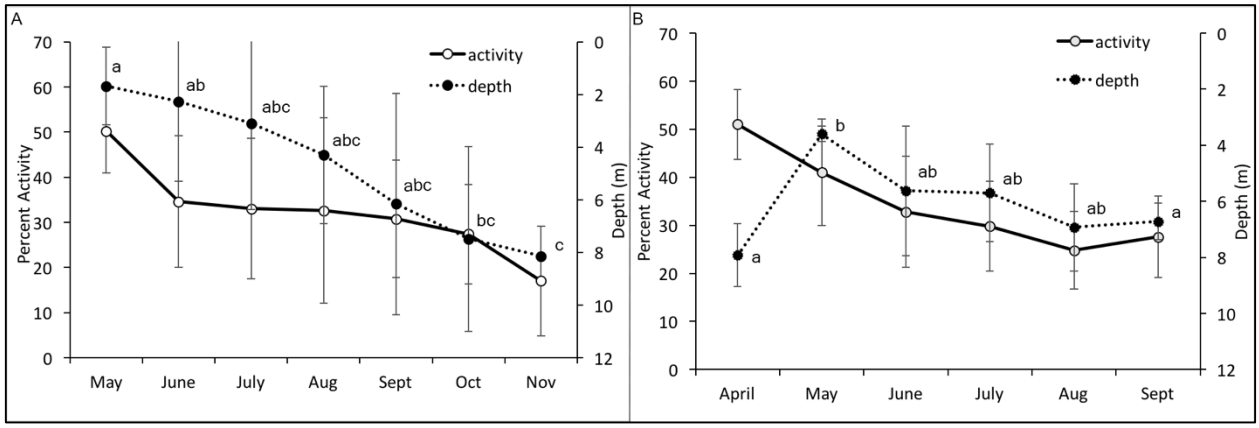

Figure 1.12. Mean (±SE) monthly horseshoe crab percent activity and depth for 2016 (A) and 2017 (B). Percent activity is the amount of time per day animals were active. Data for these analyses were based on individual's that were detected for at least one week for each month. Sample sizes for activity and depth for each month were (2016, 2017): April (0, 5); May (4, 9); June (10, 2); July (10, 3); Aug (11, 4); Sept (4, 3); Oct (5, 0), and Nov (3, 0). Different letters associated with them means represent significant differences for monthly depths (ANOVA, Tukey's HSD *post hoc; p* < 0.05).

Temperature Effects on Seasonal Depth and Activity

Animals that exhibited a higher percentage of activity > 50% of the time from May-August, and water temperatures during these months were warmer (≥ ~18°C). There was no difference in percentage of activity between bled and control animals (unpaired Student's t-test, *p* = 0.4). As water temperatures started to decrease in the fall, animals moved deeper and exhibited a lower percentage of activity (Fig. 1.12, Table 1.4). Bled animals spent significantly

more time in deeper waters in 2016 than control animals (Fig. 1.13, Table 1.4). Finally, 15 of the 21 animals that migrated from Great Bay to Little Bay moved in late July/early August when temperatures peaked at 22°C. The following year, 14/15 of those animals moved back into Great Bay when temperatures reached 11.2°C in May.

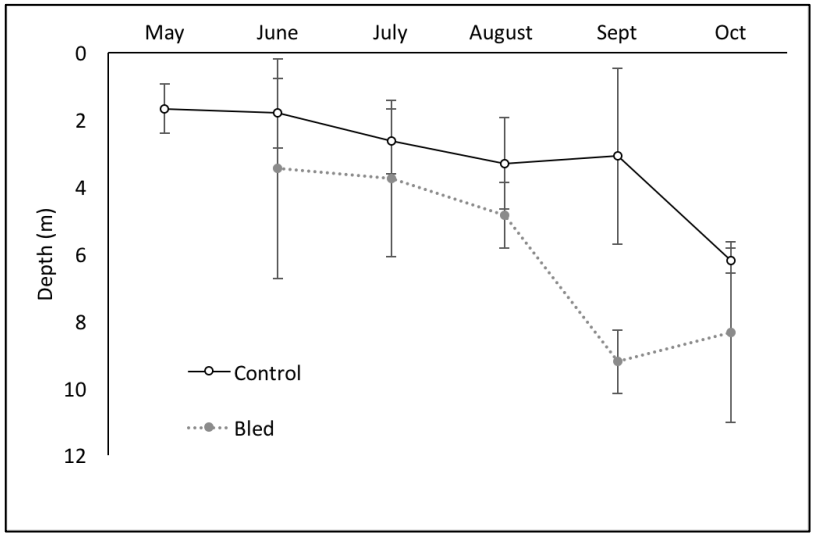

Figure 1.13. Mean depth (± SEM) of horseshoe crabs from May-October 2016. Bled horseshoe crabs on average remained deeper at all months than control horseshoe crabs. Data are not shown for bled horseshoe crabs in May because there was no data from any bled animal that was sufficient for analysis. Sample sizes for activity and depth for each month were (Control, Bled): May (4, 0); June (6, 3); July (6, 4); Aug (5, 4); Sept (2, 2); Oct (2, 3), and Nov (2, 1).

2017 Seasonal Changes in Depth and Activity

In 2017, only 5 animals were detected continuously for at least seven days in a month, so a different analysis was conducted for each year. Out of the five animals that were detected for at least seven days, one bled female was not included in the analyses because she remained at 14.1 m and was detected for 30% of the time for all four months while she was detected, causing this animal to be an outlier. This allowed for only four animals to be used for analysis, resulting in an extremely low sample size. Out of the four animals that were considered for activity and depth analyses, three of the animals were males and one was a female. Therefore, sex and treatment

could not be compared. However, there was a significant difference between month and depth (MANOVA; $F_{(6,19)} = 3.32$, p =0.021; Fig. 1.12), but there was no significant difference between activity and month (MANOVA; df = 6, F = 1.410, p = 0.262). Most of the animals in this study followed the same seasonal trend of activity and depth that was reported in two previous studies in the Great Bay Estuary (Schaller *et al.*, 2010; Watson *et al*., 2016). Animals over-wintered in deep water and remained deeper until water temperatures started to exceed ~11°C. At this time, they moved shallower and had a higher percentage of activity, and then in the fall they returned to deeper water as temperatures began to drop. However, it should also be noted that when water temperatures exceeded 20°C, eight of the animals moved from Great Bay to Little Bay, which is exactly what these same animals did at the same time in 2016 (late July/early August).

Seasonal Movements Across Years

As mentioned above, while there was no significant difference in the annual range of movements between bled and control animals, or in the overall seasonal migration trends, there were some distinct changes in the seasonal movements of some animals from one year to the next. Most of the control animals followed similar patterns, but there were several bled animals that had very different migration routes from 2016 to 2017. One noticeable trend was that in May and June, the bled animals did not approach shallower areas in Great Bay, preferring to remain in deeper channels (Fig. 1.14). Out of all of the animals that were detected in 2017 (regardless of if they were also detected at the same time in 2016), bled animals remained in deeper waters than controls (unpaired Student's t-test, $p < 0.001$). However, between 2016 and 2017, there were no major differences in the distribution of bled or control animals throughout the estuary during each year (Fig. 1.15).

Table 1.4. MANOVA results from depth and activity data for animals tracked in 2016 comparing different sexes (males and females), treatments (bled and controls), and months (May-November). Significant differences are shown in bold.

Depth and Activity

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

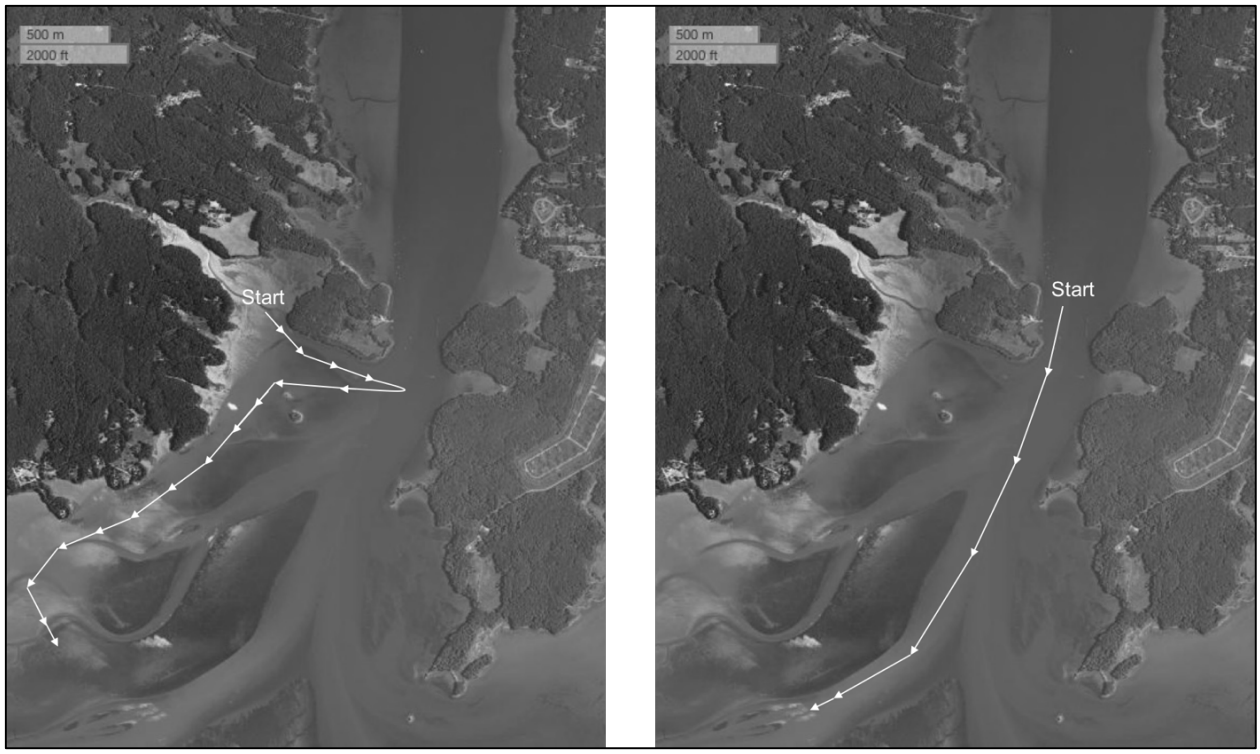

Figure 1.14. Paths of bled animal during the mating season (May-June) of 2016 and 2017. The individual remained shallower (average depth: 2.7 ± 1.2 m) in 2016 than in 2017 (average depth: 14.07 ± 2.3 m) during the same seasonal time.

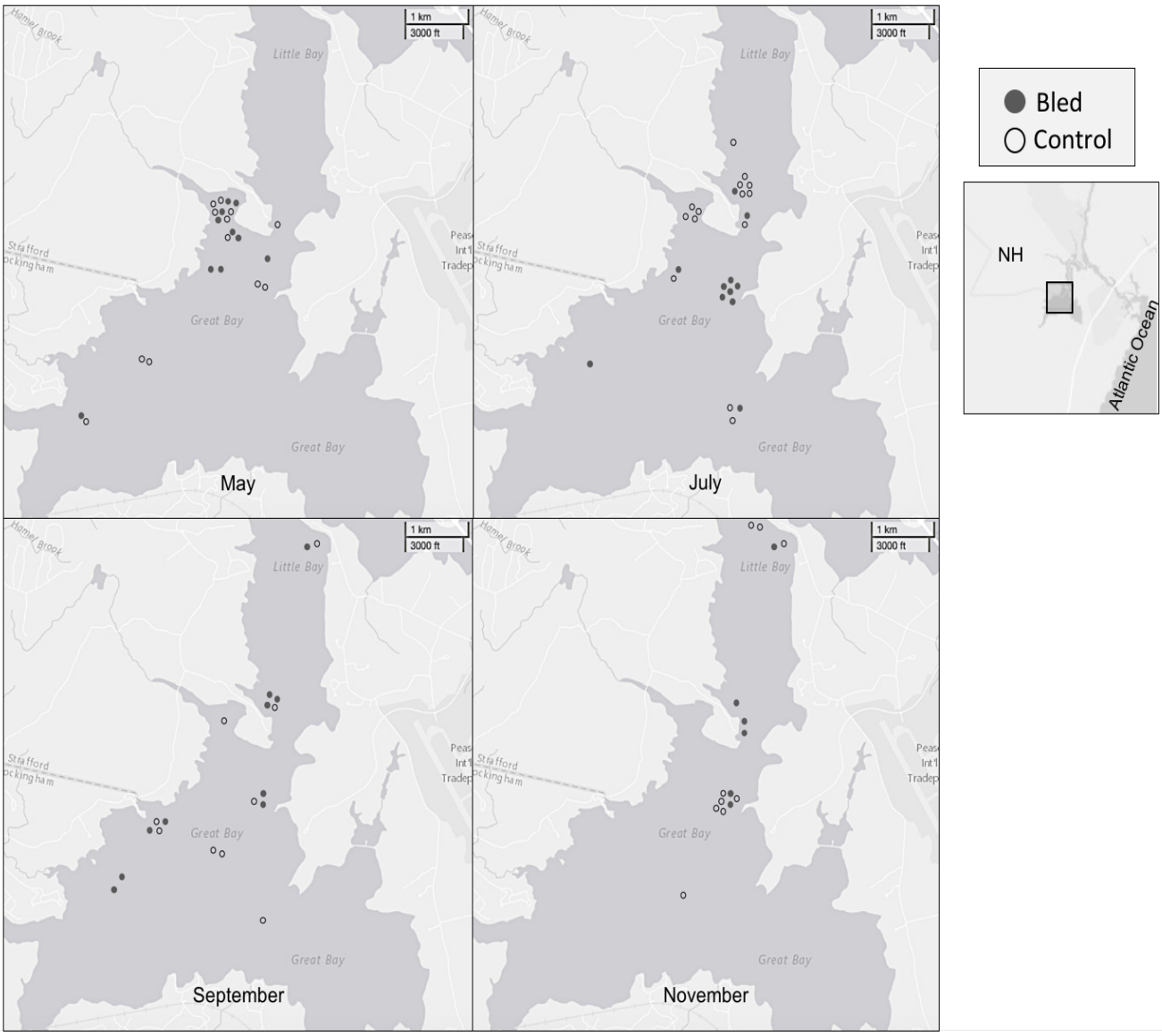

Figure 1.15. Maps of 2016 seasonal locations of animals. Solid gray dots represent bled animals, and hollow black dots represent control animals. There is no clear trend of animals over time for seasonal movements. It should be noted that these locations are based on detections by VR2s, and thus do not necessarily represent the exact locations of each animal.

Discussion

While the impacts of the biomedical bleeding process have been studied in the laboratory and the mortality rates and orientation of bled horseshoe crabs have been studied in the field (Rudloe, 1983; James-Pirri *et al.* 2012), this was the first comprehensive study to determine the behavioral effects of the bleeding process has on the horseshoe crabs that are released back into their natural environment. We found that: 1) bled females appeared to mate less than control females; 2) bled animals remained deeper throughout the year overall than control animals and had different seasonal movements from one year to the next; 3) there was no difference in ranges of bled vs control animals, or their locations in the GBE across months or years. Therefore, it appears as if the biomedical bleeding process can have sublethal impacts on horseshoe crab movements and migrations, which might have ecological implications for foraging and mating.

MATING

Since it is readily known that horseshoe crabs mate at high tide at spawning beaches during the spring and summer, it was possible to use a combination of depth, tide, and accelerometer data to determine the likely mating events of tagged individuals. Also, we collected and released crabs at a known mating location so that we could readily monitor them through appropriate placement of the receiving stations.

We did find that control animals mated more often than bled animals in 2016, especially when comparing females. Although the mating season lasted for about a month, we examined the effects of bleeding on mating during the peak two weeks (which also happened to be immediately post-release). During this time, 5 bled females spawned on average of 2.0 ± 0.7 times, whereas 5 control females spawned 4.8 ± 2.5 times. Therefore, these data suggest that the

bleeding process might have an immediate impact, as well as a long-term impact, on the reproductive output of female horseshoe crabs. Since female horseshoe crabs are preferentially selected for in the biomedical bleeding process due to their size (James-Pirri *et al.*, 2012), this can lead to negative impacts on the overall size distribution and sex-ratio of the population. For example, in several harvested habitats, such as Pleasant Bay, Massachusetts, there has already been a 20% decrease in female appearances at spawning beaches (Carmichael *et al*., 2003; Malkoski, 2010), and egg abundances have also significantly decreased at spawning sites (James-Pirri, 2012). This is consistent with some of our findings from the second year of telemetry data in 2017 during the spawning season. There was one bled female that did not seem to mate at all, considering it remained in over 14 m of water and was only located at receivers in the middle of the estuary that were far from beaches. Also, as mentioned before, bled animals did not approach shallower beaches as frequently as control animals in 2017. Overall, there are immediate sublethal and even potential prolonged effects of bleeding on horseshoe crabs affecting mating events and reproductive output.

Moreover, males are more frequently detected at mating beaches than females leading to a skewed sex-ratio (Loveland and Botton, 1992), and females usually only spawn several times within one week and do not return until the next year; whereas males return to these beaches more often (Rudloe, 1980; Brockmann and Penn, 1992; Leschen *et al.*, 2006). We did see this shown in our data (males mated 80%, females mated 61%). However, Watson and Chabot (2010) did not see any significant differences between mating events exhibited by males and females. They suggested that this could be due to the fact that only a subset of the female population mates at a certain time while males partake in the entire mating season, thus disrupting the sex ratio (Cohen and Brockmann, 1983; Brockmann, 2003; Brockmann and Smith,

2009). This explanation should not be disregarded, considering that we found that bled females significantly mated less than control females, thus, potentially altering our sex ratio.

Lastly, mating times were examined in one-week increments, allowing for around 14-15 potential mating times (accounting for two high tides per day) during one single week. Control animals appeared to mate on average 3-4 times a week, whereas bled animals mated 1-2 times per week. For control animals, this meant that they were likely to be mating during around 22-29% of the potential high tides, while bled animals only appeared to be mating during 7-14%. This is consistent with the data found by Watson and Chabot (2010), where they estimated animals approached spawning beaches about 30.5% of the high tides. Our numbers are slightly lower than Watson and Chabot (2010) and those reported by (Brousseau *et al.*, 2004), but this could be due to several reasons. Since our animals were released mid-way into the spawning season and we only looked at two weeks of data, we could have missed several spawning events. Environmental factors could have also affected horseshoe crab spawning. During our spawning surveys in both years, we noted that there were less horseshoe crabs counted on mating beaches due to heavy rain, increased detritus on beaches on days immediately following large storms, and overcast conditions. This is congruent with data collected in spawning surveys in the GBE in 2012 and 2013 where periods of heavy rain were correlated with periods of decreased spawning activity (Cheng *et al.*, 2015). Heavy rain events can decrease salinities in semi-enclosed estuaries, such as the GBE (Short, 1992), and the cost of osmoregulating can deter them from mating and conditions during these times are unfavorable for egg development (Robertson, 1970; Mangum *et al.*, 1976; Jegla and Costlow, 1982; Ehlinger and Tankersley, 2003).

BEHAVIORAL RHYTHMS

Horseshoe crabs possess endogenous clocks that allow them to anticipate changes in environmental factors, specifically changes in tides, so they can synchronize their spawning and foraging activities to specific phases of the tides (Cohen and Brockmann, 1983; Barlow *et al*., 1986; Watson and Chabot, 2010). Any disruptions to the rhythms controlled by these clocks could have negative implications for reproduction and survival. In 2016, all animals that expressed activity in June had tidal rhythms. This is likely because this was the spawning season when they approach mating beaches at high tide (Rudloe, 1980; Shuster and Botton, 1985; Barlow *et al*., 1986). During the following months, few animals expressed tidal rhythms, and many switched to either a daily rhythm or became arrhythmic. This same seasonal transition was reported in previous studies, and it is likely due to the fact that this is the time when they move into deeper waters and prepare for overwintering (Chabot and Watson, 2010).

In 2016, animals that had daily rhythms were more active during the day than during the night. It has been previously argued that horseshoe crabs spawn more during the night and increase their percentage of activity during the night (Cavanaugh 1975; Rudloe, 1980, 1981; Barlow, 1983; Barlow *et al.*, 1986; Finn *et al*., 1990; Swan *et al.*, 1991, 1993; Smith *et al.*, 2010). In the GBE, this hypothesis has not been supported and horseshoe crabs have actually been shown to be significantly more active during the day (Watson *et al*., 2009; Watson and Chabot, 2010). Our study supports the latter argument. We rarely observed animals that were more active in the night vs the day (Fig. 1.8).

In 2017, there appeared to be some impact of the bleeding process on the expression of biological rhythms in their natural habitat. For example, more bled animals were arrhythmic (4/9 animals) in 2017 than control animals (0/9 animals). For example, Animal 70, a bled female,

remained arrhythmic throughout all of the months that it was active, including during the spawning season. The animals that were arrhythmic were also deeper and mostly manifested these behaviors in April, before the start of the spawning season, which is not too unusual. They usually do not begin to express clear rhythms until temperatures warm (10-11°C; Schaller *et al.,* 2010). However, two other bled animals that were arrhythmic in May and June during the spawning season, and this could be an effect from the bleeding process that could negatively affect their mating and foraging behaviors. Also, considering that *all* of the animals expressed tidal rhythms in June in 2016 (May was not accounted for rhythm expression in 2016 due to lack of data), but only three animals in 2017 (2/5 bled animals in May and 1/3 control animals in June) exhibited tidal rhythms, suggests that bleeding might have had a long-term impact on mating behavior. This delayed impact is similar to the findings by Anderson *et al*., (2013) in the laboratory. In general, the bleeding process can have delayed and extended impacts on the expression of rhythms, and thus potential foraging and mating, on these animals.

MOVEMENTS/MIGRATIONS

The 28 animals that were tracked in 2016 appeared to remain within the GBE, along with the 23 that were detected in 2017. In fact, 21 of the 23 animals detected in 2017 were first detected at the same location where they were last detected in 2016 (the other 2 animals were still located in Little Bay, but not where they detected in 2016). This is consistent with previous findings in the GBE (Schaller *et al.*, 2010), as well as in other embayments including Pleasant Bay, MA (James-Pirri, 2010). Horseshoe crabs are thought to be philopatric to the embayments where they spawn, and in New England specifically, horseshoe crab populations appear to be more localized and do not seem to migrate offshore (Baptist *et al*., 1957; Botton and Ropes,

1987; James-Pirri *et al*., 2005; Moore and Perrin, 2007). Therefore, if horseshoe crabs are harvested from a particular area, it is unlikely they would be replenished from adults or larvae from a different area. In addition, if they are removed from one area, bled, and returned to a different area, they might not have the ability to adjust their migration and spawning behaviors to the new region.

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

Although there was no significant difference in the annual range of movements between bled and control animals, or in the overall seasonal migration trends, there were some distinct changes in the seasonal movements of some animals from one year to the next. Most of the control animals followed similar patterns, but there were several bled animals that had very different migration routes between 2016 and 2017. One noticeable trend was that in May and June, the bled animals did not approach shallower areas in Great Bay, but remained in the deeper channels. This trend is consistent with data showing that bled animals remained deeper throughout the year in 2016 and that bled females mated less than control females. In other words, the immediate impacts of bleeding that we observed in 2016 continued in 2017. There are several potential reasons that caused these bled animals to remain in deeper water. First, in the laboratory, disrupted orientation and a random direction of movement has been observed in bled horseshoe crabs (Anderson *et al*., 2013). Furthermore, bled animals may not have been as motivated to spawn, and therefore they did not move towards spawning beaches as often as controls. In one of the previous field studies comparing the impact of biomedical bleeding on movement patterns in Cape Cod, MA; it was found that the bled group had a random movement pattern compared to the movement expressed in control animals (Kurz and James-Pirri, 2002). This disorientation could prevent horseshoe crabs from locating spawning beaches and, ultimately, could be an explanation for the bled animals to remain deeper and away from spawning beaches. Furthermore, it is possible that bleeding could be influencing energy going into reproduction vs. stress remediation (i.e. recovery), thus the following year there is not as much motivation to spawn. It was difficult to compare mating behaviors in 2017 to 2016, but there were explicit examples of bled animals appearing to mate less or not at all (Animal 70 remained deep all throughout April-July, etc.). Also, as is the case in telemetry studies (Kurz and

James-Pirri, 2002; Brousseau *et al*., 2004; Moore and Perrin, 2007; Watson and Chabot, 2010; Schaller *et al*., 2010), our receivers may not have been showing accurate locations of animals. Bled animals may have been passing receivers on their way to shallower locations elsewhere or a receiver may not have been in the exact vicinity of an animal. Regardless, based on the available data, bled animals were shown to vary more in their seasonal movements from one year to the next more so than control animals.

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

In addition, there were no significant differences in percentage of activity and months in bled animals in 2016 (even in animals that were detected in both years), which was different than what we expected to find. Although some bled animals (but also some control animals) did decrease in their percentage of activity between years, it is not necessarily indicative of direct effects of the bleeding process. For example, it has been suggested that animals become less active after foraging (usually this occurs at high tides in months following the spawning season; June-July), because after individuals ingest their prey, they bury in the sediment until they have completely digested their prey (which can take up to several days; Watson and Chabot, 2010). Anderson *et al*., (2013) found that there were reductions in the percentage of activity exhibited in

bled horseshoe crabs in the laboratory. This discrepancy in our data could be due to several factors. First, in the laboratory environment, there is not sediment for animals to bury in after foraging, thus any reduction in activity could most likely be attributed to effects from the bleeding process. Also, there is only limited space for animals to move in tanks, whereas in the natural environment, animals are not enclosed in a small area and capable of freely moving around. Furthermore, it appeared that bled horseshoe crabs did not approach shallower spawning beaches, but instead they moved around in deeper waters. This shows that their behavior might have been affected, but did not prevent them from moving elsewhere, and, ultimately, not impacting their overall activity. In this regard, it is difficult to ascertain if the percentage of activity in their natural environment was impacted by the bleeding process.

Overall, not only were there immediate impacts on mating behaviors and expression of biological rhythms, but there appeared to be some sustained impacts into the second year. If bled animals, especially females that are preferentially selected for by several biomedical companies, have alterations in their rhythms and mating behaviors, it is likely to further alter the sex-ratio on spawning beaches, reduce reproductive output, lower population levels, and decrease the fitness and survival of this keystone species. Further studies should look into the immediate effects of the bleeding process on horseshoe crabs in the mating season to obtain a more precise and accurate data assessment of their behavior in the vicinity of mating beaches. Also, as compared to studies done in the laboratory, the orientation of bled animals in their natural habitat should be examined. As a whole, this study provided a glimpse into these effects, and suggests that there is a need for mitigations and improvements in this industry to lessen these behavioral impacts on this species.

CHAPTER 2: THE EFFECTS OF DIFFERENT STRESSORS ASSOCIATED WITH THE BIOMEDICAL BLEEDING PROCESS ON THE BEHAVIOR AND HEMOCYANIN LEVELS OF HORSESHOE CRABS (*LIMULUS POLYPHEMUS*)

Abstract

As horseshoe crab blood is harvested by the biomedical industry to create Limulus Amebocyte Lysate, horseshoe crabs are exposed to a variety of stressors, in addition to blood loss. The major goal of this study was to investigate the impacts of the three main stressors (air exposure, increased temperatures, and blood loss) on the locomotor activity and hemocyanin levels of horseshoe crabs. Experiments in 2016 and 2017 typically lasted three weeks. Freshly collected animals were used for each experiment and blood samples were obtained at the beginning, and then once every week. After collecting control behavioral data for one week, animals were exposed to an individual stressor, or different combinations of stressors, and then two additional weeks of behavioral data were obtained. Our findings revealed that the full bleeding procedure typically used commercially (i.e., all three stressors) had the largest impact on mortality, hemocyanin levels, overall activity, and expression of rhythms, followed by bleeding along with at least one of the other stressors. We also saw a seasonal trend in hemocyanin levels and a strong, significant relationship between hemocyanin levels and overall activity. Our data also revealed that animals with starting hemocyanin levels of 0.13 mg/mL or less were more likely to die or be impaired by the bleeding process. These data could be helpful in the development of alternative measures and regulations for biomedical facilities to implement to reduce some of the negative impacts of the process.

Introduction

The American horseshoe crab, *Limulus polyphemus,* is an economically and ecologically important species that have existed for over 450 million years (Sekiguchi, 1988; Rudkin and Young, 2009). One potential explanation for the longevity of this species is that they have a highly sensitive immune system that protects them from harmful bacterial endotoxins found in their environment (Du *et al*., 2011). These crabs are found in bays and estuaries along the Atlantic coast in North America, including the Great Bay Estuary (GBE), New Hampshire (NH), where gram-negative bacteria are numerous (up to one billion gram negative bacteria per gram of sand and over one million bacteria per milliliter of seawater; Novitsky, 1991), a physiological mechanism to protect them from these bacteria seems crucial for this organism. The immune

reaction to invading pathogens includes several responses, but most importantly, hemolymph coagulation. A number of companies that produce biomedical products have capitalized on the horseshoe crabs' immune system to create a commercially valuable product, Limulus Amebocyte Lysate (LAL).

Since producers of pharmaceuticals and medical implants are required to use LAL to ensure the sterility of their products, the demand for this valuable product has continued to grow, causing a concomitant increase in harvest rates of horseshoe crabs (ASMFC, 2013). When horseshoe crabs are harvested and transported back and forth to blood extraction, they are subjected to a variety of stressors, in addition to loss of blood. For example, they spend a significant amount of time in air when they are left on the decks of boats, and on docks, or in the trucks that transport them to and from bleeding facilities (ASMFC, 2012). Also, they are exposed to warm temperatures, since they are often in direct sunlight or poorly temperature-controlled trucks (ASMFC, 2012). Finally, the blood extraction process itself can compromise the health of the horseshoe crab, as up to 30% of the estimated blood volume of individual horseshoe crabs is removed (Novitsky, 1984; James-Pirri *et al*., 2012). Each of the four major biomedical companies has slightly different collection, handling, and bleeding processes, but, nonetheless, horseshoe crabs must endure varying amounts of increased air exposure, elevated temperatures, and significant blood loss. One of the major goals of this study was to identify which of these factors contributes to the behavioral and physiological deficits that have been observed in some horseshoe crabs (described below) that have been subjected to the entire process.

Several studies have demonstrated that blood extraction or the entire bleeding process induces increased mortality rates. When animals were just bled, there was a 10-20% increase in mortality rate compared to control animals (Rudloe, 1983; Kurz and James-Pirri, 2002). In a

study that quantified mortality rates of strictly hemolymph extraction at set levels in horseshoe crabs there was an increased mortality rate in bled animals (8%) compared to control animals (3%) that were subjected to 10-30% hemolymph extraction, but found a 29% rate in animals that had 40% of their hemolymph extracted (Hurton and Berkson, 2006). Mortality rates associated with the full bleeding procedure range from 5-30% (Thompson, 1998; Walls and Berkson, 2000; Walls and Berkson, 2003; Hurton and Berkson, 2006; Leschen and Correia, 2010; Anderson *et al*., 2013), with a differential mortality rate between the sexes (15% mortality in males and up to 29% in females; Leschen and Correia, 2010; James-Pirri, 2012). While these data show that mortality often occurs as a result of the biomedical bleeding process, it is not clear what part(s) of the process have the greatest impacts on the probability that animals will die during or after the procedure. Hurton and Berkson (2006) found that mortalities significantly increased in horseshoe crabs when they exposed crabs to a combination of effects, and studies of harvest techniques in other marine species revealed that mortality is affected by a synergistic combination due to multiple stressors (Schisler *et al.,* 2000; Schulz and Dabrowski, 2001; Hatch and Blaustein, 2003). Thus, Hurton and Berkson (2006) highlighted the need for studying the cumulative impact of multiple stressors that horseshoe crabs experience during the bleeding process.

Sublethal impacts from the bleeding process on horseshoe crabs include delayed blood volume recovery, reduced blood protein levels, and behavioral deficits. Novitsky (1984) found that it takes 3-7 days for a bled horseshoe crab to regain its total blood volume and up to 4 months for amebocytes to return to baseline levels, however, these findings were discovered through captive aquarium studies. Captive bled animals also have lower blood protein values and bled crabs released back into their natural environment displayed a random direction of

movement compared to the movement patterns in control animals (James-Pirri *et al.,* 2012). Finally, and most recently, Anderson *et al.* (2013) examined the impacts of bleeding on locomotion and hemocyanin levels in horseshoe crabs in the laboratory and found changes in the bled animals' activity levels, expression of circatidal rhythms, linear and angular movement velocities, and reductions in hemocyanin levels.

The negative impacts from the bleeding process likely impacts the ecology of horseshoe crabs in several ways. First, mortality induced by the biomedical industry can clearly affect the overall status of the population and horseshoe crab availability to commercial fishermen. Second, any reduction in protein levels or hemocyanin concentration in horseshoe crabs can cause serious health issues. The functions of hemocyanin are numerous and diverse including: transporting oxygen, eliciting the primary immune response (Coates *et al*., 2011), contributing to wound repair and cuticle hardening (Adachi *et al*., 2005a), molting (Adachi *et al*., 2005a,b; Kuballa and Elizur, 2008; Kuballa *et al*., 2011; Glazer *et al*., 2013), hormone transport (Jaenicke *et al*., 1999), and protein storage and osmoregulation (Paul and Pirrow, 1998). Different environmental conditions, such as increased temperatures, limited oxygen supplies, and air exposure, can also affect hemocyanin concentrations (Magnum *et al*., 1975, 1976; Burnett, 1988; Coates *et al*., 2012). Thus, poor environmental conditions and stress can cause alterations in hemocyanin levels, and, accordingly, increasing their susceptibility to infection along with other negative consequences. Lastly, changes in the activity levels, rhythms, and orientation of horseshoe crabs (Anderson *et al.*, 2013) can limit their ability to find spawning beaches, mate, and forage for food. Altogether, the survival and fitness of this vital species may be at risk when enough of the population is exposed to the different stressors of the bleeding process. Mitigating these effects

would help to conserve the horseshoe crab population and sustain horseshoe crab availability to multiple user groups.

In this study, our main objective was to determine which of the three major stressors (or combination of these stressors: Table 2.1) had the largest impact on the behavior and physiology of horseshoe crabs. Animals were collected from the GBE, baseline hemocyanin and activity levels were obtained, subsets of the animals were exposed to each stressor combination (bled and exposed to air, unbled and exposed to air, bled and elevated temperatures, etc.), and then we monitored their behavior and hemocyanin levels for another two weeks post-treatment in the laboratory. Overall, the data obtained indicate that all three stressors (blood loss, increased air exposure, and increased temperatures) associated with the full bleeding procedure has the largest impact on hemocyanin levels, mortality rates, *and* overall activity.

Methods

ANIMAL COLLECTION AND EXPERIMENTAL SET-UP

In 2016, a total of 48 male horseshoe crabs were collected by scuba-diving at Fox Point in the Great Bay Estuary, Durham, New Hampshire (Fig. 2.1) from June-October. In 2017, 63 female horseshoe crabs were collected by scuba-diving at Fox Point in June and July. All captured crabs were brought back to the University of New Hampshire Jackson Estuarine Laboratory (JEL) where an initial blood sample was collected and animals were fitted with HOBO acceleration data loggers (Onset Computer Corporation, Bourne, MA; Fig. 2.2). The HOBO accelerometers were used to record triaxial orthogonal measurements of the acceleration (g) of individual horseshoe crabs, and they were programmed to record a reading every minute. The accelerometers were attached to the dorsal carapace of each individual by first creating a

harness by attaching two large cable ties to the center of the prosoma with duct tape and cyanoacrylate and then placing the accelerometer face-up in the harness and fastening the cable ties over it. This arrangement made it relatively easy to remove the accelerometers weekly, download the data, and then replace them again with minimal disturbance of the animals.

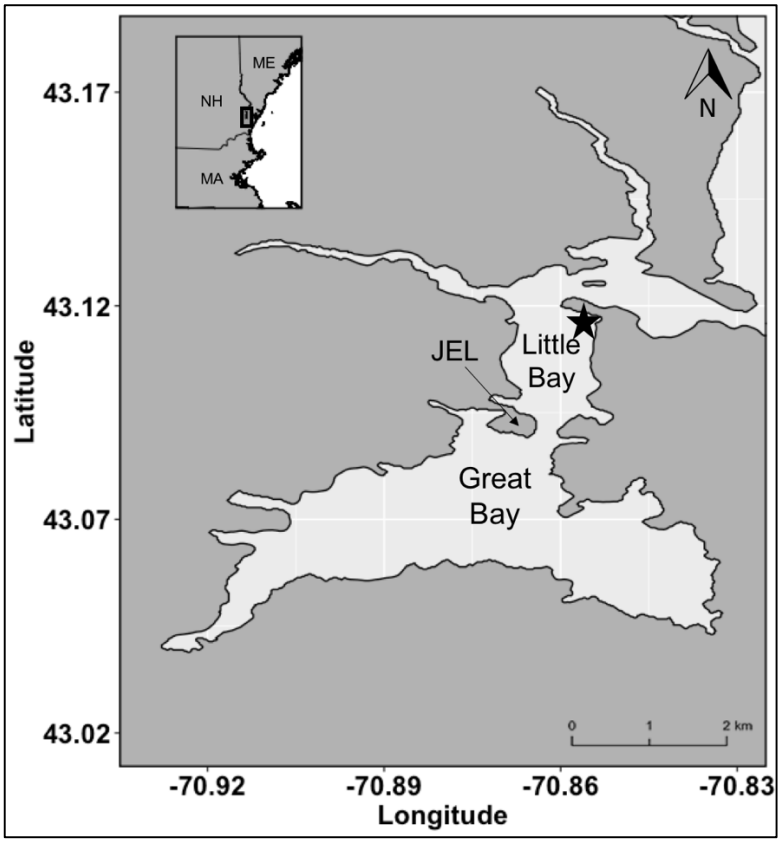

Figure 2.1. Map of study location in the Great Bay Estuary, NH, USA. Black star represents collection site at Fox Point, Durham, NH. The black arrow indicates the location of the Jackson Estuarine Laboratory (JEL) as a reference point.

Figure 2.2. HOBO accelerometer attachment on dorsal carapace of individual horseshoe crab (photo credit: Win Watson).

Animals were then placed into individual 1-m diameter circular mesh enclosures within larger flow-through estuarine-water 850-l tanks (183 cm x 92 cm x 50 cm) outside of JEL. There were two enclosures per tank and were exposed to natural light/dark cycle (enclosures were not visually shielded). Temperature data loggers (HOBO, Onset Computer Corporation, Bourne, MA) were placed in the tanks to monitor the temperature throughout the duration of each of the experiments. Behavioral data were collected for one week prior to exposing animals to a given treatment. Then, blood samples were taken and the accelerometers were downloaded. Once the accelerometers were reattached, individuals underwent their designated treatment (described below) and then they were returned to their tanks. Animals were kept in these tanks for two more weeks and their activity was recorded. Additional hemolymph samples were taken from each animal at 7 and 14 days after their treatments.

TREATMENT GROUPS

Animals were assigned to different treatment groups based on the stressors to which they were exposed, which included: bleeding, air exposure, and heat (Table 2.1). These experiments

were carried out during June-October of 2016 and June and July of 2017. In 2016, only two treatment groups were completed at one time (n=8 per group). In 2017, we pooled all of the groups together (June and July) to be examined at one time in 2017. Due to limited tank space, we repeated the pooled experiments twice in 2017, once in June (n=5 per group) and once in July (n=4 per group).

Table 2.1. Treatment groups were exposed to different combinations of the major stressors in the biomedical bleeding process. Several of the treatments were repeated in 2016 and 2017. The average water temperature (°C) for each month is also indicated.

	Stressors				Month/Year				
	Bled	Air	Heat	Burlap	June 2016	August 2016	October 2016	June 2017	July 2017
Avg. Water Temp.					18.4°C	22.3°C	14.0°C	17.7°C	20.7°C
Control					X			X	X
Bled	X				X			X	X
Air		X				X			
Air (Burlap)		X		X				X	X
Air & Bled	X	X				X			
Air & Bled (Burlap)	X	X		X				X	X
Air & Heat		X	X				X		
Air & Heat (Burlap)			X	X				X	X
Air, Heat, & Bled	X	X	X				X	X	X
Air, Heat, & Bled (Burlap)	X	X	X	X				X	X

BIOMEDICAL BLEEDING PROCEDURE

After all animals were monitored for baseline activity for one week prior to treatment and another 1 mL hemolymph sample was taken, animals were exposed to their randomly designated stressors (Table 2.2). The full bleeding procedure, replicating industry-standard procedures, took a total of three days. First, the animals were distributed between 50-gallon plastic barrels (since this is comparable to how the biomedical facilities transport the animals), and HOBO temperature loggers were placed in each of the barrels to record temperature. Then, the barrels were placed outside of JEL in direct sunlight for 4 h or next to a space heater in the JEL greenhouse (depending upon the temperature and ambient sunlight during the selected day; 31.5 ± 1.9 °C) to replicate the duration of time spent on the deck of a boat or on a dock prior to transport to a biomedical bleeding facility. After the first 4 h, the barrels were placed in the back of a car and driven around for an additional 4 h to simulate time spent in a truck traveling to a bleeding facility (23.3 ± 2.4 °C). Then, the barrels were placed indoors for 16 h at JEL to simulate time spent overnight at a bleeding facility (20.0 ± 2.1 °C). Finally, 30% of their hemolymph was extracted, and they were returned to their barrels where they remained for another 24 h to replicate a second night at a bleeding facility (19.4 ± 1.9 °C). The barrels were then placed back into a car for 4 h (22.3 ± 2.2 °C) to simulate transportation back to the dock, where they would be loaded on vessels and returned to their capture location. Lastly, all animals were returned to their respective flow-through estuarine water tanks for two weeks.

Control animals remained in their tanks throughout the entire duration of the experiment, except when a blood sample was collected. Animals that were in the just bled group also remained in their tanks, except for when 30% of their hemolymph was extracted on Day 10 and for the brief periods of time when blood samples were collected every ~7-10. The animals that

were exposed to one or more of the stressors were treated as outlined below, and each stressor was provided in the same manner as described above for the full procedure:

- **Bled**: Animals were subjected to hemolymph extraction on the second day of treatment.
- **Air**: On the first day of treatment, animals were placed inside the barrels inside JEL for the first 4 h. Thus, they were not left outside in direct sunlight or next to a space heater.
- **Heat**: Animals were exposed to direct sunlight or next to a space heater for the first 4 h.
- **Burlap**: Animals were treated as with Air, but covered with moist burlap for the entire duration of the treatment when they remained in barrels.
- **Two Stressors**: These animals were treated just as those exposed to the full procedure, except at least one of the stressors was omitted. For example, animals exposed to air and bled were treated the same as the full procedure, but they were not exposed to heat while they were in the barrels.
- **Three stressors (full bleeding procedure)**: Outlined above.

Table 2.2. Overall summary of schedule for each experiment.

Day	Treatment Schedule
1	-1 mL hemolymph sample taken -Placed in individual tanks for one week for baseline behavioral data collection
8	-1 mL hemolymph sample taken -First day of 'treatment'
9	-Second day of 'treatment' -Hemolymph extracted if bled
10	-Third and last day of 'treatment' -Returned to tanks
17	-1 mL hemolymph sample taken -Accelerometers downloaded
24	-1 mL hemolymph sample taken -Experiment ended

Hemolymph Extraction

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

$$H = 25.7\, e^{0.1928(IO)}$$ *[H= hemolymph volume (mL); IO= inter-ocular width (cm)].*

Hemocyanin concentrations (mg/mL) were measured using the procedure of Coates *et al.* (2012). Hemolymph samples were centrifuged for 10 minutes at 3000 g and 4°C. Then, an aliquot of hemolymph was diluted 1:100 in 0.1 M Tris-HCl buffer (pH 7.5) in a quartz cuvette with a path-length of 1 cm. The absorbance was measured at 280 nm on a Ultrospec 3100 pro UV/Visible spectrophotometer (Artisan Technology Group, Champaign, IL). Values were standardized using a pre-determined value of 1.39 mg/mL hemocyanin solution. This value was verified using a Pierce Modified Lowry Protein Assay Kit (ThermoFisher Scientific Inc, Waltham, MA).

DATA ANALYSES

Due to individual variation, data were converted and are shown as percent change from baseline for hemocyanin levels and overall activity. This also allowed us to pool treatment groups together (Table 2.3), except for the Air, Heat & Bled groups between 2016 and 2017.

There were significant differences in baseline hemocyanin and activity levels between these groups in different years, as well as a large difference in water temperature (2016: 14.0°C ± 1.9; 2017: 19.2°C ± 1.7) and overall behavioral patterns.

Table 2.3. Grouping of animals (last column) from different treatment groups for analyses. Every treatment that was repeated more than one time was grouped together, except for the full bleeding procedure (Air, Heat, & Bled) due to baseline differences and water temperature differences between 2016 and 2017. However, only the two experiments in 2017 of this treatment were grouped together due to similarities in the variables mentioned above.

	Month/Year					Group
	June 2016	August 2016	October 2016	June 2017	July 2017	
Avg. Water Temp.	18.4°C	22.3°C	14.0°C	17.7°C	20.7°C	
Control	X			X	X	Yes
Bled	X			X	X	Yes
Air		X				-
Air (Burlap)				X	X	Yes
Air & Bled		X				-
Air & Bled (Burlap)				X	X	Yes
Air & Heat			X			-
Heat (Burlap)				X	X	Yes
Air, Heat, & Bled			X	X	X	2017:Yes
Air, Heat, & Bled (Burlap)				X	X	Yes

Two-way ANOVAs were used to test for effects of time and treatment groups on hemocyanin levels and on overall activity. Two-way ANOVAs were also used to test for effects of different months and years, as well as different months and sexes, on hemocyanin levels. Tukey's HSD *post hoc* analyses were used to examine significant differences between means of treatment groups for hemocyanin levels and overall activity levels across different months, years, and sexes, with a threshold significance level at $\alpha = 0.05$. Unpaired Student's t-test were used to quantify changes in baseline hemocyanin levels in animals that died vs. those that survived.

Correlation and linear regression analyses were used to determine relationships between hemocyanin levels and overall activity levels.

A previously determined threshold value of 0.1 m s^{-2} (Watson *et al.*, 2016) was used to classify an animal as active or inactive. Data were lumped into 5 min bins, and if an animal exceeded the threshold value during any of the minutes in that 5 min period, the animal was considered to have been active for that 5 min period. These values were entered into the program ActogramJ to create actograms that could be used to determine the types of rhythms expressed (Schmid *et al*., 2011). Periodograms using the Lomb-Scargle method were used to determine when animals expressed significant circatidal (~12.4 h) or circadian (~24 h) rhythms (peaks exceeding α=0.001; tidal: 10-14 h range, daily: 22-26 h range; arrhythmic: no significant peaks in either range).

Results

MORTALITY

In 2016, when we used all males, there were only two mortalities throughout all of the treatment groups (Table 2.4). One mortality was in the Bled treatment group (n=7) and the second was in the Air & Bled treatment group (n=7). In 2017, we used all females and there were 13 mortalities. In June 2017, there were four mortalities, all in groups that received the full bleeding procedure. In July 2017, there were nine mortalities, including 6/8 of the animals in the full bleeding procedure groups (with and without burlap). It should be noted that the water temperatures in July were warmer than in June (average ± SD: 20.7°C ± 1.23, June: 17.7°C ± 2.1), and this might have been one reason why there were more mortalities in July.

Combined mortality rates (see Table 2.3) varied across the different experimental groups and, with the exception of the control group, there was at least one mortality resulting from each of the different individual stressors (air, heat, and bleeding). The overall mortality rate from all of the treatment groups, not including controls, was 17%. All mortalities occurred 1-2 days post-treatment, except for one animal in the Air, Heat, & Bled group that died 12 days post-treatment (Fig. 2.3).

Table 2.4. Mortalities in each of the different treatment groups. Several of the treatments were repeated in 2016 and 2017. The average water temperature (°C) per each month in each year is given. Bold numbers indicate mortality rates.

	Month/Year					
	June 2016	August 2016	October 2016	June 2017	July 2017	Combined mortality rate
Avg. Water Temp	18.4°C	22.3°C	14.0°C	17.7°C	20.7°C	
Control	0/8			0/4	0/4	**0%**
Bled	1/7 = **14%**			0/5	0/4	**6%**
Air		0/8				**0%**
Air (Burlap)				0/5	2/4 = **50%**	**22%**
Air & Bled		1/7 = **14%**				**14%**
Air & Bled (Burlap)				0/4	1/4 = **25%**	**13%**
Air & Heat			0/7			**0%**
Air & Heat (Burlap)				0/4	0/4	**0%**
Air, Heat, & Bled			0/8	2/4 = **50%**	4/4 = **100%**	2016:**0%,** 2017:**75%**
Air, Heat, & Bled (Burlap)				2/5 = **40%**	2/4 = **50%**	**44%**

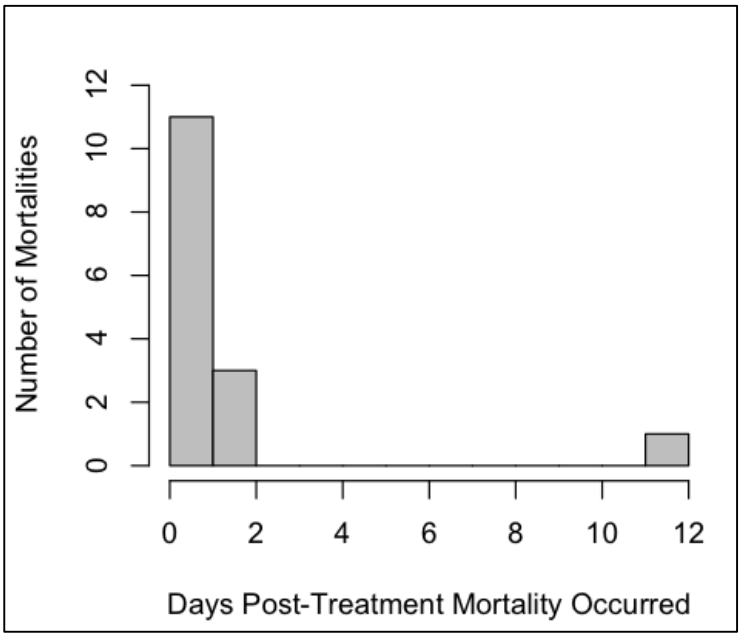

Figure 2.3. Histogram showing the number of days post-treatment when mortalities occurred. Most mortalities (n=15) occurred 1-2 days post-treatment, except for one that occurred 12 days post-treatment.

HEMOCYANIN LEVELS

Impacts of Different Treatments

The average baseline hemocyanin level for all animals was 0.42 ± 0.20 mg/mL with a maximum value of 0.96 mg/mL and a minimum value of 0.02 mg/mL. Due to this variability in the initial hemocyanin levels, data are expressed as a percent change from baseline. Every treatment group had a decrease in hemocyanin levels (mg/mL) from baseline, including the control group (Table 2.5), but changes were more pronounced in certain groups (Fig. 2.4, Table 2.5). There was a significant interaction between treatment and time on hemoycanin levels (Table 4). *Post hoc* analyses revealed that the hemocyanin concentrations in all groups were significantly lower than the Control, Heat (Burlap), and Air groups over time (Tukey's HSD test; $p < 0.05$).

Table 2.5. Two-way ANOVA results for hemocyanin levels for animals comparing all different treatments and time (baseline, week 1, and week 2). Bold represents significant difference ($p < 05$). Animals from the Air, Heat, & Bled group in 2017 were omitted from analyses due to low sample size resulting from mortalities.

Hemocyanin Levels

Source	df	MS	F	p
Treatment	9	3863.155	6.089	**<0.0001**
Time	2	28230.432	44.498	**<0.0001**
Treatment*Time	18	1339.613	2.112	**0.006**
Error	240	634.424		
Total	270			

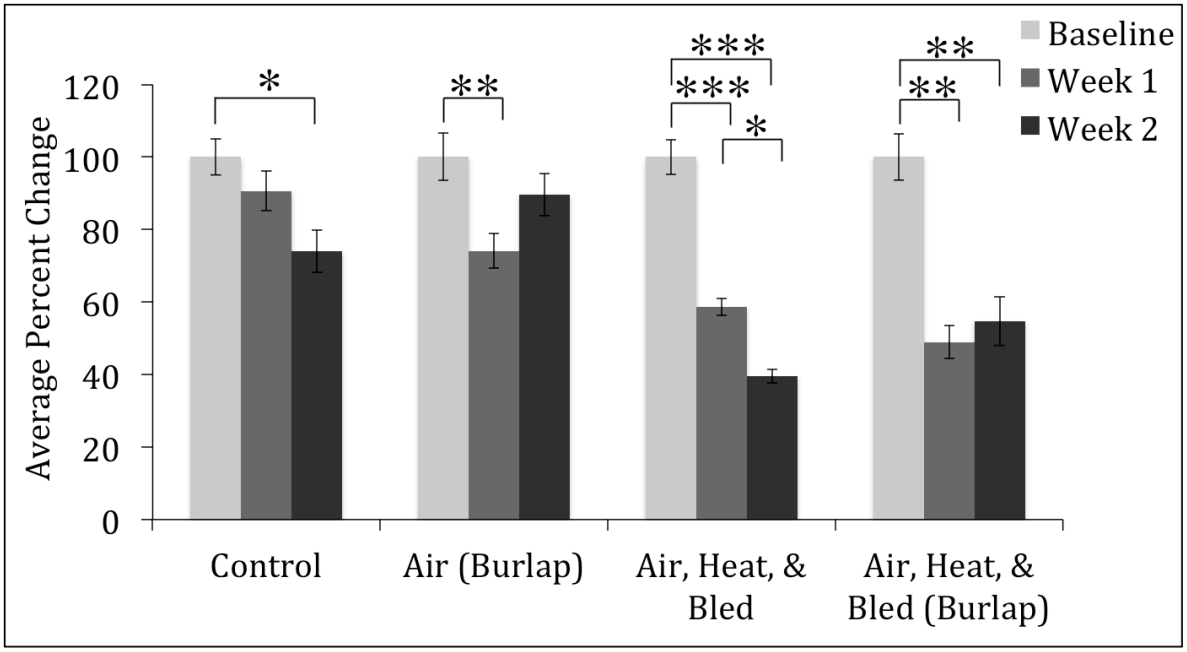

Figure 2.4. Average percent change in hemocyanin concentrations (± SEM) over time in different treatment groups. The Air, Heat, & Bled group, as well, as the Air, Heat, & Bled (Burlap) group had the greatest percent decrease in hemocyanin over time. The Control and the Air (Burlap) groups also had a decrease over time, even though they were not bled. Light gray bars are baseline (before treatment), and darker bars are hemocyanin levels at one- and two-weeks post-treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Control (n=16); Air (Burlap) (n=7); Air, Heat, & Bled (n=8); Air, Heat, & Bled (Burlap) (n=5).

Seasonal Differences

Animals were collected from June-October in 2016 and May-October in 2017. Only males were collected in 2016, so only males were used for inter-annual comparisons. Animals in

the fall had significantly higher hemocyanin levels than those collected in the spring in both years, and overall higher levels in 2017 (Table 2.6, Fig. 2.5). *Post hoc* analyses showed that animals collected in May and June had similar levels, animals collected in July were significantly higher than May and June, and animals collected in August through October were similar and had the highest average hemocyanin levels of around 0.7 mg/mL (Tukey's HSD test, $p < 0.05$). August animals had the highest peak in hemocyanin levels out of any month, and May had the lowest.

Table 2.6. Two-way ANOVA results for hemocyanin levels, comparing months (May-October) and years (2016 and 2017). Bold represents significant difference ($p < 05$).

Seasonal Hemocyanin Levels

Source	df	MS	F	p
Month	5	1.271	39.305	**<0.0001**
Year	1	2.469	76.348	**<0.0001**
Month*Year	4	0.032	0.980	0.418
Error	290	0.032		
Total	301			

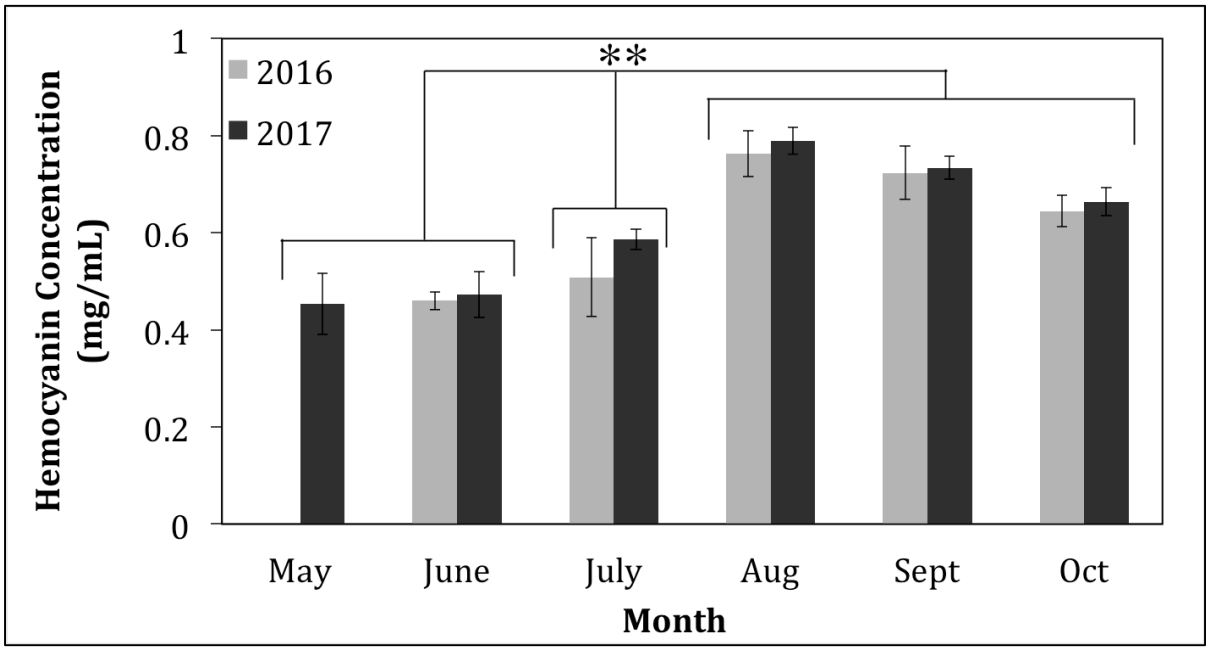

Figure 2.5. Monthly hemocyanin concentrations (mean ± SEM) of male horseshoe crabs collected during different times of the year. August animals had the highest hemocyanin concentrations and May had the lowest concentrations. **, $p < 0.001$. n=(2016, 2017); May=(12), June=(16,10), July=(3,31), August=(16,38), September=(16,15), October=(16,8).

In 2017, hemolymph samples were collected from both males and females. The overall trend of hemocyanin concentrations followed the same seasonal variation as shown above. However, animals that were collected in July were similar to those that were collected in September and October, as compared to how animals collected in July were significantly different from all months as shown above in Figure 3 (Tukey's HSD, $p < 0.05$). There were some interesting differences in monthly hemocyanin levels between males and females (Table 2.7, Fig. 2.6), with males having significantly higher hemocyanin levels in all months, except in males that were collected in July and October. Furthermore, while females had the lowest hemocyanin levels that were collected June, as compared to males that were collected in May, the hemocyanin levels peaked in animals that were collected in August, which was similar to males.

Table 2.7. Two-way ANOVA results from hemocyanin levels for animals comparing months (May-October) and sex (males and females). Bold represents significant difference ($p < 05$).

Sex Hemocyanin Levels

Source	df	MS	F	p
Month	5	0.647	26.630	**<0.0001**
Sex	1	0.565	23.272	**<0.0001**
Month*Sex	5	0.011	0.472	0.797
Error	222	0.024		
Total	234			

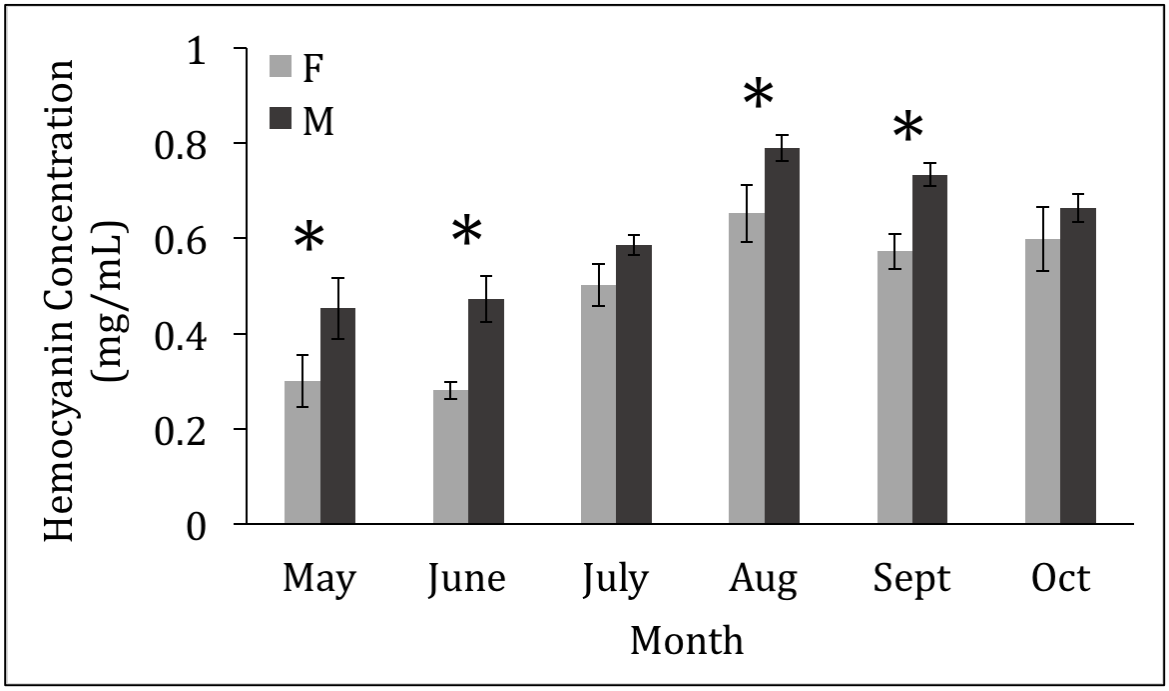

Figure 2.6. Comparison of male and female monthly hemocyanin concentrations (mean ± SEM) in 2017. Females had significantly lower hemocyanin levels than males in all months shown, except for July and October (*, $p < 0.05$ between hemocyanin levels of males and females at each month). Both sexes had their highest average hemocyanin levels occur in August. n=(female, male); May=(11,12), June=(74,10), July=(6,31), August=(13,38), September=(12,15), October=(4,8).

The Relationship Between Hemocyanin Levels and Mortality

Due to the higher number of mortalities in 2017 (13) compared to 2016 (2), only data from 2017 were used for this analysis. The average hemocyanin concentration for all other groups that did not have mortalities in 2017 was 0.23 mg/mL ± 0.14 mg/mL, with the minimum

baseline value being 0.04. The baseline hemocyanin levels in animals *that died* (n = 14; average 0.13 mg/mL ± 0.09 mg/mL) was significantly lower than animals that survived (n = 21; 0.30 ± 0.14) within each treatment group where there was at least one mortality (unpaired Student's t-test, p = 0.008; Fig. 2.7). In general, there was a trend in mortalities where animals that low baseline levels to start and were exposed to a combination of stressors seemed to be at a greater risk of mortality.

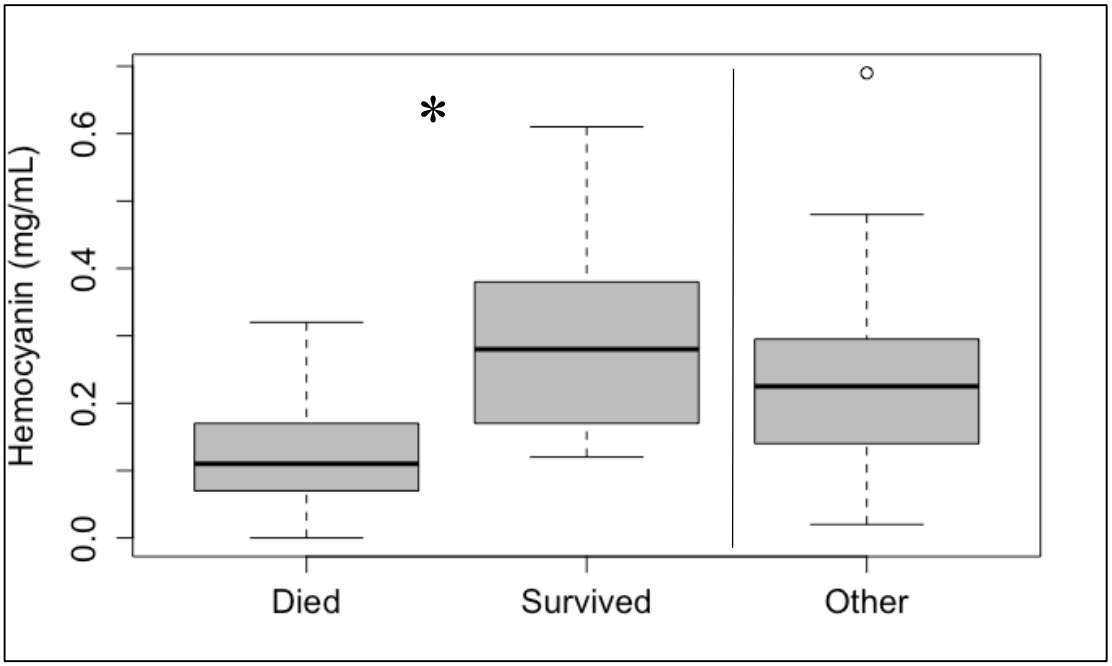

Figure 2.7. Baseline hemocyanin levels in animals from different treatment groups. The animals to the left of the dotted line represent hemocyanin concentrations in animals that were in treatment groups that had at least one mortality. There was a significant difference ($p < 0.05$) between the starting concentrations for these animals. However, in other treatment groups where there were no mortalities (right of the solid line), there were still lower starting levels but no mortalities in these groups due to different stressors imposed on the individuals.

Combined Impacts of Stressors and Hemocyanin Levels on Mortality

Using all of the data represented above, we were able to determine a mortality threshold value for animals that are collected for the bleeding process (Fig. 2.8). Out of all of the animals

that were collected between 2016 and 2017, the maximum baseline hemocyanin level was 1.2 mg/mL, and the average starting baseline level was 0.24 mg/mL. Animals that died as a result of being exposed to one of the treatments tended to have lower levels (average = 0.13 mg/mL; range 0.02-0.32 mg/mL) than those that survived (0.46 ± 0.19 mg/mL). Animals that are in captivity had decreases in their hemocyanin levels, which causes them to fall below these threshold values over time. For example, in control animals, we saw an average 10% decrease in hemocyanin levels over a one-week period (7 days) and a 27% decrease over a two-week period (14 days), as compared to baseline levels (Day 0; Fig. 2.4).

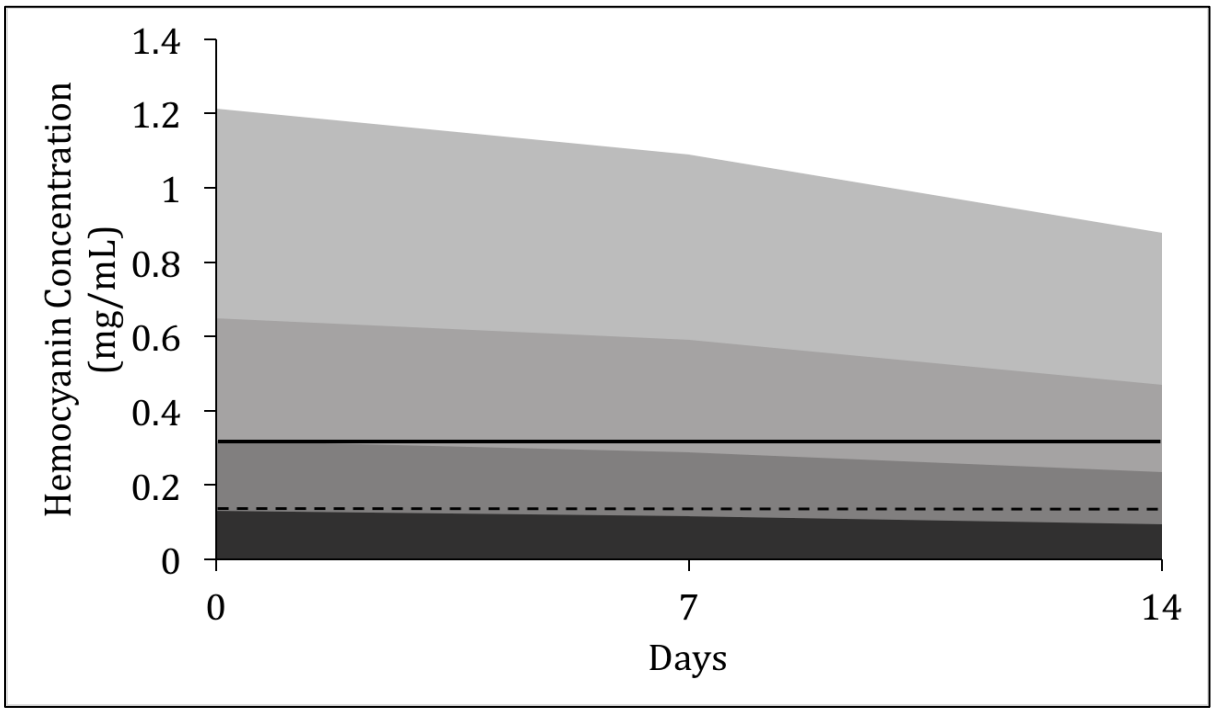

Figure 2.8. The relationship between hemocyanin levels and mortalities. The mortality threshold is represented by the solid black line (constant value of 0.32 mg/mL). Animals with hemocyanin levels above this value rarely died during the process. The dotted black line represents the most critical threshold (constant value of 0.13 mg/mL). Animals that have a baseline hemocyanin concentration of 0.13 mg/mL on Day 0 are most likely to die as a result of the bleeding process. As an animal is held in captivity over time before they are exposed to the bleeding process, they are more likely to be subjected to greater mortalities, potentially even falling below the critical threshold, as their levels decrease over time (10% decrease at Day 7 and 27% decrease at Day 14). Animals are likely to not be as affected by the bleeding process in the range from 0.32-0.65 mg/mL, and especially from 0.65-1.2 mg/mL. Shades of gray indicate health status before the bleeding process, with darker shades being less healthy.

IMPACTS OF BLEEDING AND ASSOCIATED STRESSORS ON LIMULUS *BEHAVIOR*

Overall Activity Levels

Overall activity levels in individuals significantly varied over time and across treatments, but there was no significant interaction between treatment and time (Table 2.8). Air, Air & Bled, and Air, Heat, & Bled (Burlap) groups were all significantly different from Air & Bled (Burlap) groups, while the other treatment groups were significantly similar to one another (Tukey's HSD, $p < 0.05$; Figure 2.9). Week 1 was significantly different than baseline and week 2, suggesting an immediate increase/decrease in activity levels after treatments (Tukey's HSD, $p < 0.05$; Fig. 2.10).

Table 2.8. Two-way ANOVA results for hemocyanin levels for animals comparing all different treatments and time (baseline, week 1, and week 2). Bold represents significant difference ($p < 05$). Animals from the Air, Heat, & Bled group in 2017 were omitted from analyses due to low sample size resulting from mortalities.

Activity Levels

Source	df	MS	F	p
Treatment	8	4768.589	2.743	**0.006**
Time	3	6914.491	3.977	**0.012**
Treatment*Time	15	1455.075	0.837	0.594
Error	210	1738.666		
Total	237			

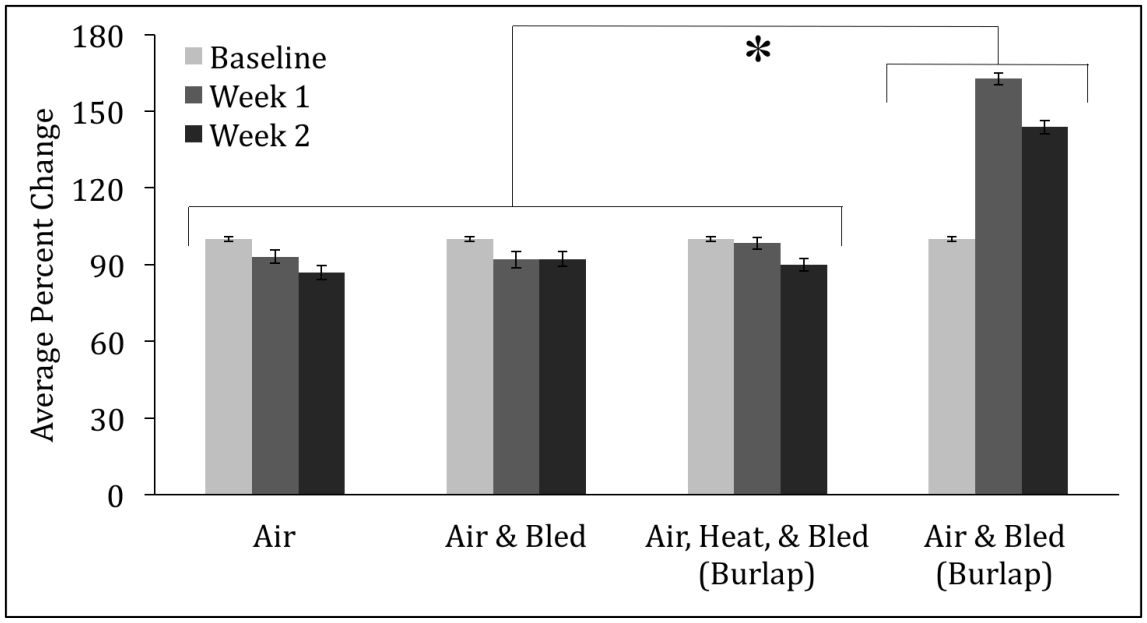

Figure 2.9. Average percent change (± SEM) from baseline (100%) for activity levels in different treatment groups over time. Animals in all the treatment groups decreased their activity after treatment, except for the Air & Bled (Burlap) group that increased activity. *, $p < 0.05$. Air (n=8); Air & Bled (n=6); Air, Heat, & Bled (Burlap) (n=5); Air & Bled (Burlap) (n=7).

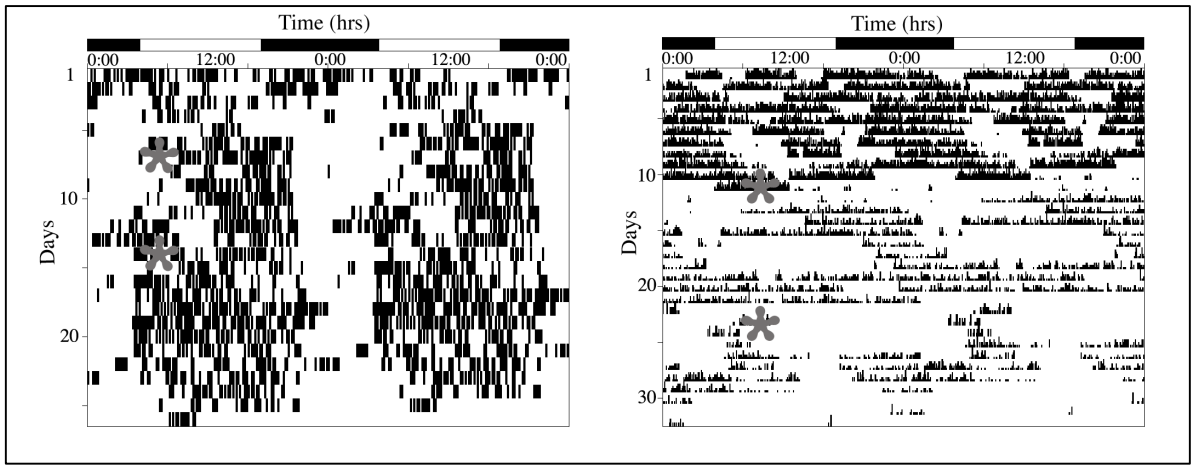

Figure 2.10. Double-plotted actograms showing activity over time. The individual on the left was from the Control group with the individual on the right being from the 2016 Air, Heat, & Bled treatment group. Activity remained constant over time for the individual on the left. Activity significantly decreased after the first week for the individual on the right (paired Student's t-test, $p < 0.0001$) and the second week compared to before the treatment ($p < 0.001$). This trend was similar across animals in this group. The x-axis is double-plotted to make rhythms appear more evident, showing a span of 48 h for each line. The gray/black horizontal bars on top represent the Light/Dark periods of the day. The y-axis represents ~three weeks of treatment for each animal (October 2016). Large gray stars indicate treatment (first star) and then two weeks post-treatment when the accelerometer was downloaded (second star). Note: this animal had a tidal rhythm during this treatment ($\tau = 12.6$ h).

Biological Rhythms

Across treatment groups and over time, there were a variety of daily, tidal, and arrhythmic behaviors expressed (Table 2.9). Initial rhythms were variable, with 37% animals exhibiting tidal rhythms, 38% expressing daily rhythms, and a lack of statistically significant rhythms in 25%. Across all treatment groups, we saw several changes in rhythms immediately after treatments and even two-weeks post-treatment. The majority of control animals (14/16) maintained their baseline rhythms throughout the duration of the study, except for two animals that switched from a tidal rhythm (τ = 12.6 h) to a daily rhythm (τ = 24.3 h) during the second week post-treatment (Fig. 2.11). The animals exposed to the full bleeding procedure had the greatest number of individuals that changed their rhythms after they were treated, while < 30% of the animals altered their rhythms as a result of all the other treatments (Table 2.9, Fig. 2.12).

Besides the full procedure, the Bled and the Air & Heat (Burlap) groups had the greatest individuals that expressed changes to their rhythms after they were treated. For the Bled group, there was not a distinct pattern in changes in rhythms from one week to the next. However, in the Air & Heat (Burlap) group, animals only switched rhythms one week post-treatment and maintained that rhythm for the duration of the study (Fig. 2.13), although rhythms expressed ranged from tidal, daily, and arrhythmic. Also, contrasting from the above two treatment groups, in the animals that switched rhythms in the full bleeding procedure in 2016, they only switched between tidal and arrhythmic behaviors. Interestingly, only the Air & Heat, and 2016 Air, Heat, & Bled group, were nocturnal (Table. 2.9). There were also some animals that were crepuscular, but this was seen across treatment groups.

Table 2.9. Summary of the rhythms expressed by each animal, in each treatment group, for each week of the study. Rhythms are only shown for animals that survived throughout the duration of the experiment. Thick black horizontal bars separates each treatment group. Light gray = tidal rhythm, dark gray = diurnal rhythm, striped= nocturnal rhythm, and black = arrhythmic. Baseline = before treatment, Week 1 = one week post-treatment, Week 2 = two weeks post-treatment.

Treatment	Sex	Baseline	Week 1	Week 2
Control	F			
Control	F			
Control	M			
Control	M			
Control	M			
Control	M			
Control	M			
Control	M			
Control	F			
Control	F			
Control	F			
Control	F			
Control	F			
Control	F			
Control	F			
Control	M			
Bled	M			
Bled	M			
Bled	M			
Bled	F			
Bled	M			
Bled	F			
Bled	F			
Bled	F			
Bled	F			
Bled	F			
Bled	M			
Bled	M			
Bled	F			
Bled	F			
Bled	F			
Air	M			
Air	M			
Air	M			
Air	M			
Air	M			
Air	M			
Air	M			
Air & Bled	M			
Air & Bled	M			
Air & Bled	M			
Air & Bled	M			
Air & Bled	M			
Air & Bled	M			
Air (Burlap)	F			

Air (Burlap)	F			
Air (Burlap)	F			
Air (Burlap)	F			
Air (Burlap)	F			
Air (Burlap)	F			
Air (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Bled (Burlap)	F			
Air & Heat	M			
Air & Heat	M			
Air & Heat	M			
Air & Heat	M			
Air & Heat	M			
Air & Heat	M			
Air & Heat	M			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air & Heat (Burlap)	F			
Air, Heat, & Bled (Burlap)	F			
Air, Heat, & Bled (Burlap)	F			
Air, Heat, & Bled (Burlap)	F			
Air, Heat, & Bled (Burlap)	F			
Air, Heat, & Bled (Burlap)	F			
2016 Air, Heat, & Bled	M			
2016 Air, Heat, & Bled	M			
2016 Air, Heat, & Bled	M			
2016 Air, Heat, & Bled	M			
2016 Air, Heat, & Bled	M			
2016 Air, Heat, & Bled	M			
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2016 Air, Heat, & Bled	M			
2017 Air, Heat, & Bled	F			
2017 Air, Heat, & Bled	F			

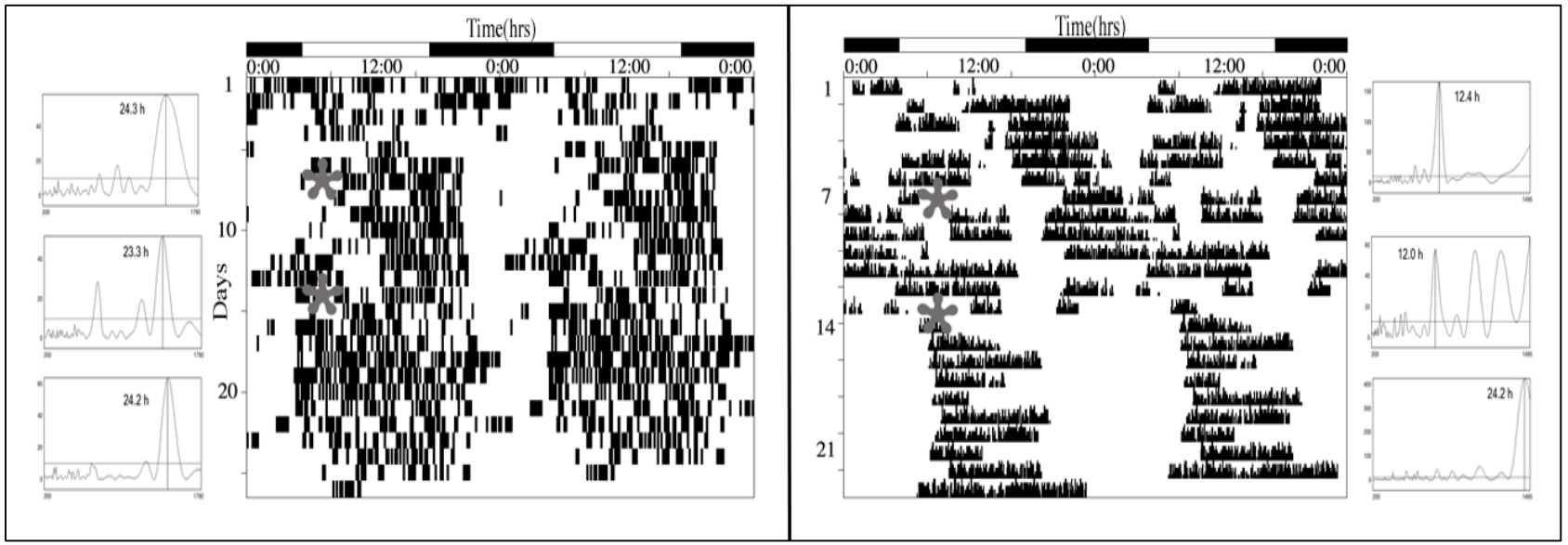

Figure 2.11. Double-plotted actograms displaying different rhythms expressed by Control animals. The animal on the left did not significantly change its rhythm over time, maintaining a daily rhythm (τ = 24.2-24.3 h) for three weeks. The animal on the right did not significantly change its rhythm until ~two weeks post-treatment (second star) on Day 15, where it switched from a tidal rhythm (τ = 12.4 h) to a daily rhythm (τ = 24.2 h). The accelerometer on the animal on the left logged at different intervals than the one on the animal on the right, causing a difference in the scale of black tick marks. See Figure 2.10 for more details about how this figure was produced.

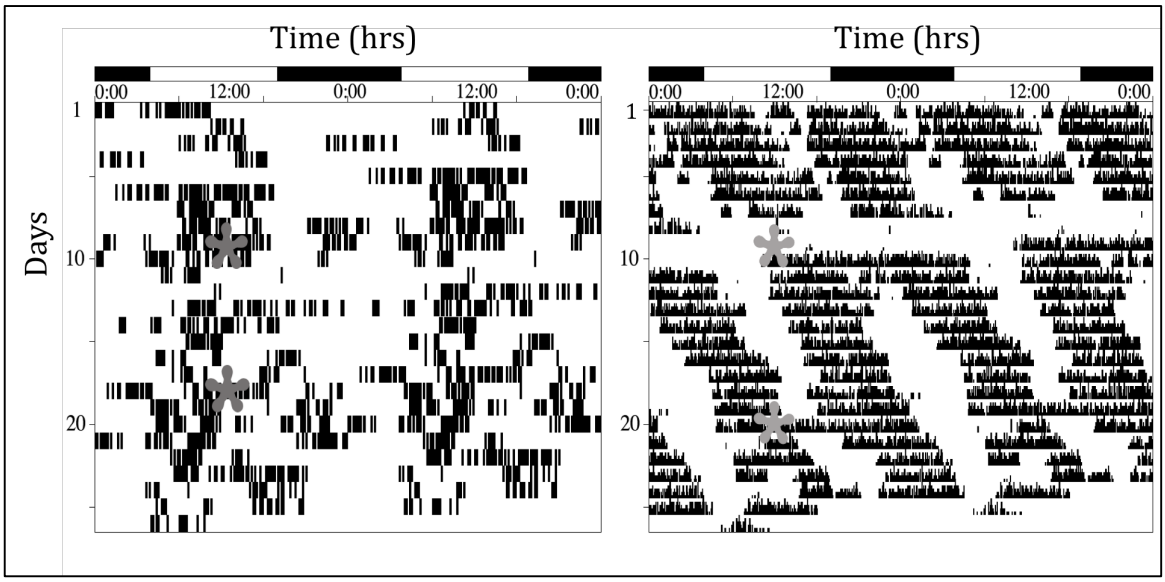

Figure 2.12. Double-plotted actograms displaying rhythms that remained constant throughout the duration of the experiment. The animal on the left was in the 2017 Heat group. It shows a daily rhythm (τ = 24.3 h), where the animal was more active during the day (especially at sunrise), with just a small decrease in activity immediately after the treatment. The animal on the right was in the 2017 Air, Heat, & Bled group and also maintained its rhythm over time, but in this case it was a significant tidal rhythm (τ = 12.4 h). However, it also expressed a brief decrease in activity immediately after treatment. The y-axis represents ~three weeks of treatment for each animal (left: October 2016; right: June 2017). See Figure 2.10 for more details about how this figure was produced.

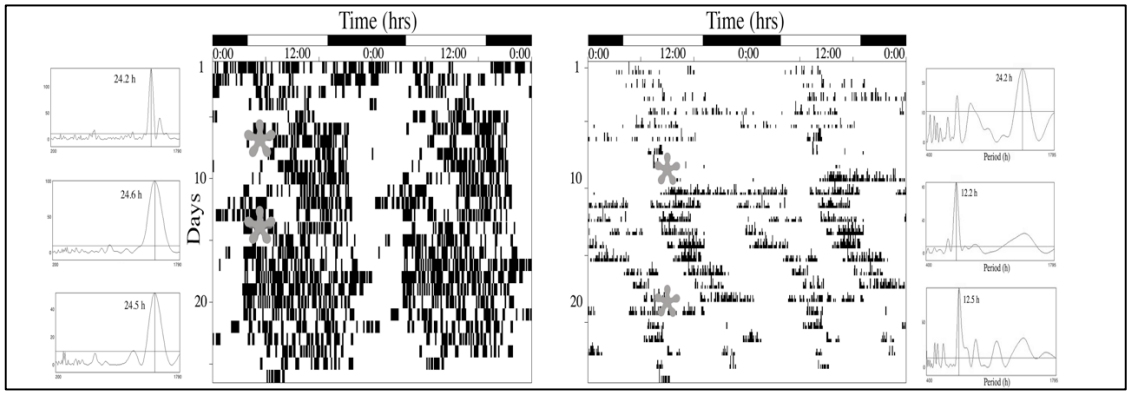

Figure 2.13. Double-plotted actograms comparing rhythms after treatments (June 2017). The individual on the left was from the Control group. It maintained a strong diurnal rhythm (τ = 24.2-24.5 h). This individual on the right was in the Air & Heat (Burlap) group. It switched from a daily rhythm (more active during the day; τ = 24.2 h) to a tidal rhythm immediately after treatment and maintained this rhythm for the duration of the study (τ = 12.2-12.5 h). See Figure 2.10 for more details about how this figure was produced.

The Relationship Between Activity and Hemocyanin Levels

Taken together, the data suggest that reduction of hemocyanin levels can cause mortality, changes in overall activity and, perhaps, changes in the types of activity rhythms they express over time. Therefore, we examined the relationship between overall activity levels and hemocyanin levels in 87 animals in both 2016 and 2017. Animals that were the most active had higher hemocyanin levels ($r = 0.41$, $p < 0.0001$; Fig. 2.14).

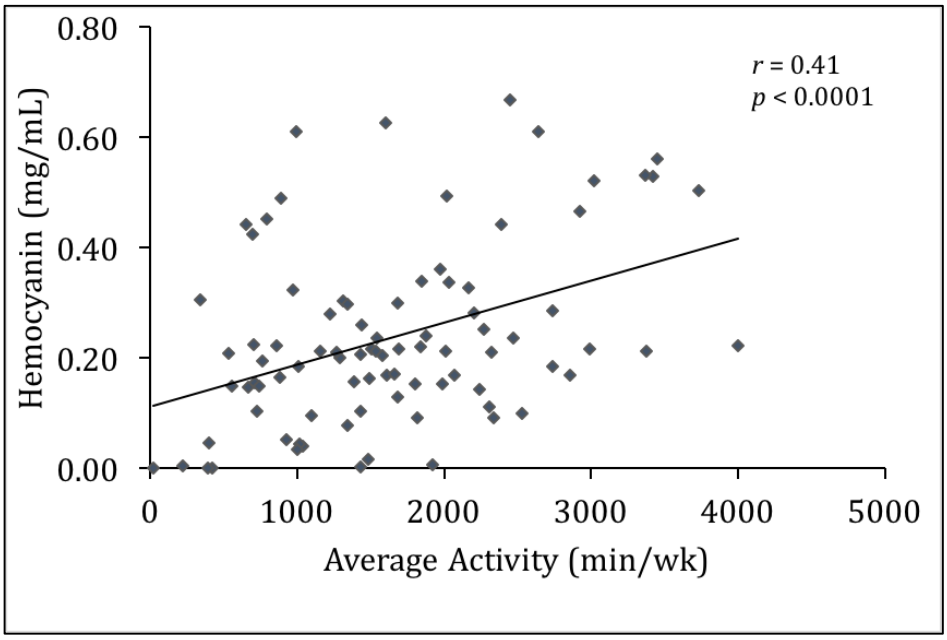

Figure 2.14. The was a significant correlation between overall activity and hemocyanin levels (n = 87; $F_{(1, 86)} = 17.01$, $p < 0.0001$, $R^2 = 0.16$).

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Discussion

The main objective of this study was to determine which of the stressors, or combination of stressors, associated with the *Limulus* biomedical bleeding process, has the greatest impact on the physiology and behavior of horseshoe crabs. Overall, we found that: 1) the full bleeding procedure (Air, Heat, & Bled) had the largest impact on mortality, hemocyanin concentrations, overall activity, and expression of rhythms, followed by bleeding along with one of the other stressors; 2) there was a seasonal trend in baseline hemocyanin levels (lower levels in spring followed by increased levels later in the summer and fall); 3) females had lower starting hemocyanin levels during all months sampled compared to males; 4) animals with a baseline hemocyanin level of < 0.13 mg/mL were most likely to die or be impaired by the bleeding process; 5) lower hemocyanin levels were correlated with lower activity levels and; 6) the findings from the laboratory studies corroborate those from the field study (see Chapter 1).

Specifically, females appear to be more impacted by the bleeding process, the full bleeding procedure has the greatest impact, and if animals are able to survive beyond the first 2 days post-treatment (especially if they are healthy), then there are few long-term impacts.

MORTALITY

Throughout all of the trials for this study, mortality rates differed between treatment groups, with an overall mortality rate of 17% (excluding Control groups). Animals exposed to the full bleeding procedure in 2017 experienced the greatest number of mortalities (75%). Our mortality rates are similar to the estimated 5-30% mortality rate seen in previous studies (Thompson, 1998; Walls and Berkson, 2000; Walls and Berkson, 2003; Hurton and Berkson, 2006; Leschen and Correia, 2010; Anderson *et al*., 2013). However, the higher end of our mortality range is extremely high compared to the numbers cited in the literature. There could be several reasons contributing to this. First, we had relatively low sample sizes for each of our treatment groups. These low sample sizes skew the mortality rates seen in each group. Also, in the group where we saw 75% mortality, this group was inflicted with the greatest stress out of any of our groups. This was the Air, Heat, & Bled group. For this treatment group, we examined the greatest potential combination of stressors possible.

We did not see any mortalities in our Control, Air, Air & Heat, and Air & Heat (Burlap) groups. This indicates that mortalities were more likely to occur in any treatment group that was bled, with the exception of two mortalities seen in the Air (Burlap) group. This supports previous findings that found a 10-20% increase in mortality rates in animals that were subjected to only hemolymph extraction as compared to control animals (Rudloe, 1983; Kurz and James-Pirri, 2002). From our findings and previous findings, it is apparent that there is a mortality rate

associated with the bleeding process, specifically when bleeding is involved, or bleeding with another stressor.

We had more mortalities (27%) in 2017 than in 2016 (4%). One potential cause for this increased rate was the use of different sexes between the two years. In 2016, we used only males, while in 2017 we used just females. This working hypothesis is supported by the studies by Leschen and Correia (2010) and James-Pirri (2012) that reported a 15% mortality in males and up to 29% mortality rate in females. It is unlikely due to temperature differences considering average water temperatures in 2017 peaked around 20.7°C, but water temperatures in 2016 peaked around 22.3°C. Also, the only difference in treatment groups between the two years was the addition of burlap in 2017 to keep the animals moist, and this was intended to mitigate any lethal or sublethal impacts.

When mortalities did occur, they took place immediately post-treatment, with 14/15 mortalities occurring 1-2 days after receiving their designated treatment. This highlights the importance of returning the crabs to their natural environment as soon as possible. The United States Food and Drug Administration mandates that crabs be returned within 24-72 hours after time of collection (ASMFC, 1998). As of today, three out of the four facilities return crabs within 48 hours of collection, with one facility distributing their bled crabs to a secondary market (bait industry; ASMFC, 2007).

HEMOCYANIN LEVELS

Change over time

We observed several different trends in hemocyanin concentrations over time and within treatment groups. First, we saw a decrease in hemocyanin levels over time, especially within the

first week in all groups, including the Control group. This is likely due to the animals being kept in captivity. According to Coates *et al.*, (2012), the immune-competence of *L. polyphemus* is negatively affected by the stress associated with holding animals in captivity. The specific effects of low protein concentration and hemocyanin levels on horseshoe crab physiology, such as maturation and egg production, as well as on their behavior has not been well-studied, except for the observation that lower protein levels have been associated with starvation and mortality (Alsberg, 1914). More recently, high mortality rates of horseshoe crabs have actually been documented in animals kept in captivity. This increased mortality appears to be caused by hepatic insufficiency, protein-losing enteropathy, nutritional imbalance/deficiency, and protein losing nephropathy (Nolan and Smith, 2009; Carmichael and Brush, 2012). These findings are the basis for the proposed new regulations that are being put forth that require biomedical facilities to use high quality water and frequent feeding to maintain the health of the horseshoe crabs (Armstrong and Conrad, 2008). However, there appears to be no published or anecdotal evidence that any of the biomedical facilities are feeding the crabs while they are held at the facilities or before they are released.

Furthermore, if mortality rates are higher, in part due to the stressors associated with being held in captivity, then every effort should be made to release them into their natural environment, or in conditions very similar to those in their natural habitat, as soon as possible after capture. In recent communications with a representative from a biomedical facility, it was discovered that at least one of the facilities (Charles River Endosafe, South Carolina) keeps their collected horseshoe crabs in aerated 'holding ponds' until they are bled or until they are released back at their collection site (J. Cooper, pers. comm., Charles River Endosafe). Although this appears to be a highly suggested alternative method than keeping the horseshoe crabs in captivity

in tanks, it has also been revealed that these holding ponds are overcrowded and there tend to be mass die-offs in these ponds. A horseshoe crab supplier stated that the die-off was caused by a major rain event that lowered the salinity levels in the pond (ASMFC, 2003). Therefore, these ponds may be causing more harm than good and should be assessed for trying to reduce the effects of captivity and holding duration, especially with the additive effect of bleeding on horseshoe crabs.

Impacts of Different Treatments

We also saw changes in hemocyanin levels due to different treatments, suggesting that captivity was not the only factor contributing to lower hemocyanin levels over time. In particular, the hemocyanin levels dropped significantly in the animals exposed to the full bleeding procedure (Air, Heat, & Bled group) when compared to other treatments. The one major stressor that caused the greatest decrease in hemocyanin levels was heat. Increased temperature may be the most significant variable because it directly affects metabolism, oxygen consumption, growth, survival and molting (Chen *et al*., 1995; Hennig and Andreatta, 1998). Coates *et al.*, (2012) found that hemocyanin concentrations decreased as temperatures increased (ranging from 8°C to 23°C). To make matters worse, the O_2 affinity of hemoycanin, like other respiratory pigments, decreases as temperature increases (Redfield, 1934; Burnett *et al*., 1988), so not only do they have less hemocyanin, but it can't hold as much oxygen. When combined with a higher metabolism at higher temperatures, this could cause long-term hypoxia, and this could be one major cause of both mortalities and reduced activity. Through a personal communication with a biomedical facility representative, I learned that one of the facilities only collects horseshoe crabs during the night when it is cool outside (ASMFC, 2003). This might be

a valuable protocol that should be adopted by all biomedical facilities and could reduce any unnecessary thermal stress on horseshoe crabs.

Changes in hemocyanin levels might also lead to mortalities or alterations in behavior due to the role that hemocyanin plays in the function of the immune system. Hemocyanin is converted into a phenoloxidase-like enzyme by hemocyte components (Adachi *et al*., 2003), and the enzymatic oxidation is important in the cuticle formation and immune reaction in arthropods (Söderhäll & Cerenius, 1998; Ashida and Brey, 1997). Thus, the impacts of different factors on hemocyte levels can also have an indirect impact on hemocyanin function, specifically in regards to temperature. For example, in a species of fiddler crabs, *Uca pugilator*, temperature also affected clotting times, levels of its plasma protein, and total hemocyte numbers (Dean and Vernberg, 1966). Another crab, *Carcinus maenas*, also exhibits seasonal changes of its hemocyte antibacterial activity by its hemocytes as temperature changes seasonally (Chisholm and Smith, 1994). All of these impacts on hemocyte levels as a result of temperature could indicate that hemocyanin levels could also be affected, especially in horseshoe crabs.

Air exposure can have negative impacts both on its own, and in combination with heat and blood loss. The major function of the book gills of horseshoe crabs is to help circulate blood throughout the lamellae, and gas exchange occurs as water flows over the lamellae. During air exposure, the book gills can become desiccated, and gas exchange cannot occur and hemolymph cannot flow properly throughout the body. As stated above, heat will increase their metabolism, so they need more oxygen, and when hemocyanin is low, they cannot carry as much oxygen. So with all of these stressors combined, it could lead to severe hypoxia. Also, amebocytes, the granular cell that plays a crucial role in defending horseshoe crabs against pathogens, are created through cell differentiation that is sourced from the gill flaps (Gibson *et al.*, 1992). Thus, any

harm to the gills can cause amebocyte growth to be hampered, and reduced immune function. However, while air exposure can have negative impacts on horseshoe crab physiology, it does not appear to be as detrimental as heat, perhaps because the placement of the gills on their ventral surface generally produces a microclimate that increases humidity and decreases desiccation. However, when both stressors are combined, they likely have a synergistic effect.

Hemocyanin comprises 90% of total hemolymph protein in *L. polyphemus* (Ding *et al*., 2005), and an estimated 30% of the hemolymph volume is extracted from horseshoe crabs during the bleeding process. This process clearly causes hemocyanin concentrations to decrease, and this can lead to some of the heath issues stated above. Although this study only observed at two weeks post-treatment, previous findings have shown that it takes many weeks for hemocyanin levels to recover following bleeding. For example, Anderson *et al.*, (2013) showed that levels were still low after six weeks, and James-Pirri *et al*., (2012) found significantly reduced total hemolymph protein levels in bled animals compared to controls 17 days after bleeding, with bled animals having 20% less hemolymph than controls. In summary, the combination of blood loss, exposure to air (so that ventilation is reduced), and exposure to heat (which increases metabolism), appears to have the most dramatic impacts on both *Limulus* mortalities and behavior. While these same stressors can also have a negative impact on the immune system, in our study, the most dramatic impacts occurred during the first week after animals were bled, and thus these changes are probably too early to be caused by immuno-deficiency and possible infection. This is probably due, in part, to the fact that they all influence the ability of horseshoe crabs to obtain sufficient oxygen, and circulate it, to all other tissues.

Seasonal and Sex Differences

Another interesting finding from this study was that we saw a significant effect of season and sex on hemocyanin levels. Hemocyanin levels were the lowest in May and June and the highest in August and September. The spawning season for 2016 was May 9-June 10 and in 2017 it lasted from May 16-June 16; which corresponded to when hemocyanin levels were significantly lower. Furthermore, during all months sampled (May-October), females had lower hemocyanin levels, than males, especially in May and June. Females have shown to have greater variability in estimated hemolymph volume for animals of the same carapace width, and this is likely due to the fact that females carry eggs which displaces the space in body cavities that could be filled with hemolymph (Hurton *et* al., 2005). Although this explains the lower hemocyanin levels in females in May and June, it does not necessarily explain the lower levels later in the year, or the low levels of males during the spawning season.

Not only is egg production an energetically expensive process (Bryant and Hartnoll, 1995; Nicol *et al*., 1995; Guillou and Lumingas, 1999; Chaparro and Flores, 2002; Lovrich *et al*., 2005; Jørgensen *et al*., 2006), but making several trips to spawning beaches within the same season, often with males attached to their carapace, can be energetically costly. If this is combined with low hemocyanin levels, it might explain, in part, why females are likely to be more heavily impacted and physiologically stressed from the bleeding process. This is certainly consistent with the data presented in Chapter One showing that females were less likely to express normal activity when bled and released into their natural habitat, especially during the spawning season.

In the past, females have been harvested more for bleeding due to their size and their associated increased hemolymph volume. In the heavily harvested Pleasant Bay, MA, 66% of the

horseshoe crabs were female, at least up until 2001 (Rutecki *et al*., 2004). Also, harvested females were larger than the average females in the Pleasant Bay populations, revealing that harvesters selected for larger females (Rutecki *et al*., 2004). In this area, over 50% of the harvest and bleeding occurs during, or shortly after, the spawning season. Since larger females are being selected for, and these animals are also more fecund (Leschen *et al*., 2006), this selection practice risks reducing the number of eggs being produced and fertilized over time and may alter population dynamics. Given that females have higher mortality rates than males when they are bled, their tendency to have lower hemocyanin levels throughout the year, and their added metabolic costs during spawning, it seems safe to say that more attention should focus on making the bleeding process less detrimental to females.

The Relationship Between Hemocyanin Levels and Mortality

In this study, we found that animals with lower baseline hemocyanin levels suffered greater sublethal and lethal impacts when exposed to multiple stressors from the bleeding process. The baseline hemocyanin levels in animals that died in different treatment groups were significantly lower than in animals that survived in those same treatment groups. The treatment groups that had mortalities included Air, Heat, & Bled (with and without burlap), Air & Bled (Burlap), and Air (Burlap). Thus, air and bleeding seemed to be the most significant stressors, because animals in poor initial health conditions that were exposed to these factors were more likely to have increased mortality rates. In order to use this information to reduce mortalities, biomedical facilities might take a hemolymph sample before bleeding animals and only proceed to bleed those animals that fall above a certain threshold. Even though facilities are required to pre-screen for injured crabs before they are bled, if they could take this precaution one step further and take an initial blood sample, it may significantly reduce mortality rates as a result of

the biomedical bleeding process. For example, from all of the hemocyanin data collected from this project, we were able to estimate a critical threshold level of hemocyanin that animals should have to be selected to undergo the bleeding process. Specifically, animals that have a baseline level of 0.32 mg/mL or below should not be bled. The absolute critical level from this study was 0.13 mg/mL. Any individual that had this baseline starting hemocyanin level and was exposed to at least two or three of the different stressors died. How close animals are to these thresholds changes across sexes (with females having lower baseline hemocyanin levels), over a seasonal time-scale (with increases in late summer and fall months as shown in our seasonal trends, see Figs. 2.5, 2.6), and the length of time an animal is held in captivity (hemocyanin decreases as the amount of time in captivity increases, and this is probably related to nutrition). The latter point is important to keep in mind because an animal that is collected and has a blood sample taken immediately, may appear to be above these suggested thresholds. However, the longer the animal is kept in captivity or in the holding ponds at the facilities, the more their hemocyanin concentration will decrease to the point where they may fall below the thresholds over time. Thus, it may be advantageous for biomedical facilities to assess the health (i.e. relative hemocyanin) just before they are bled, and bleed them, and subsequently release them, in a timely manner to alleviate any impacts from the bleeding process.

IMPACTS OF BLEEDING AND ASSOCIATED STRESSORS ON LIMULUS *BEHAVIOR*

Overall Activity Levels

Along with observed mortalities and impacts to hemocyanin concentrations, we saw other sublethal impacts on changes in the behavior of horseshoe crabs once they were exposed to different stressors from the bleeding process. Most treatment groups had an immediate decrease

in activity after treatment, with activity levels remaining lower than baseline levels even two weeks post-treatment. Note that the decrease in overall activity was positively associated with a decrease in hemocyanin levels over time, with the exception of the Air & Bled (Burlap) group discussed above (Fig. 2.9). In that group, we observed an almost a two-fold increase in activity during the first week post-treatment. This is not uncommon to see in horseshoe crabs and other crustaceans. Anderson *et al*., (2013) suggested that this increase in initial activity could have been indicative of an escape response (Rudloe and Hernnkind, 1976), foraging efforts in order to replenish lost energy reserves as has been shown in *Cancer pagarus* (Patterson *et al*., 2009), or disorientation from the bleeding process (Kurz and James-Pirri, 2002). However, Rudloe (1983) did not see any difference in overall activity levels between bled and control animals, but in that study animals were exposed to the full bleeding procedure, and in this case, the animals with increased activity were only in the ones exposed to the Air & Bled treatments.

Changes in the expression of activity rhythms also occurred, along with the decrease in overall activity, in animals exposed to the various treatments, with the most clear-cut changes taking place in three treatment groups: Bled group, Air & Heat(Burlap) group and the 2016 Air, Heat, & Bled group. The 2017 Air, Heat, & Bled group had a large number of mortalities, so it was difficult to discern any trends seen in this group. There were no overall trends in switches to different rhythms in the different groups. Initial rhythms were variable, with 37% animals having tidal rhythms, 38% expressing daily rhythms, and 25% being arrhythmic. Also, there was not a distinct pattern in changes in rhythms from one week to the next. However, we did see a decrease in activity over time. These findings are only slightly similar to those found by Anderson *et al*., (2013) in that they saw a decrease in activity, but in contrast they saw a decrease in the expression of tidal rhythms.

Another potential reason for seeing a change in rhythmic behaviors is handling impacts. For every treatment group, we picked the animals up out of the water to either treat them according to the different stressors they were assigned, or to take a blood sample (during the treatment period and one week post-treatment). Therefore, when we saw changes in the expression of rhythms two weeks post-treatment, it may not have been a delayed impact from the bleeding process, but rather a behavioral response to being handled. For example, even 2/16 control animals switched from a tidal rhythm to a daily rhythm after they were handled, and in cases where treated animals changed rhythms after about two weeks, the switches almost always occurred right after they were handled. This switch in behavior following handling is not uncommon and it took place in a number of cases in a previous study of feeding and handling (M. Owings, unpublished data). Nevertheless, despite the occasional animal that changed activity immediately following handling late in a trial, there were many other animals that were clearly impacted by the treatments and which expressed changes following their treatments that lasted throughout the duration of the experiment and these did not change following the handling associated with the two-week blood sample. Therefore, as demonstrated previously by Anderson *et al.,* (2013), it appears as if the biomedical bleeding process has both lethal and sublethal impacts. Moreover, based on the data obtained from this study, exposure to excess heat, along with losing a significant amount of blood, appear to have the greatest impacts on their behavior.

CHAPTER 3: EFFECTS OF FOOD SUPPLEMENTATION ON THE PHYSIOLOGY AND BEHAVIOR OF HORSESHOE CRABS SUBJECTED TO THE BIOMEDICAL BLEEDING PROCESS

Abstract

Previous studies have demonstrated that horseshoe crabs held in captivity, even if they are not bled, will experience a decline in their hemocyanin levels. The goal of this study was to test a food supplement that might be able to reduce these sustained decreases in hemocyanin levels, and perhaps the associated behavioral impacts. In order to do this, we measured hemocyanin levels over time in animals that were bled and fed different dietary supplements, specifically focusing on using a by-product from the bleeding process (post-processed *Limulus* hemolymph) and an ingredient from an invasive species (green crab extract). We also monitored the overall activity and expression of rhythms in a subset of animals that were bled and fed. Our findings showed that animals that were fed had an increase in their hemocyanin levels and overall activity, and maintained the same biological rhythms they had prior to being bled. This study indicates that providing dietary supplements to horseshoe crabs either before or after bleeding them might be a logistically realistic way to improve physiological status and maintain a healthy population of an important species.

Introduction

As seen in our previous findings from Chapter One and Chapter Two, there are sublethal and lethal impacts of the biomedical bleeding process on horseshoe crabs. Specifically, there are sustained decreases in hemocyanin levels within the first couple of weeks, and the decreased hemocyanin levels are intensified with air exposure and increased temperatures. Similarly, previous studies in the laboratory have observed reduced hemocyanin levels six weeks post-bleeding (Anderson *et al*., 2013), and that it takes 3-7 days for a bled horseshoe crab to regain its total blood volume and up to 4 months for amebocytes to return to baseline levels (Novitsky, 1984). Given that hemocyanin has a wide variety of functions, including transporting oxygen, eliciting the primary immune response (Coates *et al.*, 2011), contributing to wound repair and cuticle hardening (Adachi *et al.*, 2005a), molting (Adachi *et al.,* 2005a,b), hormone transport (Jaenicke *et al*., 1999), and osmoregulation (Paul and Pirow, 1998), it can be detrimental for horseshoe crabs to have prolonged low levels of hemocyanin. Thus, determining a way to

increase hemocyanin levels might accelerate the recovery time in horseshoe crabs after they have been bled, and this might be a beneficial next step in mitigating impacts of the process prior to release and improving their performance once released.

One potential method might be providing the animals with a food supplement before, or even after, they are bled to help them replenish their energy reserves and synthesize new hemocyanin. The major goal of this study was to develop and test a food supplement that could be used for this purpose.

Several other studies have looked at dietary supplements on growth performance and immune responses in the Chinese mitten crab, *Eriocheir sinensis*. These studies have shown that total hemocyte counts and survival rates in these species increased with increasing supplementation of dietary copper (Sun *et al*., 2013). Also, Qin *et al.*, (2016) found that hemocyte counts, antioxidant capacity, and hemocyanin expression were strengthened as dietary nanoSe (elemental particles of Se that are important in immune systems and functions) increased. Thus, supplementing horseshoe crab's diet with ingredients such as copper, hemocyanin, or nanoSe could be a particular adaptive strategy for overcoming the negative impacts associated with the bleeding process.

In addition to mitigating the effects of the bleeding process on horseshoe crabs, it might be valuable to target an invasive species potentially containing useful 'ingredients' by using them in our supplement. The European green crab, *Carcinus maenas*, was introduced into the US in 1817 (Chilton, 1910; Almaça, 1963; Vermeij, 1982; Cohen *et al.,* 1995; Carlton & Cohen, 2003; Eastwood *et al*., 2007; Darling *et al*., 2008), and is native to the Atlantic coastline from Norway to Mauritania (Crothers, 1968; Elner, 1980; Carlton & Cohen, 2003; Roman & Palumbi, 2004). More specifically, the green crab spread into New Hampshire in the 1890s or early 1900s

(Smith, 1879; Rathbun, 1905, 1930; Glude, 1955; Grosholz & Ruiz, 1996; Behrens Yamada, 2001; Audet *et al.,* 2003; Carlton & Cohen, 2003). The green crab is an opportunistic omnivore (Cohen *et al*., 1995) that feeds on numerous prey items, altering food webs in intertidal areas. It causes damage to mussel beds (DeGraaf & Tyrrell, 2004) and eelgrass beds (Davis *et al*., 1998; Malyshev & Quijón, 2011; Garbary *et al.,* 2014; Neckles, 2015) in New England. Therefore, using this species as an ingredient in a food supplement might encourage fishermen to remove them and reduce their impacts on estuarine and coastal ecosystems. In addition, it is important to note that *Carcinus maenas* has hemocyanin in its hemolymph, thus directly providing a source of hemocyanin and copper for horseshoe crabs.

Not only is using green crabs an ideal option for a food supplement, but using *Limulus* hemolymph from biomedical facilities that is a by-product from the bleeding process may also be a useful addition. This is left over after the removal of LAL. This by-product would be easily accessible and inexpensive for facilities to use to make a potential food supplement. With these options in mind, we created a food supplement to test the impacts on hemocyanin levels in crabs post-bleeding. In order to do this, we had several trials where we tested a food supplement and measured the hemocyanin levels in animals that were controls, bled, or bled and fed the supplement. We also measured the hemocyanin levels in animals that were bled and held in cages in their natural environment to determine if there was an additional key nutritional source that could only be provided in their habitat. Lastly, we monitored the behavior of bled horseshoe crabs, and bled and fed horseshoe crabs, to see if the food supplement would also have a positive impact on their behavior.

Methods

To assess the influence of food supplements on the hemocyanin levels and behavior of horseshoe crabs, we performed several experiments in which we fed selected horseshoe crabs different food supplements and measured their changes in hemocyanin levels and behavior. All animals were collected by scuba-diving at Fox Point in the Great Bay Estuary, New Hampshire. Animals were brought back to the Jackson Estuarine Laboratory (JEL), Durham, New Hampshire and kept in flow-through estuarine tanks that received flow-through water directly from the estuary throughout the duration of the experiment. HOBO temperature loggers (Onset Computer Corporation, Bourne, MA) were placed in the ambient environment (tanks, enclosures, etc.) to record temperature over the course of each experiment (average water temp ± SD : 19.4 ± 1.3°C). Animals were tagged with identification numbers to be able to discern individuals from one another.

FOOD SUPPLEMENT:

I. Green Crab and *Limulus* Blood Supplement (GCLB): This food supplement was designed, created, and provided to us by Dr. Jury. It was primarily made up of green crab extract (18%), *Limulus* hemolymph (9%), water, poultry byproduct meal, brewers spent grains, sodium alginate, and calcium carbonate. After the supplement was made, it was frozen overnight to harder, and then left in the refrigerator for up to two weeks. The supplement was not heated in the processing.

II. Green Crab Supplement (GC): This food supplement was designed, created, and provided to us by Dr. Jury. It was made up in the same manner as the GCLB supplement, but there was no *Limulus* hemolymph. Instead, the amount of green crab extract was doubled.

FEEDING PROTOCOL:

Animals that were given either GCLB or GC were picked up out of the water and placed ventral side up on a flat surface. A small piece of the food supplement was placed into their mouths (no more than 2g). Some ambient water from their tanks was squirted into their mouths to help dissolved the food and make it easier to be pushed down into their mouths with their legs. The animals were fed until satiation (when the animals stopped eating), and then they were returned to their designated tanks until they were to be fed again (fed every 2 days).

HEMOLYMPH EXTRACTION:

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

$$H = 25.7\, e^{0.1928(IO)}$$ *[H= hemolymph volume (mL); IO= inter-ocular width (cm)].*

Hemocyanin concentrations were measured using the procedure of Coates *et al.* (2012). Hemolymph samples were centrifuged for 10 minutes at 3000 g and 4°C. Then, an aliquot of hemolymph was diluted 1:100 in 0.1 M Tris-HCl buffer (pH 7.5) in a quartz cuvette with a path-length of 1 cm. The absorbance was measured at 280 nm on a Ultrospec 3100 pro UV/Visible spectrophotometer (Artisan Technology Group, Champaign, IL). Values were standardized using

a pre-determined value of 1.39 mg/mL hemocyanin solution. This value was verified using a Pierce Modified Lowry Protein Assay Kit (ThermoFisher Scientific Inc, Waltham, MA).

EXPERIMENTAL DESIGN

For a summary of each experiment, see Table 3.1.

Experiment 1:

For this experiment, we used 20 animals (10 males and 10 females). A baseline blood sample (1 mL) was collected from all animals on Day 0 (June 19). Then, half of the animals (5 males, 5 females) were randomly selected to be bled according to the biomedical procedure outlined above. The following day (Day 1), blood samples were collected and all animals were fed the GCLB food supplement. Blood samples and feeding occurred every two days until the end of the experiment (July 7, 2017).

Experiment 2:

In this experiment, which was carried out from July 28-August 9, 2017, we used 15 animals (all males). A baseline blood sample (1 mL) was collected from all of them on Day 0 (July 28), and then 10 of them were randomly selected to be bled according to the biomedical procedure. On Day 2, blood samples were taken from all animals, and then five bled animals and five animals that were not bled were fed the GCLB food supplement. The remaining five animals that were bled were not fed anything for the entirety of the experiment. Blood sampling and feeding (for selected animals as described above) occurred every two days until the end of the experiment.

Experiment 3:

For this experiment, which took place from August 23-September 5, 2017, we used 18 animals (all males). A baseline blood sample (1 mL) was collected from each on Day 0 (August 23), and then 12 were randomly selected to be bled according to the biomedical procedure. On Day 2, blood samples were taken from all animals, and then they were divided into three groups: six of the bled animals were fed the GC food supplement, the six controls that were not bled were also fed the same food supplement, and the remaining six animals that were bled were not fed anything for the entirety of the experiment. Blood samples and feeding (for selected animals) occurred every two days until the end of the experiment.

Experiment 4:

In this experiment, which took place from August 17-September 27, 2017, 16 male horseshoe crabs, both bled and unbled, were held in their natural habitat rather than in tanks in the laboratory. A baseline blood sample (1 mL) was collected from all animals on Day 0 (August 23), and then half of them (n=8) were randomly selected to be bled according to the biomedical procedure. Animals were then randomly placed in 1-m diameter mesh enclosures (2 animals per enclosure) or 3-m diameter mesh enclosures (3 animals per enclosure) that were distributed throughout the cove next to JEL. The mesh enclosures did not have bottoms so the animals had access to the sediment. They were pushed into the sediment to ensure that animals were not able to burrow their way out, and to stabilize the enclosures. The enclosures were partially exposed at low tide, but water covered the animals during all tidal cycles. Hemocyanin levels were taken from all animals every week, for four weeks after the start of the experiment.

Experiment 5:

In this experiment, the behavior of 20 animals (all females), along with hemocyanin levels, were monitored from September 18-October 5, 2017. A baseline blood sample (1 mL) was collected from all animals on Day 0 (September 18), and then all the animals were fitted with HOBO acceleration data loggers. The accelerometers were attached to the dorsal carapace of each individual by first creating a harness by supergluing two large cable ties to the center of the prosoma, and duct-taping over the cable ties to affix them to the carapace and then placing the accelerometer face-up in the harness and fastening the cable ties over it. This arrangement made it relatively easy to remove the accelerometers, download them, and then replace them again without disturbing the animals too much.

Animals were placed individually into flow-through estuarine water tanks outside of JEL in 1-m mesh enclosures so they could move around, but not interact with each other. Baseline behavioral data were collected for ~one week, and then all the animals were bled according to the biomedical bleeding procedure and returned to their tanks. Half of the animals (n=10) were randomly designated to be fed mussels, the chosen 'food supplement' in this experiment. For each animal, ~3-5 mussels were crushed up and placed in the respective enclosures of the animals chosen to be fed. Feeding occurred twice a day, every other day, for four days (two days of actual feeding; assuming this would be the maximum amount of time biomedical facilities would keep crabs to feed animals before they are released back into their natural environment). Blood samples were taken from all animals on the following day after feeding. Behavioral data were collected throughout the feeding portion of the experiment and then for another week after they were fed.

Table 3.1. Summary of each experimental set-up in 2017. Exp = Experiment; HCY = hemocyanin measured; ACT= activity measured.

Exp	Dates	Males	Females	Fed	Bled	Bled & Fed	Option to Forage	Location	HCY	ACT	Food
1	6/19-7/7	X	X	9		9		Tank	X		GCLB
2	7/28-8/9	X		5	5	5		Tank	X		GCLB
3	8/23-9/5	X		6	6	6		Tank	X		GC
4	8/17-9/27	X		7			6	Cove	X		Sediment
5	9/18-10/5		X		10	10		Mesh Enclosure	X	X	Mussels

DATA ANALYSES

Three-way ANOVAs were used to test for effects of treatment groups, sex, and time on hemocyanin levels. Two-way ANOVAs were used to test for effects of treatment groups and time on hemocyanin levels and, also, on activity levels. Tukey's HSD *post hoc* analyses (with a level of significant difference set at $p < 0.05$) were used to examine differences between means of treatment groups, hemocyanin levels, time, and activity levels. Data were represented in percent change from the baseline values for hemocyanin and activity levels.

A previously determined threshold value of 0.1 m s^{-2} (Watson *et al.*, 2016) was used to classify an animal as active or inactive based on accelerometer tag output. Data were lumped into 5 min bins, and if an animal exceeded the threshold value during any of the minutes in that 5 min period, the animal was considered to have been active for that 5 min period. These values were entered into the program ActogramJ to create actograms that could be used to determine the types of rhythms expressed (Schmid *et al*., 2011). Periodograms using the Lomb-Scargle method, were used to determine when animals expressed significant circatidal (~12.4 h) or

circadian (~24 h) rhythms (peaks exceeding α=0.001; tidal: 10-14 h range, daily: 22-26 h range; arrhythmic: no significant peaks).

Results

Experiment 1:

There was a significant difference between time, treatment, and sex for the hemocyanin levels of animals in this trial, but there was no significant difference between the interactions of any of these variables (Table 3.2). Tukey's HSD *post hoc* analyses revealed that bled animals had lower hemocyanin levels than those that were fed the GCLB food supplement (Fig. 3.1) and females in both treatments remained significantly lower than males (Fig. 3.2).

Table 3.2. Three-way ANOVA results comparing the hemocyanin levels by sex, treatment, and time of animals in the first feeding experiment. Comparisons included different days, males and females, and bled vs control animals. Bold values indicate significant difference ($p < 0.05$).

Experiment 1—Hemocyanin Levels

Source	df	MS	F	p
Time	9	5791.33	15.91	**<0.001**
Treatment	1	3616.13	9.94	**0.002**
Sex	1	3940.16	10.83	**0.001**
Time*Treatment	9	191.47	0.53	0.854
Time*Sex	9	385.54	1.06	0.397
Treatment*Sex	1	213.97	0.59	0.445
Time*Treatment*Sex	9	623.121	1.71	0.092
Error	140	363.962		
Total	180			

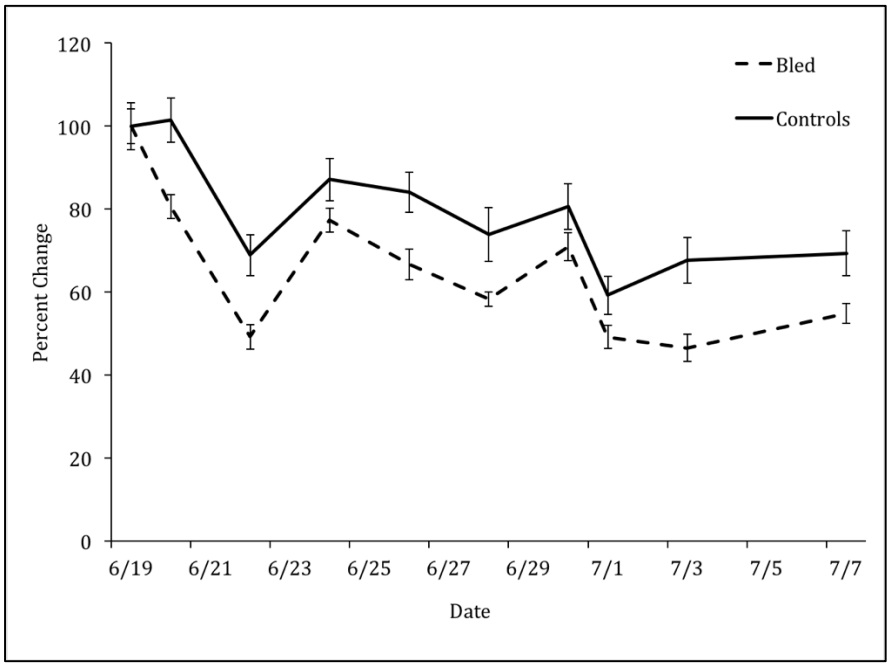

Figure 3.1. Percent change (± SEM) of hemocyanin levels in bled and control animals in the first feeding experiment. Bled and fed animals (n=9) remained significantly lower than control (fed only and not bled) (n=9) animals.

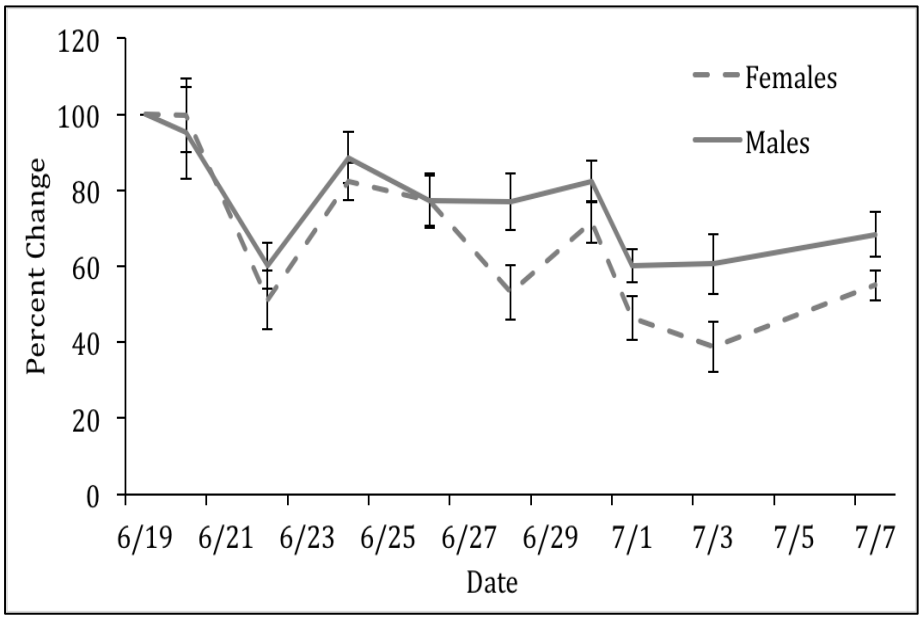

Figure 3.2 Percent change (± SEM) of hemocyanin levels in females and males in the first feeding experiment. Female hemocyanin levels (n=9) were significantly different than males (n=9).

Experiment 2:

There was a significant difference in treatment and time for the hemocyanin levels of animals that were fed the GCLB food supplement (Table 3.3). Tukey's HSD *post hoc* analyses revealed that the bled treatment group was significantly different from the fed, and the bled and fed groups (Fig. 3.3). There was no significant difference between the fed, and the bled and fed, groups.

Table 3.3. Two-way ANOVA results comparing the hemocyanin levels by treatment and time of animals in the second feeding experiment. Bold values indicate significant difference ($p < 0.05$).

Experiment 2—Hemocyanin Levels

Source	df	MS	F	p
Treatment	2	3140.33	9.31	**<0.0001**
Time	6	2246.49	6.66	**<0.0001**
Treatment*Time	12	169.44	0.51	0.908
Error	84	337.42		
Total	105			

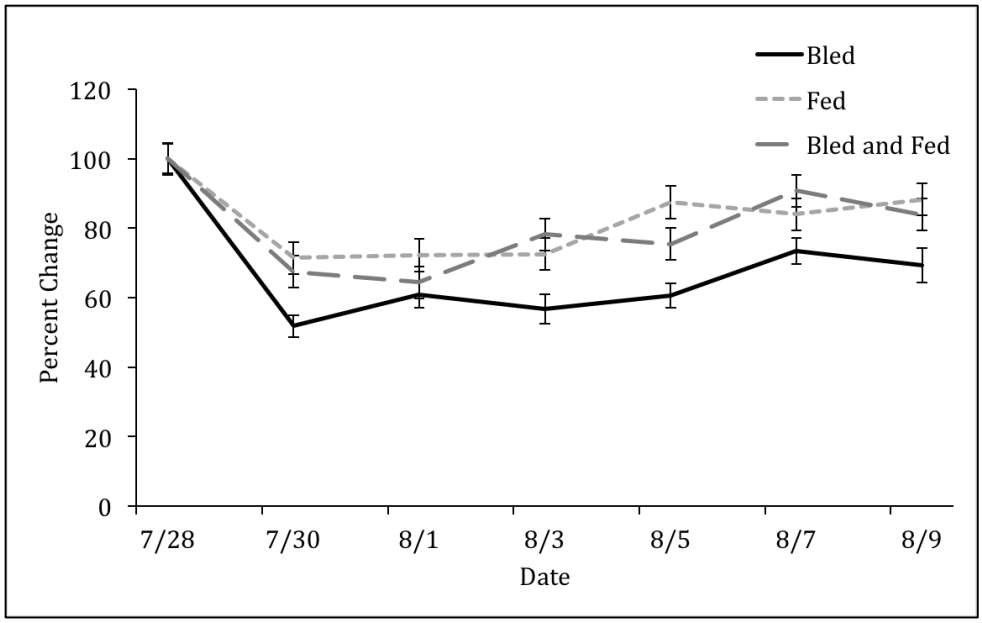

Figure 3.3. Percent change (± SEM) of hemocyanin levels in different treatment groups (n=5 per group) in the second feeding experiment. Animals that were not fed remained significantly lower.

Experiment 3:

There was a significant difference in treatment and time for the hemocyanin levels of animals that were fed the GC food supplement (Table 3.4). Tukey's HSD *post hoc* analysis showed that the fed group was significantly different from the fed and bled, and the bled group (Fig. 3.4). The latter were not significantly different from each other.

Table 3.4. Two-way ANOVA results comparing the hemocyanin levels by treatment and time of animals in the third feeding experiment. Bold values indicate significant difference ($p < 0.05$).

Experiment 3—Hemocyanin Levels

Source	df	MS	F	p
Treatment	2	2893.72	18.691	**<0.0001**
Time	6	3585.84	15.08	**<0.0001**
Treatment*Time	12	163.127	0.85	0.599
Error	105	191.85		
Total	126			

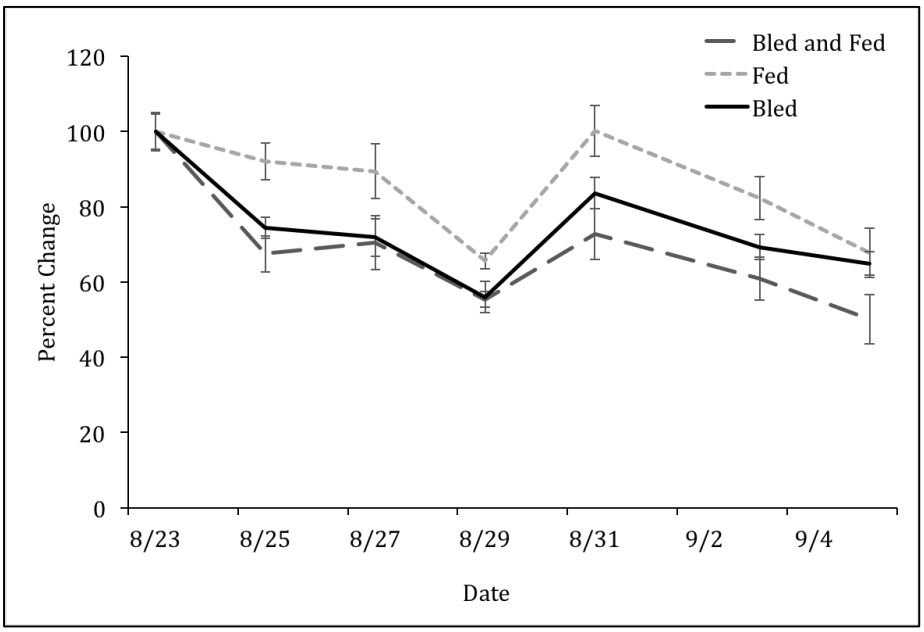

Figure 3.4. Percent change (± SEM) of hemocyanin levels in different treatment groups (n=6 per group) in the third feeding experiment. Animals that were fed (and not bled) had the highest hemocyanin levels over time.

Experiment 4:

There was a significant difference in treatment and time for the hemocyanin levels of animals that were held in their natural environment (Table 3.5). There was also a significant difference in the interaction between treatment and time for the hemocyanin levels. Tukey's HSD *post hoc* analyses revealed that control animals remained significantly higher throughout the duration of the experiment, while bled animals remained ~40% lower than baseline levels throughout the duration of the study (Fig. 3.5).

Table 3.5. Two-way ANOVA results comparing the hemocyanin levels by treatment and time of animals in the fourth feeding experiment. Bold values indicate significant difference ($p < 0.05$).

Experiment 4—Hemocyanin Levels

Source	df	MS	F	p
Treatment	1	5177.91	22.81	**<0.0001**
Time	4	4454.65	19.62	**<0.0001**
Treatment*Time	4	778.71	3.43	**0.014**
Error	55	227.02		
Total	65			

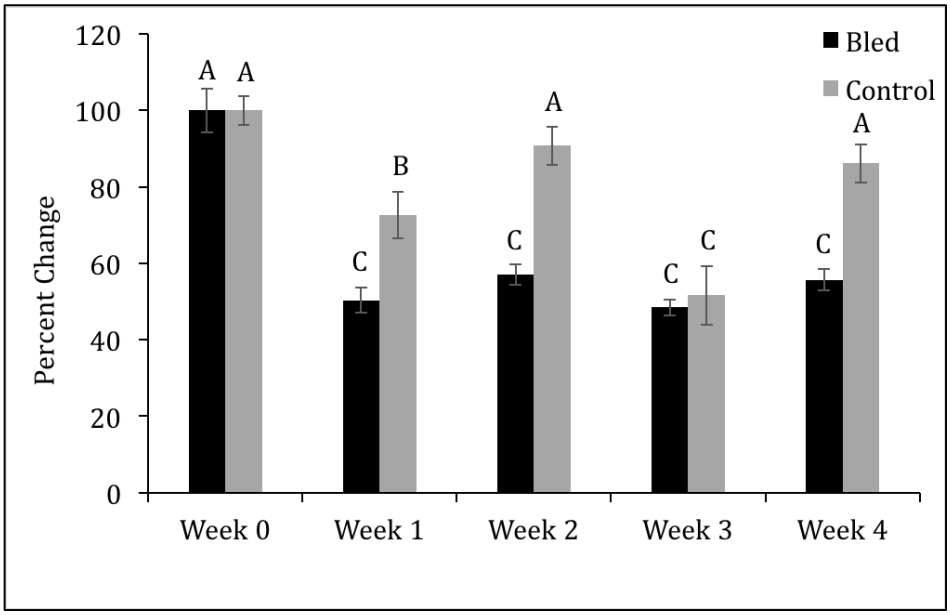

Figure 3.5. Percent change (± SEM) of hemocyanin levels of bled (n=6) and control (n=7) animals over time when they were released back into their natural environment in enclosures. A Tukey's HSD *post hoc* analysis showed significant differences, as represented by the different letters.

Experiment 5:

There was one mortality in this group, an animal that was bled and not fed. The animal died one day after hemolymph extraction.

There was a significant difference in treatment and the interaction between treatment and time for the hemocyanin levels of animals that were fed mussels and held in tanks to monitor their behavior (Table 3.6). Tukey's HSD *post hoc* analyses revealed that the animals that were bled and fed had greater hemocyanin levels eight and ten days post-bleeding than animals that were not fed (Fig. 3.6).

There was also a significant difference in time, treatment, and the interaction between time and treatment in the overall activity of animals in this experiment between baseline and one-week post-treatment (Table 3.7). The animals that were bled had significantly lower activity levels after treatment than animals that were bled and fed (Fig. 3.7).

Table 3.6. Two-way ANOVA results comparing the hemocyanin levels by treatment and time of animals in the fifth feeding experiment. Bold values indicate significant difference ($p < 0.05$).

Experiment 5—Hemocyanin Levels

Source	df	MS	F	p
Treatment	1	1500.93	31.57	**<0.0001**
Time	2	92.03	1.94	0.162
Treatment*Time	2	376.55	7.92	**0.002**
Error	30	47.54		
Total	36			

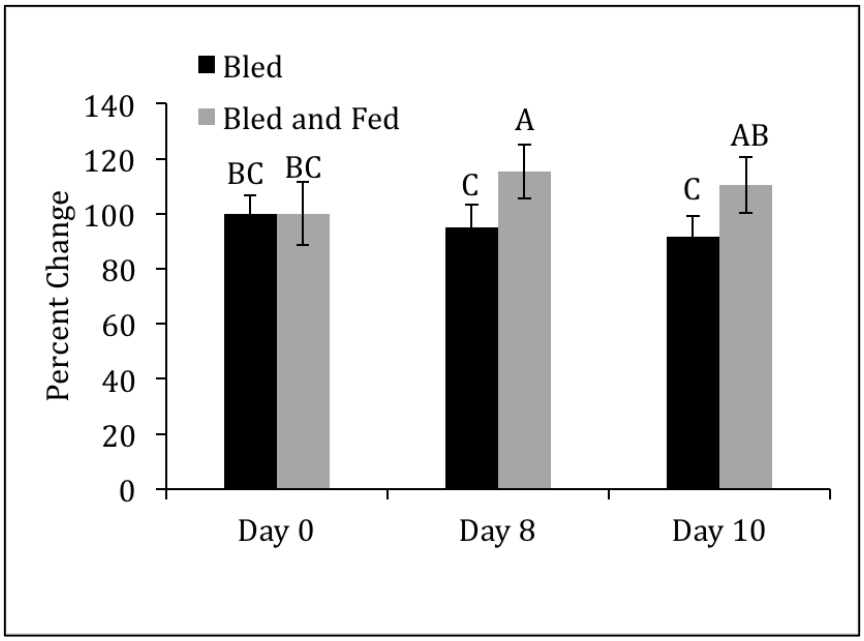

Figure 3.6. Percent change (± SEM) of hemocyanin levels of different treatment groups over time (bled: n=5, bled and fed: n=5). A Tukey's HSD *post hoc* analysis showed significant differences, as represented by the different letters.

Table 3.7. Two-way ANOVA results comparing the behavior in different treatment groups and over time of the animals in the fifth feeding experiment. Bold values indicate significant difference ($p < 0.05$).

Experiment 5—Overall Activity

Source	df	MS	F	p
Treatment	1	3509.11	4.34	**0.049**
Time	1	7259.65	8.98	**0.008**
Treatment*Time	1	3509.11	4.32	**0.049**
Error	18	807.98		
Total	22			

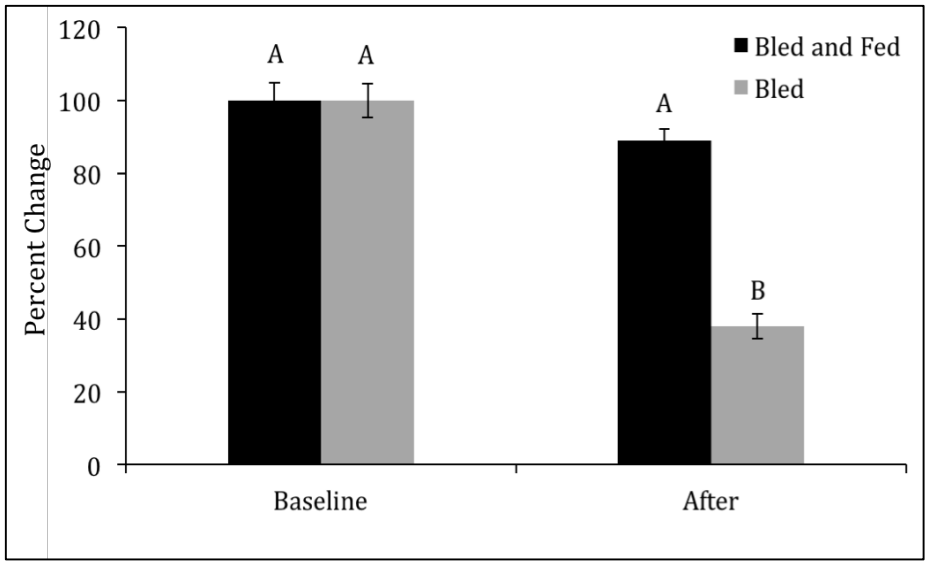

Figure 3.7. Percent change (± SEM) of daily activity of different treatment groups over time (bled: n=5, bled and fed: n=5). A Tukey's HSD *post hoc* analysis showed significant differences, as represented by the different letters. Baseline = average percent of time active before being bled; After = average percent of time active five days after being bled.

Every animal that was fed (5/5) maintained its type of activity after it was bled (Table 3.8). For animals that were not fed, 3/5 of the animals switched rhythms (Table 3.8). In both groups, 3/5 of the animals started with tidal rhythms (Fig. 3.8). Also, every animal that expressed a daily rhythm was more active at night.

Table 3.8. Summary of the rhythms expressed by each animal in each treatment group before and after treatment. Rhythms are only shown for animals that survived throughout the duration of the experiment. Light gray = tidal rhythm, dark gray = diurnal rhythm, striped= nocturnal rhythm, and black = arrhythmic. Baseline = before treatment, After = one week post-treatment.

Treatment	Baseline	After
Bled		
Bled		
Bled		
Bled		
Bled		
Bled & Fed		
Bled & Fed		
Bled & Fed		
Bled & Fed		
Bled & Fed		

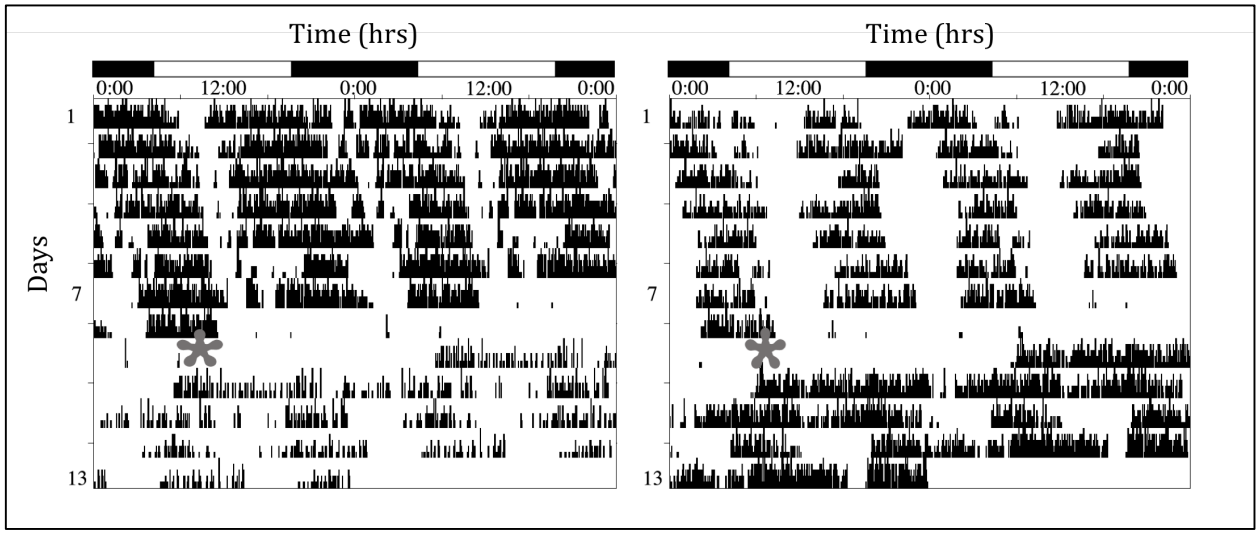

Figure 3.8. Double-plotted actograms showing difference in activity between treatment groups. The animal on the left was only bled. It expressed a tidal rhythm throughout, but was significantly less active after treatment. The animal on the right was bled and fed. It also was expressing a tidal rhythm, but increased its activity after treatment. The x-axis is double-plotted to make rhythms appear more evident, showing a span of 48 h for each line. The gray/black horizontal bars on top represent the Light/Dark periods of the day. The y-axis represents ~two weeks of treatment for each animal. Large gray stars indicate treatment (first star, Day 8).

Discussion

The main objective of this study was to determine if supplemental food could influence the sublethal effects of the biomedical bleeding process, specifically by decreasing the recovery time for hemocyanin levels and decreasing behavioral alterations following bleeding. Overall, we found that: 1) the GCLB food supplement led to the greatest increases in hemocyanin levels in animals that were fed and bled as compared to those that were bled but not fed as compared to the other food types suggesting a possible component of *Limulus* hemolymph that is beneficial (experiment 1 vs. experiment 2&3); 2) female hemocyanin levels remained lower than males over time (experiment 1); 3) holding animals in their natural environment seemed to increase (or maintain) hemocyanin levels better than compared to being held in captivity (experiment 4); 4) animals that were fed after bleeding maintained their overall activity levels and biological

rhythms, better than animals that were bled but not fed (experiment 5). Overall, we saw potential beneficial effects on the hemocyanin levels and behavior in horseshoe crabs that were fed directly after being bled that are worth further consideration.

In determining ways to alleviate lethal and sublethal impacts from the bleeding process, we theorized that providing these animals with a food supplement would benefit them because they would be better equipped to produce new hemocyanin to replace what they lost. In addition, we thought that using an easily accessible by-product, 'spent' *Limulus* hemolymph, that is present at the biomedical facilities, as well as an invasive species that is a nuisance and readily found in the GBE, might be an advantageous way to achieve our research goals and aims. We achieved the most promising results from the supplement made up of green crab extract and *Limulus* blood (GCLB). This suggests that horseshoe crabs may be increasing their hemocyanin levels directly from the hemolymph that is being given to them through this food supplement. Although green crab extract also appears to increase hemocyanin levels, the *Limulus* blood might provide an ingredient specific benefit to help recover depleted hemocyanin reserves as a result of the bleeding process. Given that there is feeding of a conspecific, ingredient biosafety (e.g. pasteurization) would need to be considered for actual implementation to ensure no pathogens were transferred.

We also found that females had lower levels of hemocyanin in all treatment groups when compared to males. This is similar to our previous findings in Chapter Two, where females had lower hemocyanin levels in all months sampled (May-October). We suggest that this could be due to females carrying eggs or developing ovaries, which displaces the space in body cavities that could be filled with hemolymph (Hurton *et* al., 2005). It is unfortunate, given their low

hemocyanin levels and increased sensitivity to the lethal and sublethal impacts of bleeding, that females are selected over males by several biomedical companies.

We performed the experiment where we returned animals to their natural habitat because we hypothesized that this would help them increase their hemocyanin levels. We thought that there is a source of nutrition in the mud that they are not receiving in captivity, since it has been known that horseshoe crabs feed on bivalves, decaying animal matter, and polychaete worms that they obtain through burrowing in marine sediment (Shuster, 1982a; Lee, 2010). Although, we did not see the increased hemocyanin levels in the bled crabs that we expected, some of the data were promising. For example, the control hemocyanin levels started to increase and remained closer to 100%. Also, the hemocyanin levels dropped immediately in the bled animals, but stabilized over time. This is contrary to the results we've seen in the lab where they are not exposed to sediment post-bleeding. As seen in Chapter Two, control animals that are held in captivity have an average 10% decrease in hemocyanin levels over a one-week period and a 27% decrease over a two-week period. This trend holds true for animals that are bled, as well. Thus, in our cove experiment, not only did control animals' hemocyanin levels not decrease, but they tended to increase and approach baseline levels. In the bled animals, although the levels decreased suddenly after being bled, they maintained those lower levels, instead of continuing to decrease which is usually typical for laboratory conditions. As a result, there might be something in the sediment that horseshoe crabs need to recover and maintain healthy hemocyanin levels, highlighting the importance of returning crabs as soon as possible to their natural environment after being bled and looking in the marine sediment for a dietary supplement to provide horseshoe crabs. This experiment should be repeated again for two reasons. First, we lost several crabs during this experiment (either from being too buried or escaping our cages), so our findings

might not be representative of a larger population. Second, the animals might not have been provided with a large enough foraging area so they were unable to find enough of their typical preferred prey.

In our final experiment, where we looked at the behavior of horseshoe crabs as a result of feeding, we also saw some interesting results. Although our sample size was small (5 animals per group) as a result of faulty accelerometers, we saw differences in hemocyanin levels and behavior between the different treatment groups. All the animals that were fed maintained or slightly increased their activity levels, as well as maintaining their rhythms. In contrast, animals that were just bled, (3/5) switched rhythms and significantly decreased their activity. In this experiment, we also fed the animals mussels so we could simply add them to the tanks and not have to handle them. Because the opened mussels remained intact, in contrast to our artificial food supplement, we could examine the tanks and tell if the animals had eaten the provided food.

Lastly, in contrast to the experiments reported in Chapters 1 and 2, we only observed one mortality in all of these feed experiments. This is likely due to the fact that we did not expose them to the full bleeding procedure; we only withdrew blood, and we also provided feed to a subset of animals. This further highlights the need for reducing the additional stressors on horseshoe crabs that they are exposed to during the full bleeding procedure.

Overall, this study showed that providing a dietary supplement to horseshoe crabs post-bleeding can positively impact their hemocyanin levels and behavior. Moving forward, there are several changes that could be done to help improve the results of this study. As previously mentioned, Qin *et al.*, (2016) found that hemocyte counts, antioxidant capacity, and hemocyanin expression were strengthened as dietary nanoSe (elemental particles of Se that are important in immune systems and functions) increased. Thus, hemocyte levels could be measured to also see

if the food supplements were improving the health of these animals or dietary nanoSe could also be included in the food supplement. Also, it might be important to try and make the food supplement hold together more firmly or a pelleted feed, so that it can be dropped into tanks where horseshoe crabs are located. This allows for animals to eat on their own, instead of having humans pick them up out of their tanks (providing them with additional stress) and pushing food into their mouths. Also, it was difficult to determine if the animals were always eating or if they were at satiation with our feeding method, potentially skewing our results. Lastly, it might be useful to use insect larvae in a food supplement. As mentioned above, gut content studies of two Asian species of juvenile horseshoe crabs revealed that they had a stronger preference for insect larvae (specifically, *Chironomous* sp.) as compared to polycheates and bivalves (Zhou and Morton, 2004). Thus, this could be another option for including in a food supplement. Finally, if a suitable food supplement could be developed, it could lead to changes in regulations, such as demanding facilities to provide a food supplement to crabs before they are returned to their natural environment, to help alleviate the impacts from the biomedical bleeding process on these animals.

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

The findings from these studies show that there are sublethal and lethal impacts from the biomedical bleeding process. Overall, we found that when animals were returned to their natural environment, there were some immediate impacts on their tendency to approach mating beaches, but few long-term effects related to their seasonal movements. In the laboratory, we determined that the full bleeding procedure, followed by bleeding along with another stressor (heat or air exposure) was the most detrimental for horseshoe crabs (in regards to hemocyanin levels, locomotor activity, and expression of biological rhythms). This suggests that there is a synergistic relationship between these stressors that is worth further investigation. Given these findings, and the fact that all the biomedical facilities have a slightly different bleeding procedures and handle the animals in different ways, it might be worth revisiting the current best bleeding practices in order to reduce the impacts on horseshoe crab behavior and physiology. Finally, we demonstrated that one possible way to remedy some of the negative impacts associated with biomedical bleeding might be to provide them with a food supplement comprised of invasive green crabs and the *Limulus* hemolymph that is a by-product of the bleeding process. When we did this, they seemed to recover hemocyanin levels faster than those in individuals that were not fed.

There are still several recommendations and alternative procedures that could be suggested to the biomedical facilities to reduce the impacts seen on horseshoe crabs as a result of this process. For example, harvest for the biomedical process could be restricted during the spawning season due to the stress that is already placed on these animals at this time (lower energy reserves, increased air exposure and temperature as they spend time on the spawning beaches, etc.) and the fact that hemocyanin levels are lowest at this time of the year. Horseshoe

crabs should also be inspected for any damage or weakness and baseline hemocyanin levels might be examined. We found that animals in the worst condition before they were bled were also the most impaired after the procedure. Animals should also be kept in temperature-controlled facilities before and after bleeding because in this study heat was shown to exacerbate the negative impacts for bleeding. Biomedical facilities, at least the ones that return crabs to their natural environment and not to the bait industry, should also return the crabs to their original collection site as soon as possible and tag the crabs to make sure to not bleed them within the same season. As stated above, animals with low initial hemocyanin levels were the most likely to die or suffer behavioral impairments following the bleeding procedure.

Determining a way to increase hemocyanin levels and speed up the recovery time in horseshoe crabs after they have been bled might be a positive next step. One potential method might be providing them with a food supplement before, or even after, they are bled. In this study food supplements appeared to help keep hemocyanin levels higher and reduce some of the behavioral impacts of the bleeding process. If the industry could develop a cost and labor effective way to do this it could help to maintain healthy populations of horseshoe crabs in regions where they are harvested.

Finally, I would like to recommend several additional follow-up studies related to this project. First, this study only looked at the impacts two weeks post-treatment in all the laboratory based trials. Therefore, it might be beneficial to look at long-term impacts to see if there are lasting impacts from this process, or if the animals eventually return to their baseline levels after treatments. Second, we only looked at hemocyanin concentrations in our study, but it might be worthwhile to look at both hemocyte and amebocyte counts as a method of addressing the physiological stress and health of this species. Third, reduced hemocyanin levels appeared to be

a common theme in this study and it would be useful to determine why this causes mortality and/or changes in their behavior. Fourth, while our data indicated that there was a reduction in the tendency of bled animals to approach mating beaches, it would be much more convincing if animals could be tracked in a manner that demonstrated this change in a more convincing manner. Finally, this study provided insight into the different effects that three major stressors (air, heat, and blood loss) from the biomedical bleeding process have on the behavior and physiology of horseshoe crabs, and can hopefully be utilized to guide and inform new regulations and better management practices for the biomedical bleeding industry. To this end, it would be useful to repeat aspects of this study while using an improved procedure based on the following recommendations: 1) keep animals in cold, well-oxygenated, water throughout most of the process; 2) do not use animals that are not healthy; 3) do not remove > 30% of their blood; 4) do not capture and bleed animals from March-June when they are recovering from overwintering and spawning; and 5) if possible, feed them before and after they are bled.

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