Potential causes of mortality for horseshoe crabs (*Limulus polyphemus*) during the biomedical bleeding process

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Biomedical companies catch and bleed horseshoe crabs for the production of *Limulus* amebocyte lysate (LAL), a product used for protecting public health (Berkson and Shuster, 1999). LAL is a clotting agent, derived solely from horseshoe crab blood cells, which is used to detect the presence of pathogenic gram-negative bacteria in injectable drugs and implantable medical and dental devices (Mikkelsen, 1988; Novitsky, 1991). In addition, LAL is used in many diagnostic tests for such illnesses as gram-negative bacterial meningitis and typhoid fever (Ding and Ho, 2001). Because the LAL test allows one to detect femtogram levels of endotoxin (Ding and Ho, 2001), it is the most effective test for detecting endotoxin contamination, and its increasing use in medical and pharmaceutical laboratories makes it a highly valued product.

The biomedical industry harvested approximately 260,000 horseshoe crabs in 1997 (HCTC[1]) for the production of LAL. By 2000, the number of horseshoe crabs bled had increased less than 1% (ASMFC[2]). However, approximately 25% of the horseshoe crabs landed for biomedical purposes were rejected for use, and about 45% of those rejected were rejected because they were injured (ASMFC[2]). Some mortality is likely related to harvest injuries, but this rate of mortality is unknown. At current harvest levels, mortalities from biomedical collection and bleeding methods may not be negligible. Mortalities inflicted by the biomedical industry on the horseshoe crab population come in addition to mortalities caused by the commercial fishery, where horseshoe crabs are harvested for use as bait for eel (*Anguilla rostrata*) and whelk (*Busycon* spp.) fisheries (Walls et al., 2002). Furthermore, numerous migratory shorebird species feed on horseshoe crab eggs (Clark, 1996) and adults (Botton and Loveland, 1989, 1993) to fuel their migration from South American wintering grounds to their Arctic breeding grounds (Clark, 1996). Concern has arisen over horseshoe crabs, in part because of local population declines, increased catch and effort, and the need for a superabundance of horseshoe crab eggs for shorebirds, all of which necessitate a conservative, risk-averse management approach (ASMFC[2]). It is important to not only investigate the effects of the commercial fishery harvest and natural predation on horseshoe crab stocks but to comprehensively assess the impact of the biomedical industry take on horseshoe crab population viability. Reducing mortalities from the biomedical industry may aid in conserving the horseshoe crab population, as well as in reducing the magnitude of conflict between this industry, the commercial fishery, and environmentalists. To achieve this goal, further investigation into the biomedical bleeding process and its effects is required.

Throughout the typical biomedical bleeding process, horseshoe crabs are subjected to a variety of potential stressors (i.e., air exposure, increased temperature, handling, blood loss, trauma, etc.). Each LAL producer has its own bleeding process involving different methods of capture, distance and method of travel to the bleeding facility, different holding times and conditions, different bleeding methods, and methods of returning bled crabs that are most appropriate to that company's location and facility set-up (Walls and Berkson, 2003). An example of one version of the biomedical bleeding process begins with the collection of the horseshoe crabs by trawling, dredge, or hand-harvest methods (HCTC[1]). Animals may be held on the deck of a boat or in containers for several hours during collection, transported to the bleeding facility in trucks (that may or may not be air-conditioned), held in the

[1] HCTC (Horseshoe Crab Technical Committee). 1998. Status of the horseshoe crab (*Limulus polyphemus*) population off the Atlantic Coast, 9 p + figures and tables. Horseshoe Crab Technical Committee, Atlantic States Marine Fisheries Commission, 1444 Eye Street, NW, Sixth Floor, Washington, DC 20005.

[2] ASMFC (Atlantic States Marine Fisheries Commission). 2002. Review of the fishery management plan for horseshoe crab (*Limulus polyphemus*). Horseshoe Crab Plan Review Team, Atlantic States Marine Fisheries Commission, 1444 Eye Street, NW, Sixth Floor, Washington, DC 20005.

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cold room of a laboratory for several hours at an air temperature of 16–18ºC, bled for a period of time, and then held in the coldroom or in a truck until transport back to the dock (Grogan[3]; Walls[4]). Horseshoe crabs that are returned to the ocean are transferred onto a boat and returned to their approximate point of capture within 72 hours of their collection (HCTC[1]). The numerous stressors to which the horseshoe crabs are exposed throughout the biomedical bleeding process likely have an effect on their mortality rates, both for horseshoe crabs used for bleeding and for those crabs collected, but rejected for use.

Additional sources of stress also may arise from the bleeding methods used by some biomedical companies. The bleeding protocols at some facilities involve gravitationally withdrawing blood from the heart (punctured with a large gauge needle) until blood flow slows to an intermittent drip (Grogan[3]; Walls[4]). One study reported a range of extracted blood to be from 8.4 mL to 218.7 mL in male horseshoe crabs (Walls, 2001) and up to 267.8 mL in females (Walls[5]). Unfortunately, biomedical companies do not know how much blood horseshoe crabs of a given size possess and how much can be safely extracted. Thus, extracting the reported upper ranges of blood volume from males and females may cause stress or mortality.

Several studies have estimated mortality associated with the biomedical bleeding process to be between 8% and 20% (Rudloe, 1983; Thompson, 1998; Kurz and James-Pirri, 2002; Walls and Berkson, 2003). These studies either simulated the biomedical bleeding process (Rudloe, 1983; Kurz and James-Pirri, 2002) or monitored mortality of a given number of horseshoe crabs bled by a biomedical company (Thompson, 1998; Walls and Berkson, 2003). Although the results provide information on mortality rates associated with a biomedical companies' procedures, they provide no guidance on how biomedical companies may reduce mortality rates.

Rather than quantifying mortality rates associated with the biomedical bleeding process as a whole, we isolated and tested certain elements of the bleeding process to gain information on the potential causes of mortality for horseshoe crabs used in the biomedical industry. The objectives of this study were 1) to quantify mortality associated with blood extraction at set levels and 2) to quantify mortality associated with stress from simulated transport and holding procedures, combined with the stress of blood extraction at the same levels as in the first objective.

[3] Grogan, W. 2003. Personal commun. Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State Univ., Blacksburg, VA 24061.

[4] Walls, B. 2001. Personal commun. Center for Environmental Studies, Virginia Commonwealth Univ., 1000 West Cary Street, Box 843050, Richmond, VA 23284.

[5] Walls, B. 2001. Unpubl. data. Center for Environmental Studies, Virginia Commonwealth Univ., 1000 West Cary Street, Box 843050, Richmond, VA 23284.

Methods

Adult horseshoe crabs were obtained from Cambrex BioScience Walkersville, Inc., a commercial biomedical company on 9 July and 28 August 2003. Horseshoe crabs were captured by using a standard trawling procedure off the coast of Ocean City, Maryland (Hata and Berkson, 2003). After capture, horseshoe crabs were transported in an air-conditioned van to the Horseshoe Crab Research Center (HCRC) at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. The animals were maintained in a recirculating aquaculture system in appropriate environmental conditions (Brown and Clapper, 1981) and monitored daily, but not fed. The animals were acclimated to the aquaculture conditions for one week, during which time they were tagged, sexed, and measured. Tagging involved drilling two 3/32″ holes into the prosoma at its thinnest point and attaching a laminated oval fish tag (Floy Tag, Seattle, WA) with two 3/32″ wide cable ties. Measurement consisted of recording the inter-ocular width (IO), the distance between the inside margins of the horseshoe crab's compound eyes, and the prosomal width (P), which is the distance across the horseshoe crab's carapace. Horseshoe crabs used for both bleeding mortality studies had a mean IO width of 14.0 cm and ranged from 10.5 to 19.0 cm; as well as, an average P width of 23.5 cm that ranged from 17.5 to 35.0 cm. Only animals that were uninjured (i.e., no cracked carapaces or missing legs, etc.) and free of epibionts were used in the two mortality experiments.

Experiment 1

Horseshoe crabs from the July collection were used in experiment 1. From this collection, 100 males and 100 females were selected to test only the effects of various levels of blood extraction on mortality. This sample of horseshoe crabs was termed the "lower-stressed" group because they were not exposed to external stressors associated with simulated holding and transport. The selected horseshoe crabs ranged in size to provide a representative sample of animals bled by some biomedical companies (Grogan[3]; Walls[4]). Horseshoe crabs were arbitrarily assigned to one of five bleeding treatments: 1) unbled crabs (control), 2) crabs bled 10% of their predicted total blood volume, 3) crabs bled 20% of total blood volume, 4) crabs bled 30% of total blood volume, and 5) crabs bled 40% of their predicted total blood volume. Each bleeding subgroup comprised equal numbers of males and females. Predicted blood volume was calculated by using the relationship between blood volume and IO width (Hurton et al., 2005):

$$H = 25.7e^{\,0.1928\,(IO)},$$

where H = hemolymph volume in mL; and
IO = interocular width in cm.

The bleeding process of experiment 1 involved removing horseshoe crabs from their holding tank, positioning

them in a bleeding rack, and disinfecting the surface of their arthrodial membrane with a 70% ethanol-soaked cotton swab. An 18-gauge needle was inserted through the arthrodial membrane into the cardiac sinus to extract the predetermined amount of blood. The animals were then placed in a holding container without water for 15–20 hours. Time out of water approximated the duration that horseshoe crabs may be out of water after bleeding at some biomedical bleeding facilities (Grogan[3]; Walls[4]). Bled horseshoe crabs were also not immediately put back in water to prevent them from absorbing water and regaining their blood volume. Temperatures to which horseshoe crabs were exposed were recorded using a temperature logger. Air temperature during this time was stable at 21°C. Subsequently, the animals were returned to their holding tanks and mortality was monitored for two weeks.

Experiment 2

Horseshoe crabs from the August collection were used for experiment 2. Because of difficulty in obtaining the desired 200 study animals, slightly fewer (195) horseshoe crabs were selected for this experiment. This sample of 110 males and 85 females was used to test the effects of various levels of blood extraction on mortality in the presence of external stressors. These animals represented the "higher-stressed" group because they were exposed to additional stressors to which the first group was not. The external stressors (i.e., air exposure, increased temperature, etc.) were those derived from simulated transport and holding procedures during a simulated biomedical bleeding process. Because there is great variability in transport and holding procedures and conditions, we simulated a protocol that would demonstrate horseshoe crab response under relatively poor conditions.

During the week of acclimation to the aquaculture system, the horseshoe crabs were held in the same conditions and handled in the same manner as group 1. Horseshoe crabs were assigned to one of five bleeding treatments used in experiment 1. The bleeding process of experiment 2 involved removing horseshoe crabs from their holding tank and placing them in holding containers located outside where they were exposed to air, sun, and increased temperatures. During this 6-hour period, the air temperature rose from 21°C to 29°C to simulate time on the deck of a trawler. Next, the animals were moved into a small moving truck, which was not air-conditioned. This phase simulated transportation to the bleeding facility and holding time until placement into the coldroom of the laboratory. During this 4-hour period, the outdoor temperature increased to 31°C and the temperature inside the closed truck peaked at 36°C. The animals were then transferred to the HCRC's tank room where they remained for 16 hours at 21°C. This phase mimicked the holding time in a coldroom prior to bleeding. The animals were bled according to their assigned treatment and in the same manner as in experiment 1. During this 8-hour period, the room temperature was 22°C. Once the bleeding was completed, the horseshoe crabs were moved back into the truck for 13 hours to simulate holding time and transport to the boat. The overnight temperature inside the truck dropped from 24°C to 20°C. In the morning, the horseshoe crabs were returned to the water in their recirculating aquaculture tanks and mortality was monitored for two weeks.

Statistical analyses

Fisher's exact test was used to evaluate 1) differences in mortality of unbled horseshoe crabs between the higher-stressed and lower-stressed groups, and 2) differences in mortality of bled crabs between the lower-stressed and higher-stressed groups. Logistic regression was used to examine the association between mortality and bleeding in the higher-stressed group. Data were also assessed to see if an increase in the volume bled was correlated with an increase in mortality by pooling the frequency of mortalities between males and females in each bleeding treatment and testing these values against bleeding percentage by employing a regression with a fitted quadratic curve.

Results

Experiment 1

No horseshoe crab mortality occurred in any of the five treatments in the lower-stressed group, indicating that post-bleeding mortality in *Limulus* did not arise solely from the effects of blood loss. Hence, our initial hypothesis that substantial blood loss was the principle cause of mortality in horseshoe crabs was rejected.

Experiment 2

There were a total of 14 mortalities distributed throughout the bleeding treatments among horseshoe crabs under higher stress conditions (Table 1). All mortalities occurred within the first seven days of the study. Bled horseshoe crabs had an overall mortality rate of 8.3% compared to the 2.6% mortality rate of unbled crabs, suggesting a relationship between mortality and bleeding under higher-stressed conditions (n=195; P=0.0088) (Table 1). The bleeding variable was significant in the logistic regression model (P=0.0160); yet, sex was not a significant variable (n=156; P=0.6100); seven deaths in total occurred in each sex category (Table 1). Mortality rates reached 13.6% for males bled 30% of blood volume and female mortality was as high as 29.4% in the subgroup bled 40% of blood volume (Table 1). With male and female mortalities pooled, the frequency of mortality increased as the percentage of blood taken from the crabs was increased (n=5; P=0.006; r^2=0.994) (Table 1).

Comparison of the two experiments

With no mortalities in the unbled treatment of the lower-stressed group and one mortality in the unbled treat-

Table 1

Comparison of mortalities observed in the higher-stressed group between unbled horseshoe crabs and horseshoe crabs bled at different levels of total blood volume (n=195).

	Mortality (no.)			Mortality (%)		
% bled	Male	Female	Total	Male	Female	Total
0%	1	0	1	4.5	0	2.6
10%	0	1	1	0	5.9	2.6
20%	2	0	2	9.1	0	5.1
30%	3	1	4	13.6	5.9	10.3
40%	1	5	6	4.5	29.4	15.4
Total	7	7	14	6.4	8.2	7.2

ment of the higher-stressed group, mortality rates were not significantly different between unbled crabs in the two bleeding experiments (n=79; P=0.4937). Of the bled crabs, there was a mortality rate of 0% in the lower-stressed group and a rate of 8.3% in the higher-stressed group, indicating a significant difference in mortality rates between the two groups (n=316; P<0.0001).

Discussion

According to our study of 395 horseshoe crabs, no impact on mortality was observed with blood extraction up to 40% of the horseshoe crab's blood volume under conditions of lower stressors. Any mortality associated with this maximum level of bleeding (40% of total blood volume) was so low that it was not captured by our sample size of 40 individuals per bleeding treatment. This result also may indicate that despite hypovolemia, or low blood volume, and altered blood chemistry (Hurton, 2003), horseshoe crabs may be relatively tolerant of the removal of a large amount of blood in the absence of other stressors.

Horseshoe crab mortality, however, was significant in the presence of higher levels of stress, including external stressors (i.e., lengthy air exposure, elevated temperatures, etc.) as applied in our study. Novitsky's (1991) aquarium studies reported that up to 30% of blood volume can be safely extracted, indicating that the animals in that study may have been similar to our lower-stress group; however, details regarding harvest method, transport, and handling procedures were not specified (Novitsky, 1991). It is likely that, under different conditions, horseshoe crab response to blood extraction may be altered. Findings from our study indicated that a combination of external effects and sublethal hemorrhaging may result in significantly increased mortalities, possibly from a synergistic interaction between the two types of stressors. Studies in other species exposed to various stressors have also indicated that mortality may be affected by a synergistic combination of effects from multiple stressors (Schisler et al., 2000; Schulz and Dabrowski, 2001; Hatch and Blaustein, 2003). Such results emphasize the importance of considering the cumulative impact of multiple stressors.

Although the data in our study indicated a significantly increased mortality rate in the higher-stressed group as opposed to the lower-stressed group, it is important to note the variability in mortality throughout the treatments. It was observed that the male and female horseshoe crabs had different and variable responses in the different bleeding treatments. For example, males had two and three mortalities in the 20% and 30% bleeding treatments, respectively; yet, only one mortality in the 40% treatment (Table 1). Females appeared to have a spike in mortality in the 40% treatment (Table 1). When viewing the data this way, the variability in mortality could be in part due to the health and precollection stress level of a horseshoe crab, which can differ from its counterparts. Different conditions may lead to different responses and not every individual will react exactly alike. More than likely, much of this variability is an artifact of a small sample size. Once the male and female data were pooled, total mortality appeared to proceed in an increasing trend as bleeding amount increased (Table 1). If sample size were increased enough, perhaps a strong trend would emerge in mortality between males and females, or perhaps not. Further investigation with larger sample sizes and a different experimental design could shed light on a possible trend in male and female mortality rates and whether one sex may be more susceptible to mortality than the other.

The combination of multiple stressors could also affect not only mortality, but possibly the amount of blood able to be extracted by the biomedical industry. It is possible that horseshoe crabs exposed to higher levels of external stress, such as greater lengths of time to air and higher temperatures, would lose moisture from their exposed gills and possibly become dehydrated and thus have a decreased blood volume such that a smaller amount of blood is available for extraction. It is unknown how this would affect the harvest of horseshoe crab blood for the production of LAL. Dehydration rates in horseshoe crabs are unknown, but have been examined in a freshwater crayfish (*Austropotamobius pallipes*) which is a facultative air breather that can survive about three days out of water (Taylor et al., 1987). Taylor et al. reported that when exposed to air (70–80% relative humidity) for 27 hours, crayfish became dehydrated and had a 25% decreased blood volume. When exposed to water-saturated air (100% relative humidity), crayfish did not have a decrease in blood volume. Similarly, horseshoe crabs exposed to water-saturated air may be less likely to become dehydrated, thereby possibly decreasing the effects of one type of stress.

The results presented in the present study provide insight into the possible combined effects of blood extraction and external stressors associated with biomedical

transport and holding methods on horseshoe crab mortality. Of the various protocols followed in the bleeding process, we simulated an example of transport, holding, and bleeding methods that provided poor conditions for the horseshoe crabs and that resulted in significant mortality rates. In the typical biomedical bleeding process, horseshoe crabs undergo time on a boat deck or in collection bins, transport, storage in a coldroom, bleeding, holding time, and transport back to the ocean. At each of these stages of processing, the animals are exposed to a number of stressors of varying magnitudes, including exposure to air for extended time periods, elevated temperatures, dehydration, hypovolemia, and likely other unknown stressors. However, these factors can be controlled to a certain extent by providing conditions for the horseshoe crabs that could alleviate much of the stress. For example, holding horseshoe crabs in an air-conditioned environment (i.e., during time in the truck and in the cold room), covering the animals with wet burlap while outside to keep them moist and shaded from the sun, and increasing the relative humidity of the air in the holding room could help to decrease the physiological stress the animals experience throughout the bleeding process. Because blood volume estimates have recently been determined (Hurton et al., 2005), the blood volume extracted by biomedical companies potentially could be optimized according to average stress conditions and attempts could be made to decrease stress as much as feasibly possible, including keeping horseshoe crabs hydrated and bleeding a prescribed amount from individuals, as opposed to withdrawing a wide range of blood volumes. Another important consideration is stress arising from harvest methods. Our study did not test the effects of this factor, but it may have an effect on the condition of horseshoe crabs collected for bleeding. Biomedical companies should include the potential effects of harvest methods when determining what may be typical stressful conditions for the horseshoe crabs, or they could consider using a harvesting method that would be the least stressful on the animals. All LAL producers can potentially implement some form of these recommendations, thereby altering bleeding process protocols to decrease the stress and mortality levels that these horseshoe crabs experience. Implementation of these recommendations would decrease the impact of the biomedical industry on the potentially declining horseshoe crab population and aid in conservation of this species.

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