REDUCING POST-BLEEDING MORTALITY OF HORSESHOE CRABS (*Limulus polyphemus*) USED IN THE BIOMEDICAL INDUSTRY

By

Lenka Hurton

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
IN
FISHERIES AND WILDLIFE SCIENCES

Approved:

Dr. Jim Berkson
Advisory Chair

Dr. Eric Hallerman
Committee Member

Dr. Stephen Smith
Committee Member

December 2003
Blacksburg, Virginia

Keywords: mortality, blood volume, hemolymph, amebocyte, *Limulus polyphemus*, horseshoe crab

Copyright 2003, Lenka Hurton

REDUCING POST-BLEEDING MORTALITY OF HORSESHOE CRABS (*Limulus polyphemus*) USED IN THE BIOMEDICAL INDUSTRY

by:
Lenka Hurton

Jim Berkson, Chair
Fisheries and Wildlife Sciences

(ABSTRACT)

The biomedical industry bleeds horseshoe crabs (*Limulus polyphemus*) to produce a product from the blood cells that is used to test the sterility of medical and pharmaceutical products. This study examined the effects of blood extraction on the survival of horseshoe crabs and performed a preliminary investigation into amebocyte maintenance *in vitro*. Hemolymph volume of *L. polyphemus* was estimated over a representative size range of adults. Hemolymph volume expressed as a percentage of wet body weight was 25 ± 2.2% (mean ± S.D.) for males and 25 ± 5.1% for females. Relationships of hemolymph volume to weight ($P = 0.0026$, $r^2 = 0.8762$), hemolymph volume to prosomal width ($P < 0.0001$), and hemolymph volume to inter-ocular width ($P < 0.0001$) were identified.

Mortality associated with blood extraction was evaluated for horseshoe crabs bled 0, 10, 20, 30, and 40% of their estimated hemolymph volume (unstressed group, $N = 200$). Mortality associated with the same bleeding levels was evaluated in horseshoe crabs that underwent simulated transport and handling procedures of the biomedical industry's bleeding process (stressed group, $N = 195$). Mortality rates of the unbled crabs were not significantly different between the stressed group and unstressed group. Of the bled animals, there was a higher (8.3%) mortality rate in the stressed group, than that (0%) in the unstressed group ($P < 0.0001$). Within the stressed group, mortality was significantly associated with bleeding ($P = 0.0088$), and overall

mortality increased with increasing levels of blood extraction ($P = 0.006$, $r^2 = 0.994$). The recovery of hemolymph volume over a 12-day period was monitored ($N = 45$). Total hemolymph protein levels in the 10, 20, 30, and 40%-bled groups all demonstrated decreased levels between days 0 and 3 ($P < 0.0001$) post-bleeding.

Horseshoe crab serum and a variety of standard invertebrate and insect cell culture media were evaluated for their effects on amebocyte morphology and viability after 7 days of maintenance *in vitro*. Horseshoe crab serum-supplemented cultures had significantly higher cell viability than serum-free cultures ($N = 6$; $P = 0.0147$). Significant differences in amebocyte viability were identified among the six insect cell culture media tested ($N = 36$; $P < 0.0001$). Amebocytes in Grace's Insect Medium without serum remained 77.2 ± 5.1% (mean ± S.D.) viable, followed distantly by Grace's Modified Insect Medium without serum with a viability of 35.1 ± 8.7%. Taking amebocyte morphology and viability into consideration, this study indicated that Grace's Insect Medium with horseshoe crab serum supplementation was the best candidate for future medium optimization for *Limulus* amebocyte requirements. Information gained from this study provides guidance on altering biomedical bleeding protocols to decrease horseshoe crab stress and mortality, and advances information on amebocyte culture medium selection, both of which contribute to decreasing the biomedical industry's impact on the horseshoe crab population.

ACKNOWLEDGEMENTS

I thank my advisor, Dr. Jim Berkson, for providing me with the opportunity to work on this project. His unwavering support, constructive advice, and dedicated effort over the course of this project are greatly appreciated. My committee members, Dr. Stephen Smith and Dr. Eric Hallerman, provided valuable guidance and input throughout this project, and their efforts are greatly appreciated as well.

This work is a result of research sponsored in part by the NOAA Office of Sea Grant, US Dept of Commerce, under Grant No. NA96RG0025 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. Cambrex Bio Science Walkersville, Inc. generously supplied my project with horseshoe crabs and cell culture media. I would like to thank Dr. Ron Berzofsky and all of Cambrex Bio Science Walkersville, Inc. (Cambrex) for their support throughout this project. I would also like to thank Jeffrey Eustler and his boat crew for his patience and support when he supplied us with horseshoe crabs on behalf of Cambrex.

Finally, I would like to thank my parents, friends, and fellow graduate students for their encouragement and invaluable assistance with my research. I would especially like to thank Vincent Caruso, Dr. Dave Hata, René Olsen, Michelle Davis, Whitney Grogan, Jay McGhee, Mary Tilton, Alison Williams, and Alison Sasnett for aiding in transporting, tagging, and/or bleeding horseshoe crabs for this study. In particular, Whitney Grogan provided much appreciated advice during the writing process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 – INTRODUCTION AND JUSTIFICATION

INTRODUCTION

The horseshoe crab, *Limulus polyphemus*, is a unique animal that has persisted for more than 200 million years (Botton and Ropes 1987). It has been ecologically important throughout its time, but is now also economically and medically significant. The horseshoe crab is a multiple use-marine resource that has become the center of controversy among its user groups (Berkson and Shuster 1999). Conflicts between stakeholders have arisen over resource allocation and have intensified due to concerns that horseshoe crab populations are declining in abundance. Information about horseshoe crab population status and dynamics is growing. However, more statistically valid population data is needed to make appropriate management decisions (ASMFC 2002). It is also necessary to continue exploring the effects of various users on horseshoe crab populations and to examine options for reducing the pressures to which this resource is currently subjected. Without a comprehensive assessment of this issue, management will continue to be difficult and confounded due to the conflicting needs of its various user groups.

Commercial Fishery

Three major stakeholders are heavily dependent on the horseshoe crab resource. The first of these groups is commercial fishermen who harvest horseshoe crabs for use as bait in the American eel (*Anguilla rostrada*) and whelk (*Busycon* spp.) fisheries (HCTC 1998). Horseshoe crab harvest increased dramatically in the 1990s. In 1996, 5,153,630 pounds of horseshoe crabs were landed compared to 908,130 pounds in 1990 (ASMFC 1999). Increases in horseshoe crab harvest are due to stricter regulations on other traditionally harvested groundfish, increasing demand for whelk and American eel, and lack of regulations in the whelk and eel fisheries

(Berkson and Shuster 1999). These commercial fisheries are economically important to coastal communities, where the total value of the eel and whelk pot fisheries is estimated at approximately $21 million per year (Manion et al. 2000).

Historically, fishing restrictions for horseshoe crabs have been minimal or nonexistent (ASMFC 1998). The Atlantic States Marine Fisheries Commission (ASMFC) initiated a fishery management plan (FMP) for the horseshoe crab in 1997 in response to mounting pressures from conservationists regarding the perceived decline in horseshoe crab populations and the continued increase in landings (Berkson and Shuster 1999). Reported coastwide horseshoe crab landings have declined each year since implementation of the FMP, falling from 3 million horseshoe crabs in 1997 to approximately 1 million in 2001 (ASMFC 2002). It is difficult to predict whether the commercial fishery's impact will be sufficiently reduced to release the population from demographic stress. Complicating the matter, the horseshoe crab has a reported maturation time of 10 years and lifespan of 20 years (Shuster 1982), creating a time lag for population recovery (Berkson and Shuster 1999).

Progress is being made in conserving the crab resource, not only in implementing restrictive regulations, but also in decreasing the fishery's dependency on horseshoe crabs. Efforts have been made to decrease the number of horseshoe crabs used for bait with the use of bait bags (Walls et al. 2002). Also, researchers have isolated the chemical cue in horseshoe crab eggs that serves as an attractant to eel and whelk (Ferrari and Targett 2003). However, further investigation is needed to formulate a cost-effective artificial bait.

Shorebirds and Conservationists

The second stakeholder group consists of migratory shorebirds, bird-watchers, and environmentalists. The arrival of migrating shorebirds in Delaware Bay coincides with the peak

of horseshoe crab spawning (Botton et al. 1994, Clark 1996). Each year, 425,000 to 1,000,000 shorebirds stop in this area during May and June (Myers 1981, 1986, Clark et al. 1993). At least 11 species of shorebirds, including red knots (*Calidris canutus*), ruddy turnstones (*Arenaria interpres*), and sanderlings (*Calidris alba*), are known to feed on horseshoe crab eggs (Shuster 1982) which comprise a significant portion of their diet during particular time periods (Tsipoura and Burger 1999). Several of the 11 species are listed as threatened under the Endangered Species Act. Most of these shorebirds migrate long distances (Myers et al. 1987) from their South American wintering grounds before stopping to replenish their fat deposits, fueling their continued journey to their Arctic breeding grounds (Myers 1986, Myers et al. 1987). Staging areas provide abundant food, which facilitates rapid weight gain in shorebirds for their continued migration (Myers et al. 1987). During their short two-to-three-week stopover, the birds can undergo weight gains of 40% (Castro et al. 1989). Their life history strategy depends in great part on the presence of horseshoe crabs at this staging area, which serves as a midway resting ground and refueling station for the birds. The birds must reach the Arctic in time to have their eggs hatch for the annual insect emergence (Clark 1996). It appears that life history synchronies of horseshoe crabs and birds play an important role in the shorebird species' survival and migration.

Female horseshoe crabs deposit most of their 88,000 eggs in clusters about 10 to 20 cm below the sand's surface (Shuster and Botton 1985). In these subsurface nests, the eggs are safe and beyond the reach of most shorebirds (Clark 1996). Wave action and burrowing by spawning horseshoe crabs move some eggs to the surface, exposing them to shorebird predation (Botton et al. 1994). With only a portion of eggs made available to the birds, a high density of spawning

horseshoe crabs is needed to ensure availability of eggs for the shorebirds (Berkson and Shuster 1999).

Environmentalists' concerns have prompted changes in state and interstate policy, and stimulated interest in gathering information on the horseshoe crab (Berkson and Shuster 1999). Interest and awareness of the horseshoe crab–shorebird connection is present among other groups. The migration phenomenon in Delaware Bay, and other areas, has become part of a large ecotourism industry. Bird-watching enthusiasts are attracted to these areas, generating between $7 million and $10 million of annual spending in the Cape May region alone (Manion et al. 2000). The relationship between high crab populations and shorebird numbers has been correlated (Botton et al. 1994, Clark 1996), and trepidation over major declines in shorebird populations are driving the issue of regulating the collection of horseshoe crabs.

Biomedical Industry

The third stakeholder for the horseshoe crab resource is the biomedical industry, which has developed a product that capitalizes on the horseshoe crab's immune response. Horseshoe crabs live in an environment that contains large amounts of Gram-negative bacteria. There are over one million Gram-negative bacteria per milliliter of seawater and close to one billion bacteria can be found per gram of sand (Novitsky 1991). Horseshoe crabs have evolved an extremely sensitive non-specific immune system to protect themselves from the harmful and ubiquitous bacterial endotoxins present in their environment. Endotoxin is present in the cell walls of Gram-negative bacteria and is released into the environment when the bacterial cell dies or disintegrates (Martin and Hine 2000). In the body, this toxin acts as an antigen, inducing an immune response (Martin and Hine 2000). Horseshoe crabs lack an adaptive immune system and rely completely on innate immunity, responding to common antigens on the surface of

potential pathogens (Iwanaga 2002). The predominant blood cell type, the amebocyte, circulates in the horseshoe crab's copper-based hemolymph (Shuster 1978) and comprises the majority of the immune system. The horseshoe crab's immune reaction includes hemolymph coagulation, cell agglutination, antimicrobial action, and phagocytic action (Iwanaga 2002). In the presence of endotoxin, the active component lipopolysaccharide (LPS) triggers amebocytes to exocytose granules containing a coagulogen/protease system (Armstrong 1985). Polymerization of the coagulogen results in the formation of a clot (Armstrong 1985). It is this immune response of the horseshoe crab amebocytes that led to the first formulation of *Limulus* Amebocyte Lysate (LAL), and the coagulation cascade is the basis of the LAL test produced by the biomedical industry.

LAL can be used to detect minute amounts of bacterial toxins (Novitsky 1991), and is sufficiently sensitive to detect 10^{-15} of a gram of endotoxin in less than one hour (Mikkelsen 1988).

Endotoxins are ubiquitous pathogenic molecules that are a bane to the pharmaceutical industry and medical community (Ding and Ho 2001). All injectable drugs and implantable medical devices must be endotoxin free. If contaminated products are inadvertently administered or used, a pyrogenic response ensues (Novitsky 1991). Pyrogens are substances that cause fever after injection (Ding and Ho 2001), but in larger amounts can induce endotoxic shock and even death (Miller and Hjelle 1990). Of the species examined to date, humans are the most sensitive to endotoxins, where concentrations as low as 1.0 ng/ml may be lethal (Miller and Hjelle 1990). In addition, endotoxin is exceptionally resistant to destruction or removal (Novitsky 1991). Recognizing this, the U.S. Pharmacopoeia (USP) requires that medications used intravenously be not only sterile but also nonpyrogenic (Novitsky 1991). With LAL's integration into the pharmaceutical industry, many widespread applications of LAL for the

detection of endotoxin were developed. Clinical applications include diagnostic tests for Gram negative bacterial meningitis, endotoxemia, Legionnaires disease, and typhoid fever (Ding and Ho 2001). Other LAL applications include testing for endotoxins in influenza vaccine, antibiotics, milk, water, and meat (Ding and Ho 2001).

Presently, there are five companies licensed by the Food and Drug Administration (FDA) to produce LAL (Walls and Berkson 2003). Lysate production is seasonal, beginning in the spring with the appearance of horseshoe crabs in coastal areas for their spawning season (Mikkelsen 1988). The FDA estimates that 260,000 horseshoe crabs were bled for the biomedical industry in 1997, contrasting the 130,000 horseshoe crabs used in 1989 (HCTC 1998). Currently, biomedical companies may sell their bled horseshoe crabs to the bait industry (ASMFC 2001), although most bled horseshoe crabs are still returned to the ocean (Walls et al. 2002). Even though bled horseshoe crabs are returned to the ocean within 72 hours of capture (HCTC 1998), there is an expected level of mortality due to the bleeding process. Studies have indicated that post-bleeding mortality is as high as 20% (Rudloe 1983, Thompson 1998, Kurz and James-Pirri 2002, Walls and Berkson 2003). However, there has been no investigation of mortality associated with trawling, handling, and transport of horseshoe crabs to and from bleeding facilities.

Horseshoe crabs are subjected to stressors (i.e., air exposure, increased temperature, handling, etc.) during the biomedical bleeding process, which may last up to 72 hours. Additionally, some biomedical companies' bleeding protocols involve gravitationally withdrawing blood via cardiac sinus puncture using a large gauge needle until blood flow ceases, either due to hemolymph coagulation or due to emptying of the cardiac sinus. Therefore, some horseshoe crabs lose more blood than others. The volume of blood extracted ranges from 8.4 ml

to 218.7 ml for male horseshoe crabs (Walls 2001) and up to 267.8 ml for females (B. Walls, Virginia Commonwealth University, pers. comm.). However, biomedical companies do not know how much blood horseshoe crabs of a given size possess and how much can be safely extracted. Thus, the upper ranges of blood extraction in both males and females may pose stress and mortality.

The biomedical industry's use of horseshoe crabs has increased over the past decade. The yearly harvest of horseshoe crabs for biomedical use in South Carolina increased over 300% since reporting requirements were instituted in 1991 (Thompson 1998). There was a slight increase in harvest of horseshoe crabs between 1998 and 2000. Interestingly, the number of horseshoe crabs bled increased less than 1%, but the number of animals rejected for bleeding increased by about 9.5%. Approximately 25% of the horseshoe crabs landed for biomedical purposes were rejected for use, with about 45% of these rejected due to injury (ASMFC 2002). With current harvest levels, cumulative mortalities from harvest and bleeding may be demographically significant.

Of the five U.S. companies licensed to produce LAL, three provide 80-90% of the LAL utilized throughout the world: Charles River Endosafe in South Carolina, Cambrex Bio Science Walkersville, Inc. in Maryland, and Associates of Cape Cod, Inc. in Massachusetts (Manion et al. 2000). The biomedical industry's production of LAL provides annual revenue of $60 million and generates a social welfare benefit of over $150 million dollars yearly (Manion et al. 2000). Currently, lysate is produced on a commercial scale primarily in the United States; however, China and Japan also export a quantity of LAL (Lakshmanan and Venkateshvaran 1999). Biomedical companies distribute LAL to both domestic and foreign markets. However, current

production cannot meet demand for LAL, resulting in a shortage of lysate in international markets (Lakshmanan and Venkateshvaran 1999).

Efforts are underway to develop alternatives to LAL and to reduce the biomedical industry's dependence on horseshoe crabs in order to meet increasing demand. Research has produced 'recombinant LAL' based on Factor C (rFC) found in LAL (Ding and Ho 2001). The rFC demonstrated greater sensitivity to endotoxin than naturally-derived LAL (Ding and Ho 2001) and could be produced continuously rather than seasonally. A patent for the compound has been filed in the United States (ASMFC 2002) and is being marketed as PyroGene™.

Another method for developing an alternative to naturally-obtained LAL is through cell culture. Amebocyte cultivation potentially could produce a lysate that is not subject to the seasonality of the natural resource (Pearson and Woodland 1979) and that could be produced continuously. Past attempts to culture amebocytes have been non-reproducible or yielded results conflicting with other studies (Pearson and Woodland 1979, Chen et al. 1986, Chen et al. 1989, Frieberg et al. 1991), but some methods have been patented (Pearson 1980, Gibson and Hilly 1992). Progress in amebocyte cultivation for production of LAL remained at a standstill for over a decade until Joshi et al. (2002) recently reported long-term *in vitro* generation of amebocytes. They used a standard insect cell culture medium to cultivate amebocytes. If Joshi et al.'s amebocyte culture method is reproducible, there is potential for cell culture medium optimization to meet the requirements of horseshoe crab amebocytes. Cell growth requirements include: supplement of all essential nutrients by the medium, acceptable physiological parameters (temperature, pH, and osmolality), absence of toxic or inhibitory effects of substances in the serum, and the presence of specific growth factors (Ham and McKeehan 1979). Modifying cell

culture media to better suit the amebocytes may enhance Joshi's (2002) amebocyte culture method, with a possible increase in amebocyte generation or longer viability of the culture.

Alternatives to LAL may contribute to the conservation of *Limulus*, but these advances are still on the horizon. Presently, the biomedical industry relies on the horseshoe crab resource and is competing for its share. The industry's actions are only part of a larger management issue that is primarily focused on regulating the commercial fishery. There are clashing user group interests, increasing harvest regulations, a lack of valid population data, and no feasible alternative to the horseshoe crab for any of the stakeholders. As a result, there is growing pressure to reduce biomedical and commercial fishery impacts on this critical species.

PROJECT PURPOSE AND OBJECTIVES

The horseshoe crab resource presently is not sufficiently abundant to sustainably meet the needs of the commercial fishery, migratory shorebirds, and biomedical industry. With the biomedical industry's lucrative interest in horseshoe crabs, a growing international demand for LAL, and LAL's vital role in protecting public health, it is critical to investigate the effects of human-induced stresses on this critical species. In addition, with population status uncertain and regulations for the biomedical industry to heed, it is essential to constructively address growing concerns among conservationists. Anticipating a move to decrease biomedical industry pressure on the horseshoe crab resource, I investigated ways to reduce post-bleeding mortality and conducted a preliminary study of cell culture as a source of LAL. This project had three objectives to address areas where information was lacking.

There are few estimates of total hemolymph volume in the horseshoe crab (Robertson 1970, Shuster 1978, Shuster 1982). None of the studies are useful for the biomedical industry as,

none examine hemolymph volume over a representative range of adult horseshoe crab size classes. Therefore, the first objective was to determine total blood volume over a representative sample of horseshoe crabs. This information supported estimation of percentage of blood extracted from individuals, information critical to conducting improved bleeding mortality studies.

Past bleeding mortality studies offered estimates of the impact of biomedical processing on horseshoe crab populations; however, they did not offer insight into decreasing mortality rates. For this reason, the second objective was to identify the relationship between the percentage of blood volume removed and mortality. Work executed under this objective specifically tested the impact of bleeding on horseshoe crab mortality, as well as the effects of simulated biomedical company transport, holding, and bleeding procedures on mortality. The results provide insight into the horseshoe crab's physiological capability and tolerance of bleeding and stresses associated with the biomedical bleeding process. This information may allow biomedical companies to optimize the blood volumes extracted from individuals while modifying their bleeding process to reduce stress levels and lower post-bleeding mortality rates.

Past amebocyte studies (Pearson 1980, Chen et al. 1989, Gibson and Hilly 1992, Joshi et al. 2002) documented varied and sometimes conflicting results with respect to defining an appropriate amebocyte culture medium with optimal physiological parameters. Consequently, this study's third objective was to identify a commercial culture medium that is well suited for the maintenance of horseshoe crab amebocytes. The information provided by this study may help to decrease uncertainty resulting from past, conflicting studies about amebocyte culture parameters. In addition, a recommendation was made for a culture medium that can be used as a base medium for future optimization to meet *Limulus* amebocyte requirements. In this way, this

study contributes to progress for amebocyte culture that has the potential for commercial production of LAL while drastically reducing the need to harvest horseshoe crabs.

Overall, results of this study should stimulate research that will provide much-needed information on the effects of the biomedical industry on *Limulus*, and on alternative approaches to decrease the industry's reliance on horseshoe crabs to help sustainably manage this invaluable resource.

REFERENCES

Armstrong, P.B. 1985. Adhesion and motility of the blood cells of *Limulus*. pp. 77-124 *In:* Cohen, E., (ed). Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology. Alan R. Liss, Inc., New York.

ASMFC (Atlantic States Marine Fisheries Commission). 1998. Interstate fishery management plan for horseshoe crab. Fishery management report No.32. Atlantic States Marine Fisheries Commission, Richmond, VA.

---. 1999. Stock assessment report No. 98-01 (Supplement). Horseshoe crab stock assessment report for peer review. Atlantic States Marine Fisheries Commission, Richmond, VA.

---. 2001. Horseshoe Crab Fishery Management Plan, Addendum II, Section D. Atlantic States Marine Fisheries Commission, Washington, D.C.

---. 2002. Review of the fishery management plan for horseshoe crab (*Limulus polyphemus*). Horseshoe Crab Plan Review Team, Atlantic States Marine Fisheries Commission, Richmond, Virginia.

Berkson, J.B. and C.N. Shuster, Jr. 1999. The horseshoe crab: the battle for a true multiple-use resource. *Fisheries* 24(11):6-11.

Botton, M.L., R.E. Loveland and T.R. Jacobsen. 1994. Site selection by migratory shorebirds in Delaware Bay, and its relationship to beach characteristics and abundance of horseshoe crab (*Limulus polyphemus*) eggs. *The Auk* 111:605-616.

Botton, M.L. and J.W. Ropes. 1987. Populations of horseshoe crabs, *Limulus polyphemus*, on the northwestern Atlantic continental shelf. *Fishery Bulletin* 85:805-812.

Castro, G., J.P. Myers and A.R. Place. 1989. Assimilation efficiency of sanderling (*Calidris alba*) feeding on horseshoe crab (*Limulus polyphemus*) eggs. *Physiological Zoology* 62:716-731.

Chen, I.-J., Y.-M. Chen, S.-J. Hong and L.-C. Chiang. 1986. Morphological changes of horseshoe crab amebocytes during *in vitro* cultivation. *Kaohsiung Journal of Medical Sciences* 2:769-773.

Chen, I.-J., S.-J. Hong, Y.-M. Chen and Y.-C. Yang. 1989. Cultivation of horseshoe crab amebocytes. *Kaohsiung Journal of Medical Sciences* 5:515-521.

Clark, K. 1996. Horseshoe crabs and the shorebird connection. pp. 23-25 *In:* Farrell, J. and C. Martin, (eds). Proceedings of the horseshoe crab forum: status of the resource. University of Delaware Sea Grant College Program, Lewes, Delaware.

Clark, K.E., L.J. Niles and J. Burger. 1993. Abundance and distribution of migrant shorebirds in Delaware Bay. *Condor* 95:694-705.

Ding, J.L. and B. Ho. 2001. A new era in pyrogen testing. *Trends in Biotechnology* 19:277-281.

Ferrari, K.M. and N.M. Targett. 2003. Chemical attractants in horseshoe crab, *Limulus polyphemus*, eggs: the potential for an artificial bait. *Journal of Chemical Ecology* 29(2):477-495.

Frieberg, J.A., P.J. Weathers and D.G. Gibson, III. 1991. Culture of amebocytes in a nutrient mist bioreactor. *In Vitro Cell. Dev. Biol.* 28A:215-217.

Gibson, D.G., III and J.B. Hilly. 1992. Patent No. 5,082,782: Production of horseshoe crab amebocytes *in vitro*. US Patent & Trademark Office, Washington, D.C., USA.

Ham, R.G. and W.L. McKeehan. 1979. Media and growth requirements. pp. 44-93 *In:* Jackoby, W.B. and I.H. Pastan, (eds). Cell Culture. Academic Press, New York.

HCTC. 1998. Status of the horseshoe crab (*Limulus polyphemus*) population off the Atlantic coast. Horseshoe Crab Technical Committee. Atlantic States Marine Fisheries Commission, Washington, D.C.

Iwanaga, S. 2002. The molecular basis of innate immunity in the horseshoe crab. *Current Opinion in Immunology* 14:87-95.

Joshi, B., A. Chatterji and R. Bhonde. 2002. Long-term *in vitro* generation of amoebocytes from the Indian horseshoe crab *Tachypleus gigas* (Müller). *In Vitro Cell. Dev. Biol. - Animal* 38(5):255-257.

Kurz, W. and M.J. James-Pirri. 2002. The impact of biomedical bleeding on horseshoe crab, *Limulus polyphemus*, movement patterns on Cape Cod, Massachusetts. *Marine and Freshwater Behaviour and Physiology* 35(4):261-268.

Lakshmanan, R. and K. Venkateshvaran. 1999. Biomedical potentials of the Indian horseshoe crab. *INFOFISH International* 1:46-48.

Manion, M., R. West and R. Unsworth. 2000. Economic assessment of the Atlantic coast horseshoe crab fishery. Prepared for the Division of Economics, U.S. Fish and Wildlife Service, Arlington, VA, by Industrial Economics, Inc., Cambridge, MA.

Mikkelsen, T. 1988. The Secret in the Blue Blood. Science Press, Beijing, China. 125 pp.

Miller, M. and J.T. Hjelle. 1990. Endotoxins and their clinical significance - an overview. pp. 1-14 *In:* Prior, R.B., Ph.D., (eds). Clinical Applications of the *Limulus* Amoebocyte Lysate Test. CRC Press, Boston.

Myers, J.P. 1981. Conservation of migrating shorebirds: staging areas, geographic bottlenecks, and regional movements. *American Birds* 37:23-25.

---. 1986. Sex and gluttony on Delaware Bay. *Natural History* 95(5):68-77.

Myers, J.P., R.G. Morrison, P.Z. Antas, B.A. Harrington, T.E. Lovejoy, M. Sallaberry, S.E. Senner and A. Tarak. 1987. Conservation strategy for migratory species. *Am. Sci.* 75:19-26.

Novitsky, T.J. 1991. Discovery to commercialization: the blood of the horseshoe crab. *Oceanus* 27(1):13-18.

Pearson, F.C. 1980. Patent No. 4,229,541: *In vitro* cultivation of horseshoe crab amebocytes. US Patent & Trademark Office, Washington, D.C., USA.

Robertson, J.D. 1970. Osmotic and ionic regulation in the horseshoe crab *Limulus polyphemus*. *Biological Bulletin* 138:157-183.

Rudloe, A. 1983. The effect of heavy bleeding on mortality of the horseshoe crab, *Limulus polyphemus*, in the natural environment. *Journal of Invertebrate Pathology* 42:167-176.

Shuster, C.N., Jr. 1978. The circulatory system and blood of the horseshoe crab. A Review. Federal Energy Regulatory Commission (FERC), United States Department of Energy, Washington, D.C.

--- 1982. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae. pp. 1-52 *In:* Bonaventura, J., C. Bonaventura, and S. Tesh, (eds). Physiology and Biology of Horseshoe Crabs: Studies on Normal and Environmentally Stressed Animals. Alan R. Liss, Inc., New York.

Shuster, C.N., Jr. and M.L. Botton. 1985. A contribution to the population of horseshoe crabs, *Limulus polyphemus* (L.), in Delaware Bay. *Estuaries* 8(4):363-372.

Thompson, M. 1998. Assessments of the population biology and critical habitat for the horseshoe crab, *Limulus polyphemus*, in the South Atlantic Bight. M.S. Thesis. University of Charleston, Charleston, South Carolina.

Tsipoura, N. and J. Burger. 1999. Shorebird diet during spring migration stopover on Delaware Bay. *The Condor* 101:635-644.

Walls, B.A. 2001. A horseshoe crab (*Limulus polyphemus*) demographic study. M.S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Walls, B.A. and J.M. Berkson. 2003. Blood extraction effects on horseshoe crabs (*Limulus polyphemus*). *Fishery Bulletin* 101:457-459.

Walls, B.A., J.M. Berkson and S.A. Smith. 2002. The horseshoe Crab, *Limulus polyphemus*: 200 million years of existence, 100 years of study. *Reviews in Fisheries Science* 10:39-73.

CHAPTER 2 - DETERMINATION OF TOTAL HEMOLYMPH VOLUME IN THE HORSESHOE CRAB

ABSTRACT

Biomedical companies extract blood from the horseshoe crab, *Limulus polyphemus*, for the production of *Limulus* Amebocyte Lysate, used worldwide for detecting endotoxins in injectable solutions and medical devices. Despite the extensive use of horseshoe crabs by the biomedical industry, total hemolymph volume in animals of a given size is not known. The hemolymph volume of 60 adult horseshoe crabs was determined using an inulin dilution technique. Blood volume of the horseshoe crab represented as a percentage of wet body weight was 25 ± 2.2% for males and 25 ± 5.1% (mean ± S.D.) for females. A linear relationship of hemolymph volume to weight ($P = 0.0026$, $r^2 = 0.8762$), an exponential relationship between hemolymph volume and prosomal width ($P < 0.0001$), and an exponential relationship between hemolymph volume and inter-ocular width ($P < 0.0001$) were observed. No significant differences were observed between males and females. The relationship of horseshoe crab size and hemolymph volume can be used to predict how much blood can be drawn from the animals used by the biomedical industry, and to predict any resulting bleeding mortality.

INTRODUCTION

The horseshoe crab *Limulus polyphemus* is a multiple-use living marine resource that has become the center of controversy among user groups (Berkson and Shuster 1999, Walls et al. 2002). This unique and ancient animal is an essential component of a healthy marine ecosystem. Horseshoe crab eggs provide an energy source for migratory shorebirds to fuel their journey from South American wintering grounds to their Arctic breeding grounds (Botton et al. 1994). Fishermen have harvested increasing numbers of horseshoe crabs to supply the demand for bait in the American eel (*Anguilla rostrata*) and conch (*Busycon* spp.) fisheries (HCTC 1998, Walls et al. 2002). In addition, the biomedical industry extracts a compound from the animal's hemolymph to produce *Limulus* Amebocyte Lysate (LAL). LAL is used by pharmaceutical and medical industries to ensure that their products (e.g., intravenous drugs, vaccines, and implantable medical and dental devices) are not contaminated with endotoxins from pathogenic Gram-negative bacteria (Mikkelsen 1988, Novitsky 1991).

Considering the extensive use of horseshoe crabs in the biomedical industry since the 1950s, relatively few studies have been conducted concerning the effects of bleeding on the animals. One of the current regulations requires biomedical companies to return bled horseshoe crabs alive to the ocean within 72 hours of capture (HCTC 1998). Mortality rates resulting from the bleeding process are reported to be as high as 20% (Rudloe 1983, Thompson 1998, Kurz and James-Pirri 2002, Walls and Berkson 2003). Amounts of blood extracted from an individual animal range from 100 to 300 ml (Rudloe 1983, Walls 2001), while the maximum available blood volume was believed to be 200 to 300 ml in the past (Rudloe 1983). However, biomedical companies do not know exactly how much blood adult horseshoe crabs of a given size possess and how much can be safely extracted, thus posing implications for stress and mortality.

A variety of methods can be used to estimate blood volume, ranging from traditional methods such as exsanguination to more popular dilution methods using dyes and radioactive isotope tracers (St. Aubin et al. 1978). Stable inulin is frequently used in dye dilution methods. Inulin, a polyfructosane (Ruhl et al. 1995), is an inert substance that mixes with the blood and body fluid (Steinitz 1938), which does not appear to complex with any portion of the circulating fluid, and is slowly excreted (Martin et al. 1958). This non-lethal and minimally invasive method is a long-established colorimetric determination of a red-colored fructose complex formed from inulin during acid hydrolysis (Steinitz 1938, Roe et al. 1949, Ruhl et al. 1995). Inulin has been used in numerous blood volume studies on other species, such as rock lobster (*Panulirus longpipes*) (Dall 1974), crab (*Carcinus maenas*) (Harris and Andrews 1982) and snail (*Littorina littorea*) (Jones and Kamel 1984).

To date, one study has used inulin estimated extracellular volume (Robertson 1970), or total hemolymph volume, in juvenile horseshoe crabs. Other data on hemolymph volume in horseshoe crabs are minimal. The few studies estimating total blood volume did not examine blood volume over a representative size range of adult horseshoe crabs. Shuster (1982) estimated that the total blood volume of one 3000 g adult female from Delaware Bay was 300 ml. Two estimates of blood volume exist for juvenile horseshoe crabs (Robertson 1970, Shuster 1978). In this study, hemolymph volume was estimated across a representative size range of adult *Limulus*.

METHODS

Adult horseshoe crabs were obtained from Cambrex Bio Science Walkersville, Inc. (Cambrex), a major LAL producer, during early fall of 2002. Horseshoe crabs were captured

using a standard trawling procedure off the coast of Ocean City, Maryland. After capture, horseshoe crabs were brought to Cambrex's bleeding facility in Chincoteague, Virginia. These specimens were then transported in an air-conditioned van to the Horseshoe Crab Research Center at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. The animals were maintained in a recirculating aquaculture system with salinity between 27-30‰ and water temperature between 21-23°C. They were allowed to acclimate for two weeks, during which they were tagged, weighed and measured. Measurement consisted of recording the inter-ocular (IO) width, which is the distance between eye-slits of the horseshoe crabs' compound eyes, and the prosomal (P) width, which is the distance across the crab's carapace.

The biomedical industry bleeds horseshoe crabs with a prosomal width greater than eight inches, or 20.5 cm. This minimum size limit tends to exclude most juvenile animals from bleeding. Therefore, sixty specimens, thirty males and thirty females, ranging from 0.90 kg to 4.40 kg were selected to provide a representative size range of adult horseshoe crabs. Hemolymph volume was estimated by a dye dilution method using stable inulin (Roe et al. 1949; J. Shields, Virginia Institute of Marine Science, pers. comm.). A working solution of 30 mg/ml of inulin (Sigma Chemical Co.) was prepared in sterile filtered seawater (Sigma Chemical Co.) and then sterile filtered using 20 μm filters. Initial experiments indicated a dosage of 200 mg of inulin per 1 kg of body weight to be appropriate (Appendix 1). Based on this dosage, a portion of the working solution was injected through the arthrodial membrane into the cardiac sinus. Preliminary experiments also showed that full mixing was achieved within 6 hours at a 21.5°C holding temperature (Appendix 2). After 7 hours, 1 ml of hemolymph was withdrawn from the cardiac sinus. The samples were centrifuged at 10,000 x g to isolate the cell-free hemolymph. Samples then underwent a colorimetric assay (J. Shields, Virginia Institute of Marine Science,

pers. comm.) and the absorbance, at 520 nm wavelength, was measured. Absorbance values of the standard and its serial dilutions were measured every 15 minutes to monitor any changes in color intensity over the course of sample evaluation (Appendix 3).

Calculations and statistical analyses

Once inulin concentration in the hemolymph samples was determined relative to the standard curve, blood volume was estimated as (Martin et al. 1958, Jones and Kamel 1984):

$$V = [d\ (c_1 - c_2) / c_2] - d$$

where V = volume, d = injected volume, c_1 = injected concentration of inulin, and c_2 = final concentration of inulin.

Data were analyzed with the aid of SAS (Statistical Analysis System, Version 8) using a significance level of $\alpha = 0.05$. The relationship between weight, sex, and blood volume was analyzed by linear regression. The effect of gender of the animals was tested using dummy variables to test for a difference in blood volume between males and females by testing the slope and intercept of their respective regression lines. The P width and blood volume data were subjected to non-linear regression fitted with an exponential curve. The IO width and blood volume data were analyzed by non-linear regression fitted with an exponential curve.

RESULTS

The linear relationship of blood volume to weight ($P = 0.0026$; $r^2 = 0.8762$) was characterized by the equation:

$$H = -5.7 + 92\ s + 257.2\ w - 41\ w\ s$$

where H = hemolymph volume in ml, s = sex (if male $s = 0$, if female $s = 1$), and w = wet body weight in kg. The relationship between weight and blood volume is presented in Figure 2.1. No

significant difference was found between males and females in either slope or intercept of their respective regression lines. However, females did demonstrate greater variability in blood volume than males (Figure 2.1). Blood volume of the horseshoe crabs represented as a percentage of wet body weight was 25 ± 2.2% (mean ± S.D.) for males and 25 ± 5.1% for females.

In the field, it is often more straightforward to measure P width rather than weight. The exponential relationship between blood volume and P width ($P < 0.0001$) is represented by the equation:

$$H = 21.6\, e^{0.1234\,(P)}$$

where H = hemolymph volume in ml and P = prosomal width in cm. This relationship is presented in Figure 2.2. Females demonstrated greater variability than males in blood volume within the same size classes (Figure 2.2).

Another relationship characterized was between blood volume and IO width ($P < 0.0001$), which is represented by the equation:

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

where H = hemolymph volume in ml and IO = inter-ocular width in cm. This relationship is presented in Figure 2.3. Females demonstrated greater variability than males in blood volume within the same size classes (Figure 2.3).

DISCUSSION

I estimated hemolymph volume in horseshoe crabs of different sizes, and found a significant linear relationship with weight. Horseshoe crab hemolymph volume as a percentage of body weight falls within the range of those in other arthropods, which share the feature of an

open circulatory system. The blue crab *Callinectes sapidus* has a blood volume of 25.5% of wet body weight (Gleeson and Zubkoff 1977) as compared to the crab *Carcinus maenas*, which has a blood volume of 33.0% of wet body weight (Harris and Andrews 1982). Other arthropod blood volume estimates are: rock lobster *Panulirus longipes* with 17.8% (Dall 1974), freshwater crayfish *Cambarus virilis* with 25.6% (Prosser and Weinstein 1950), and scorpion *Heterometrus fulvipes* with 33.4% (Kumari and Naidu 1987).

Shuster's (1982) single blood volume estimate from a 3000 g adult female horseshoe crab was 300 ml, or 10% of body weight. My study presents blood volume estimates of both adult male and female horseshoe crabs spanning a representative size range of the species ($N = 60$). The results indicate that the mean blood volume is 25% of wet body weight, or 2.5 times that of Shuster's (1982) estimate. One explanation for the discrepancy between Shuster's (1982) results and those of my study is that Shuster (1982) estimated blood volume by exsanguination. Because horseshoe crabs have an open circulatory system, a significant amount of blood can remain in the body within the muscle, tissue spaces, and sinuses. Clotting can also occur, triggered by trauma to the arthroidial membrane during exsanguination, and would hinder measurement of total hemolymph volume. The inulin dye-dilution method is capable of measuring the total blood volume, including the blood that empties into the blood sinuses bathing the muscles and organs. In addition, clotting is not a factor as long as the inulin injection solution is endotoxin free.

Even though the inulin dye-dilution method is more accurate in estimating hemolymph volume than exsanguination, various factors may have influenced the recorded measurements. First, inulin was believed not to penetrate into the cells (Steinitz 1938). However, intracellular penetration of inulin was reported to occur slowly in some tissues, but not in others (McIver and

Macknight 1974, Foglietta and Herrera 1996). These observations stem from work on several mammalian species, the toad *Bufo marinus*, and the holothurian *Isostichopus badionotus* Selenka (McIver and Macknight 1974, Foglietta and Herrera 1996). Despite this, inulin is regarded as a suitable extracellular marker (McIver and Macknight 1974, Foglietta and Herrera 1996). It is not known if inulin penetrates any *Limulus* cell types, or how quickly inulin is taken up into cells. Inulin penetration was assumed to be negligible over the time course of our experiment, since reports of inulin penetration stated that it occurred gradually (McIver and Macknight 1974, Foglietta and Herrera 1996). The impact of inulin penetration into cells would be an apparent greater dilution of inulin, creating an upward bias for estimated blood volume.

Second, inulin is slowly excreted (Martin et al. 1958, Harris and Andrews 1982). In this study, the fluid volume of the inulin injection was nearly 2% of the calculated blood volumes. It is unknown whether this additional fluid input increased excretion rates. Tank water sampled 12 hours after the animals were injected with inulin tested positive for inulin, providing evidence of inulin excretion during this time period. Hence, some amount of inulin may have been excreted before sampling as well as after sampling. This would have resulted in a decreased level of inulin in the blood, giving the impression of greater dilution and therefore leading to a higher blood volume estimate.

Third, the water volume of the blood varies with the salinity of the horseshoe crab's environment and with the length of time the animal is out of the water (Shuster 1978). Lower salinity would increase blood volume and exposure to air would decrease blood volume. During the two-week acclimation period, salinity ranged from 27-30‰ and was 28‰ on the day of the study. During injection and sampling periods, the animals were out of the water less than 30 minutes. These two factors were assumed to have negligible influences on blood volume during

the course of our study. In view of all the evidence at hand, it may be well to regard all the volumes measured with inulin as somewhat greater than the true blood volume.

It is important to note that females had a greater variability in estimated hemolymph volume for the same size classes (Figure 2.3). For example, of the 30 females injected with inulin, 10 females had an inter-ocular width of 17 cm. These females were estimated to have hemolymph volumes from 548 to 951 ml. Yet, the prediction for this size class is 681 ml. Hence, the estimated hemolymph volumes were up to 270 ml greater and 133 ml less than the predicted value. Variability of blood volume among females may be attributable to whether the female is carrying eggs. Eggs in a gravid female would displace space in the body that would otherwise be filled with hemolymph in the absence of eggs. Therefore, it is possible for blood volume to vary seasonally in females corresponding to the spawning season from May to July. Determining if this explanation is correct would improve blood volume predictions for females.

Data from this study did not address the issue of changing blood volume in horseshoe crabs. Information pertaining to horseshoe crab blood volume change is currently unknown or anecdotal. However, some of these factors have been documented to influence blood volume in other species. Blood volume was influenced by seasonal changes in shore crabs *Carcinus mediterraneus* (Devescovi and Luču 1995), salinity in the gastropod *Littorina littorea* (Taylor and Andrews 1988), nutritional state in various decapod crustaceans (Dall 1974, Depledge and Bjerregaard 1989), and parasitism in *Littorina littorea* (Jones and Kamel 1984). Therefore, my results may not be reflective of horseshoe crab blood volumes at other time periods and locations, or in other health conditions.

This study examined blood volume of horseshoe crabs in a controlled environment. This may not be completely reflective of blood volumes in a natural population. Even though these

values should be considered approximations, they are the first blood volume estimates for a range of sizes of adult horseshoe crabs. These results decrease the uncertainty regarding the total blood volume of adult horseshoe crabs and can be used to conduct improved studies on post-bleeding mortality in horseshoe crabs used in the biomedical industry.

It is understood that mortality rates increase as greater blood volume is extracted; however, the exact relationship is unknown in horseshoe crabs, thus the accurate determination of hemolymph volume in *Limulus* is applicable to this topic. This relationship would be particularly useful to biomedical companies producing LAL. Bleeding protocols could then be modified to reflect a maximum threshold level of bleeding based on size, thereby significantly reducing post-bleeding mortalities.

ACKNOWLEDGEMENTS

This work is a result of research sponsored in part by the NOAA Office of Sea Grant, US Dept of Commerce, under Grant No. NA96RG0025 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. Cambrex Bio Science Walkersville, Inc. generously supplied us with horseshoe crabs for the study. I wish to thank Dr. Stephen Smith of the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech for the use of his laboratory. I also thank Dr. Eric Hallerman of the Department of Fisheries and Wildlife Sciences at Virginia Tech for his helpful advice throughout this project. In addition, I thank Penelope Pooler and Keun Pyo Kim of the Department of Statistics at Virginia Polytechnic Institute and State University for statistical consulting. A special thanks is extended to Jay McGhee for his assistance in tagging horseshoe crabs for this study.

REFERENCES

Berkson, J.M. and C.N. Shuster, Jr. 1999. The horseshoe crab: The battle for a true multiple-use resource. *Fisheries* 24(11):6-11.

Botton, M.L., R.E. Loveland and T.R. Jacobsen. 1994. Site selection by migratory shorebirds in Delaware Bay, and its relationship to beach characteristics and abundance of horseshoe crab (*Limulus polyphemus*) eggs. *The Auk* 111:605-616.

Dall, W. 1974. Indices of nutritional state in the western rock lobster *Panulirus longipes*. I. Blood and tissue constituents and water content. *Journal of Experimental Marine Biology and Ecology* 16:167-180.

Depledge, M.H. and P. Bjerregaard. 1989. Haemolymph protein composition and copper levels in decapod crustaceans. *Helgoländer Meeresuntersuchungen* 43:207-223.

Devescovi, M. and Č. Luču. 1995. Seasonal changes of the copper level in shore crabs *Carcinus mediterraneus*. *Marine Ecology Progress Series* 120:169-174.

Foglietta, L.M. and F.C. Herrera. 1996. Ionosmotic response of respiratory trees of the holothurian *Isostichopus badionotus* Selenka preincubated in hyper-, iso- and hypo-osmotic sea water. *Journal of Experimental Marine Biology and Ecology* 202:151-164.

Gleeson, R.A. and P.L. Zubkoff. 1977. The determination of hemolymph volume in the blue crab, *Callinectes sapidus*, utilizing ^{14}C-thiocyanate. *Comparative Biochemistry and Physiology* 56A:411-413.

Harris, R.R. and M.B. Andrews. 1982. Extracellular fluid volume changes in *Carcinus maenas* during acclimation to low and high environmental salinities. *Journal of Experimental Biology* 99:161-173.

HCTC (Horseshoe Crab Technical Committee). 1998. Status of the horseshoe crab (*Limulus polyphemus*) population off the Atlantic coast. Horseshoe Crab Technical Committee, Atlantic States Marine Fisheries Commission, Washington, D.C.

Jones, H.D. and E.G. Kamel. 1984. The effect of *Cryptocotyle lingua* infection on the blood volume of *Littorina littorea*. *Comparative Biochemistry and Physiology* 79A:493-494.

Kumari, J.P. and B.P. Naidu. 1987. Effect of temperature on whole-animal weight changes, blood water content and blood volume of the scorpion. *Environment and Ecology* 5:584-586.

Kurz, W. and M.J. James-Pirri. 2002. The impact of biomedical bleeding on horseshoe crab, *Limulus polyphemus*, movement patterns on Cape Cod, Massachusetts. *Marine and Freshwater Behaviour and Physiology* 35:261-268.

Martin, A.W., F.M. Harrison, M.J. Huston and D.M. Stewart. 1958. The blood volumes of some representative molluscs. *Journal of Experimental Biology* 35:260-279.

McIver, D.J. and A.D. Macknight. 1974. Extracellular space in some isolated tissues. *Journal of Physiology* 239:31-49.

Mikkelsen, T. 1988. The Secret in the Blue Blood. Science Press, Beijing, China. 125 pp.

Novitsky, T.J. 1991. Discovery to commercialization: The blood of the horseshoe crab. *Oceanus* 27(1):13-18.

Ott, R.L. and Longnecker, M. 2001. An introduction to statistical methods and data analysis. Duxbury, Washington.

Prosser, C.L. and S.J. Weinstein. 1950. Comparison of blood volume in animals with open and with closed circulatory systems. *Physiological Zoology* 23:113-124.

Robertson, J.D. 1970. Osmotic and ionic regulation in the horseshoe crab *Limulus polyphemus*. *Biological Bulletin* 138:157-183.

Roe, J.H., J.H. Epstein and N.P. Goldstein. 1949. A photometric method for the determination of inulin in plasma and urine. *Journal of Biochemistry* 178:839-845.

Rudloe, A. 1983. The effect of heavy bleeding on mortality of the horseshoe crab, *Limulus polyphemus*, in the natural environment. *Journal of Invertebrate Pathology* 42:167-176.

Ruhl, A., H. Lubke and M. Wienbeck. 1995. Effects of nutrient solutions on concentration analysis of non-absorbable dilution markers - implications for studies of gastric emptying. *Neurogastroenterology and Motility* 7(2):79-88.

SAS (Statistical Analysis System). 1999. The SAS/STAT user's guide, version 8. SAS Institute, Cary, NC.

Shuster, C.N., Jr. 1978. The Circulatory System and Blood of the Horseshoe Crab. A Review. Federal Energy Regulatory Commission (FERC), United States Department of Energy, Washington D.C., 44-60 pp.

--- 1982. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae. pp. 1-52 *In:* Bonaventura, J., C. Bonaventura, and S. Tesh, (eds). Physiology and Biology of Horseshoe Crabs: Studies on Normal and Environmentally Stressed Animals. Alan R. Liss, Inc., New York.

St. Aubin, D.J., J.R. Geraci, T.G. Smith and V.I. Smith. 1978. Blood volume determination in the ringed seals, *Phoca hispida*. *Canadian Journal of Zoology* 56:1885-1887.

Steinitz, K. 1938. A colorimetric method for the determination of inulin in blood plasma and urine. *Journal of Biochemistry* 126:589-593.

Taylor, P.M. and E.B. Andrews. 1988. Osmoregulation in the intertidal gastropod *Littorina littorea*. *Journal or Experimental Marine Biology and Ecology* 122:35-46.

Thompson, M. 1998. Assessments of the population biology and critical habitat for the horseshoe crab, *Limulus polyphemus*, in the South Atlantic Bight. M.S. Thesis. University of Charleston, Charleston, South Carolina.

Walls, B.A. 2001. A horseshoe crab (*Limulus polyphemus*) demographic study. M.S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Walls, B.A. and J.M. Berkson. 2003. Blood extraction effects on horseshoe crabs (*Limulus polyphemus*). *Fishery Bulletin* 101:457-459.

Walls, B.A., J.M. Berkson and S.A. Smith. 2002. The horseshoe crab, *Limulus polyphemus*: 200 million years of existence, 100 years of study. *Reviews in Fisheries Science* 10:39-73.

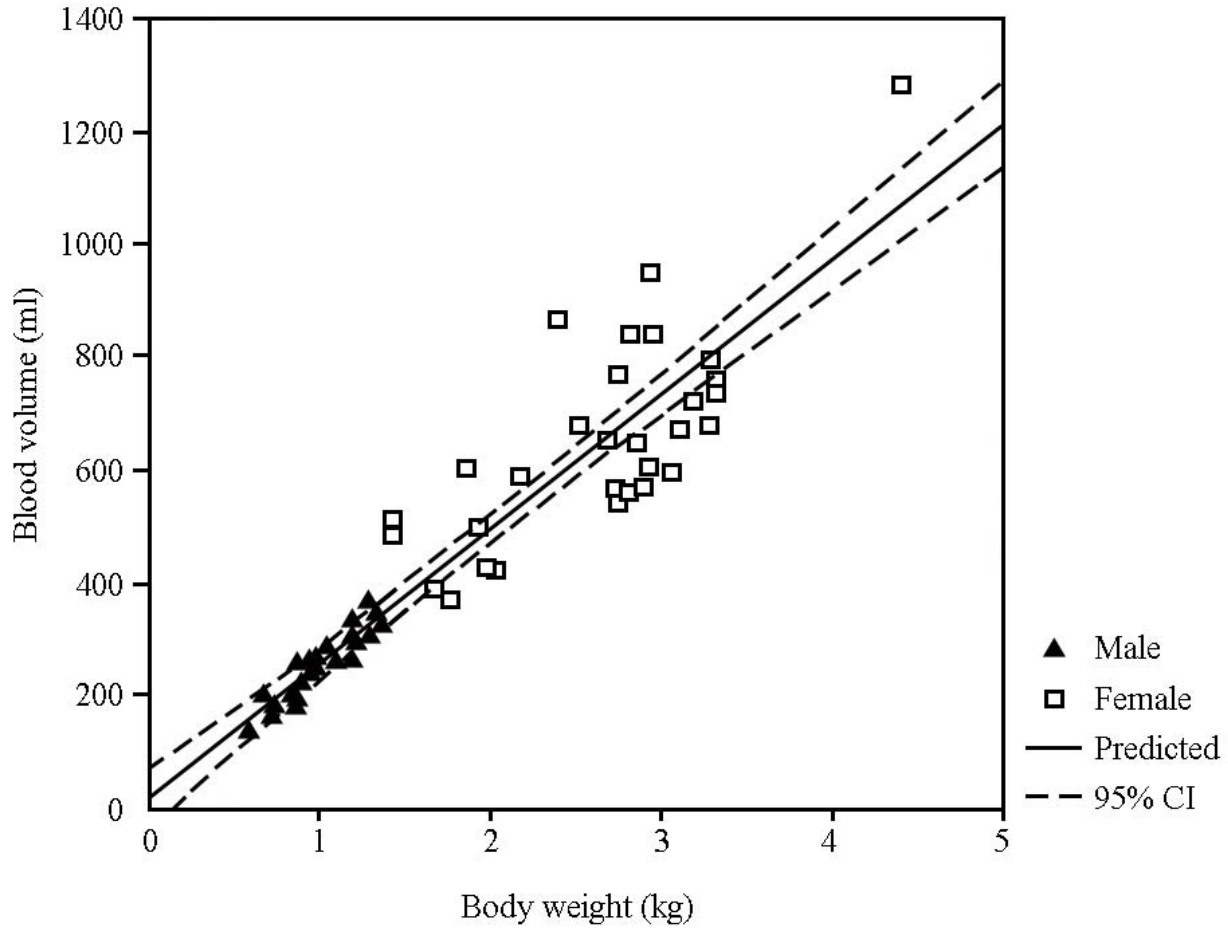


Figure 2.1. Linear regression of wet body weight against estimated hemolymph volume (N = 60; r^2 = 0.874; P = 0.0026). The population mean is enclosed by a 95% confidence interval of the mean.

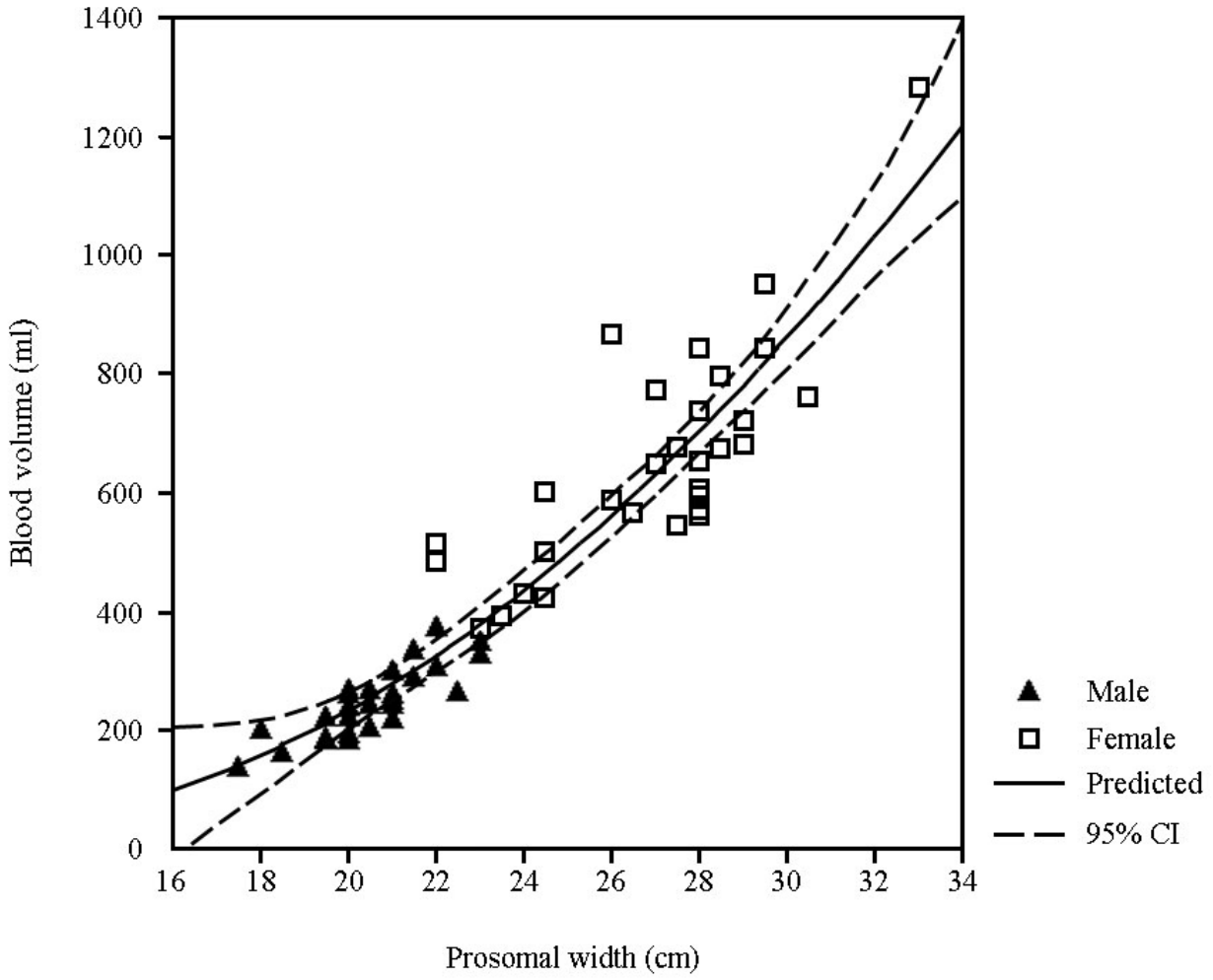


Figure 2.2. Non-linear regression of P width against estimated hemolymph volume with an exponential fit ($N = 60$; $P < 0.0001$). The population mean is enclosed by a 95% confidence interval of the mean.

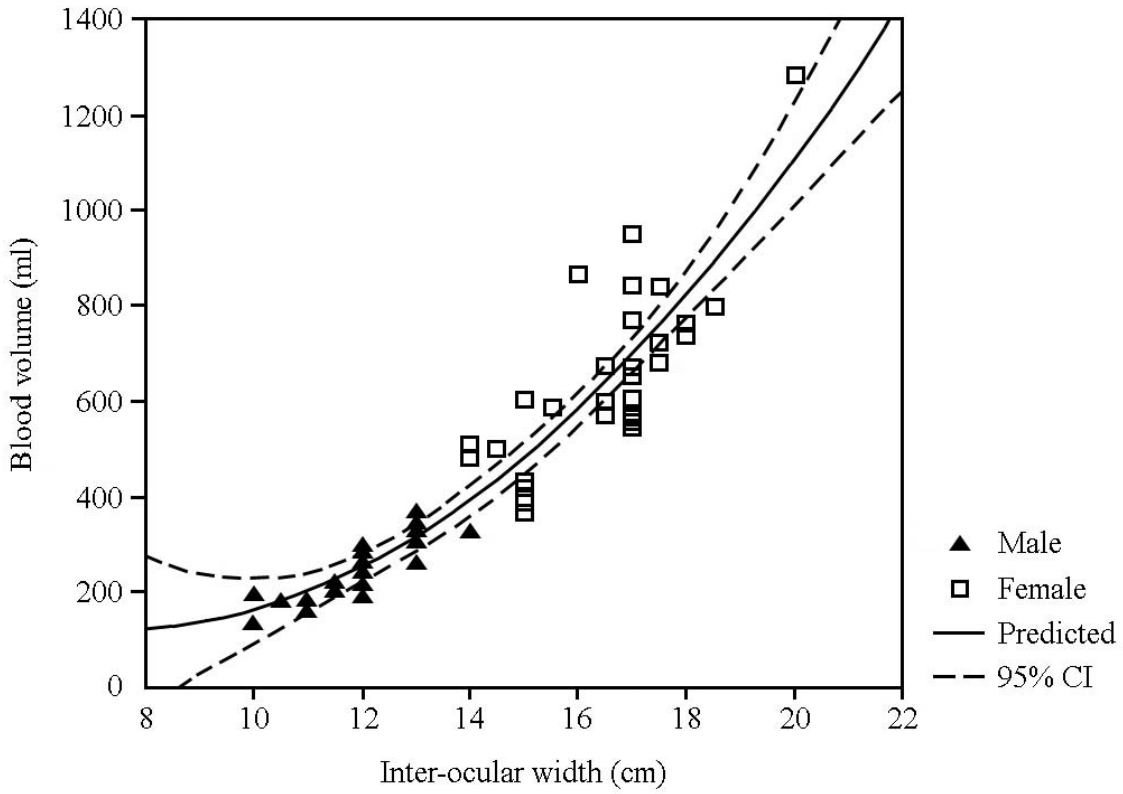


Figure 2.3. Non-linear regression of IO width against estimated hemolymph volume with an exponential fit ($N = 60$; $P < 0.0001$). The population mean is enclosed by a 95% confidence interval of the mean.

CHAPTER 3 - POST-BLEEDING MORTALITY OF HORSESHOE CRABS

ABSTRACT

Biomedical companies extract blood from the horseshoe crab, *Limulus polyphemus*, for the production of *Limulus* Amebocyte Lysate. This compound is used worldwide for detecting endotoxins in injectable solutions and medical devices. Despite the extensive use of horseshoe crabs by the biomedical industry, it is not known how much blood can be safely extracted. Mortality associated with blood extraction was evaluated for horseshoe crabs bled 0, 10, 20, 30, and 40% of their predicted blood volume ($N = 200$). Mortality associated with those same bleeding levels was evaluated in horseshoe crabs that were exposed to multiple stressors before and after bleeding ($N = 195$). These "stressed" animals underwent simulated transport and handling procedures, which commonly occur before and after biomedical bleeding. Mortality data were collected over a 2-week observation period following bleeding. No mortalities occurred in the unstressed group. Of the bled animals, there was an 8.3% mortality rate in the stressed group as compared to 0% mortality in the unstressed group ($P < 0.0001$). Within the stressed group, the mortality rate of bled animals was 8.3% as compared to 2.6% in unbled animals, indicating a relationship between mortality and bleeding under stressed conditions ($P = 0.0088$). Overall mortality in the stressed group increased with increasing levels of blood extraction ($P = 0.006$; $r^2 = 0.994$). In addition, the recovery of blood volume was monitored over a 12-day period in a subsample of horseshoe crabs during the first mortality study ($N = 45$). Total hemolymph protein levels in the 10, 20, 30, and 40% bled groups all demonstrated decreased levels between days 0 and 3 ($P < 0.0001$), with further protein level decrease to day 8 in the 20% ($P = 0.0004$) and 30% ($P = 0.0042$) bled groups. This information can provide guidance on altering transport, holding, and bleeding protocols to decrease the stress and mortality associated with those processes.

INTRODUCTION

Various groups are heavily reliant on *Limulus polyphemus* for a number of diverse purposes. Commercial fishermen harvest horseshoe crabs for use as bait in eel and whelk fisheries (HCTC 1998, Walls et al. 2002). Horseshoe crab eggs serve as an energy source for migratory shorebirds that helps fuel their journey from South American wintering grounds to their Arctic breeding grounds (Clark 1996). Birdwatchers are drawn to these feeding areas in such numbers that an ecotourism industry has grown around this phenomenon (Manion et al. 2000). Biomedical companies catch horseshoe crabs in order to produce a product used for protecting public health (Berkson and Shuster 1999).

There are 5 biomedical companies licensed by the Food and Drug Administration (FDA) to produce *Limulus* Amebocyte Lysate (LAL). Horseshoe crabs are caught, bled, and may be sold to the bait industry (ASMFC 2001); yet, most bled horseshoe crabs are returned to the ocean (Walls et al. 2002). LAL is a clotting agent, derived 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). The LAL test is the most effective test for detecting endotoxins, and its increasing medical and pharmaceutical applicability makes it a highly valued product. The biomedical industry is striving to keep up with the increasing demand for this product. However, there is a reported shortage of lysate in the international markets (Lakshmanan and Venkateshvaran 1999).

Presently, the horseshoe crab resource is under intense pressure to meet the needs of its user groups. The commercial fishery, environmentalists working on behalf of migratory

shorebirds, and the biomedical industry are all competing for their share. Recent concerns of overharvest have led to conflicts among commercial fishermen, environmentalists, and biomedical companies (Berkson and Shuster 1999, Walls et al. 2002). Regulations have been implemented in an effort to protect the possibly declining horseshoe crab populations. Regulations affecting the biomedical industry include a mandate from the FDA stating that biomedical companies release bled horseshoe crabs within 72 hours of capture (HCTC 1998). Each LAL producer has its own bleeding process involving a unique method of capture, distance and method of travel to the bleeding facility, different holding time and conditions, bleeding method, and method of return of the bled crabs that is most appropriate to that company's setting and situation (Walls and Berkson 2003). As a result, there is a mandate from the Atlantic States Marine Fisheries Commission (ASMFC) stating that each company estimate mortality rates resulting from their bleeding process (Schrading et al. 1998).

Several previous 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) or monitored mortality of a given number of horseshoe crabs bled by a biomedical company (Thompson 1998, Kurz and James-Pirri 2002, Walls and Berkson 2003). While the results provide useful information on mortalities reflective of the companies' procedures, they provide no guidance on how biomedical companies can reduce mortality rates.

The biomedical industry harvested approximately 260,000 horseshoe crabs in 1997 (HCTC 1998). By 2000, the number of horseshoe crabs bled had increased less than 1%. However, approximately 25% of the horseshoe crabs landed for possible biomedical use were rejected for use, with about 45% of those rejected due to injury (ASMFC 2002). Mortality likely

is associated with harvest and associated injuries, but the rate of mortality is unknown. With current harvest levels, cumulative mortalities from biomedical collection and bleeding 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. The biomedical source of mortality has an overall impact on the horseshoe crab population, affecting horseshoe crab population viability, horseshoe crab availability to the commercial fishery, and horseshoe crab egg availability to the shorebirds. Reducing mortalities from the biomedical industry would aid in conserving the horseshoe crab population as well as reducing the magnitude of conflict among user groups. To achieve this goal, further investigation into the biomedical bleeding process is required.

In general, the biomedical bleeding process begins with collection of the horseshoe crabs using either trawling or hand-harvest methods. During this time period, the animals may be held on the deck of a boat or in collection containers for an extended period of time. Next, they are transferred to trucks, which may or many not be air-conditioned, and are transported to the bleeding facility. In some cases, they are held in the truck until being moved into the laboratory's cold room. This room is air-conditioned and generally has a stable air temperature of approximately 16 – 18°C. The crabs are held in the coldroom until bleeding, are bled for a period of time, and then remain in the coldroom or in the truck until transport back to the dock. Following their transfer back to the boat, they are returned to their approximate point of capture. Throughout the typical biomedical bleeding process, horseshoe crabs are subjected to a variety of stressors (i.e., air exposure, increased temperature, handling, trauma, etc.), which may last up to 72 hours. This stress likely has an impact on the mortality rates of horseshoe crabs used for biomedical bleeding as well as those harvested, but rejected for use.

Additional sources of stress also may arise from the bleeding methods used by biomedical companies. Some facilities' bleeding protocols involve gravitationally withdrawing blood via heart puncture using a large gauge needle until blood flow ceases, either due to hemolymph coagulation or emptying of the heart sinus. Therefore, some horseshoe crabs lose more blood than others. A recent study reported a range of blood extracted to be from 8.4 ml to 218.7 ml in male horseshoe crabs (Walls 2001) and up to 267.8 ml in females (B. Walls, Virginia Commonwealth University, pers. comm.). However, biomedical companies do not know how much blood horseshoe crabs of a given size possess and how much can be safely extracted. Thus, the reported upper ranges of blood extraction in both males and females pose stress and mortality.

Monitoring recovery from bleeding provides information on the effects of bleeding on the horseshoe crab. One approach is to monitor total blood protein levels after blood is extracted. The fall in concentration of total protein in the hemolymph may be used to monitor the uptake of fluid replacing the lost blood. A similar method using hemocyanin was used by Wells and Wells (1993). Tracking how long it takes blood volume to be regained may provide insight into the duration of the physiological stress associated with hypovolemia, or low volume, which may be a major factor affecting post-bleeding mortality.

To decrease the mortality rates associated with the biomedical bleeding process, several key questions must be addressed:

- How much blood do these animals contain?
- How much blood can be safely withdrawn?
- How does physiological stress from bleeding influence mortality?

- How does stress from transport and holding influence mortality when combined with blood loss?

With the determination of blood volume by size and sex in horseshoe crabs (Chapter 2), the percentage of blood extracted from bled individuals can now be estimated. This information is critical to conducting a bleeding mortality study designed to provide information on decreasing the mortality of horseshoe crabs used in the biomedical industry. The objectives of this study are: (1) to quantify mortality associated with blood extraction at various amounts, (2) to quantify mortality associated with simulated transport and holding stress with blood extraction at the same levels as in the previous objective, and (3) to monitor the recovery of blood volume after blood extraction.

METHODS

Adult horseshoe crabs were obtained from Cambrex Bio Science Walkersville, Inc., a commercial biomedical company, on July 9 and August 28, 2003. Horseshoe crabs were captured 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 appropriate environmental conditions (Brown and Clapper 1981) and monitored daily. They were held in a recirculating aquaculture system with salinity between 31-33‰ and water temperature between 20-21°C. The animals were allowed to acclimate for 1 week, during which time they were tagged, sexed, and measured. Tagging involved drilling two 3/32″ holes into the corner of their prosoma 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 eye-slits of the horseshoe crab's compound eyes.

Bleeding mortality study 1

The first bleeding mortality study used horseshoe crabs from the July 9th collection. From this sample, 100 males and 100 females were selected to test only the effects of various levels of blood extraction on mortality and blood volume replacement. This sample of horseshoe crabs was termed the "un-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 the biomedical industry. Horseshoe crabs were arbitrarily assigned to one of five bleeding treatments (Appendix 4). Each bleeding group was comprised of equal numbers of males and females. According to the bleeding group, horseshoe crabs were bled 0 (control), 10, 20, 30, or 40% of their predicted blood volume. Predicted blood volume was calculated using the relationship between blood volume and IO width (Chapter 2):

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

where H = hemolymph volume in ml and IO = inter-ocular width in cm.

The bleeding process of this component involved removing horseshoe crabs from their holding tank and positioning them in a bleeding rack (Figure 3.1). Their exposed arthrodial membranes were swabbed with a 70% ethanol-soaked cotton swab to disinfect the surface. An 18-gauge needle was inserted through the membrane into the cardiac sinus to extract the predetermined amount of blood (Figure 3.1). The animals were then placed in a holding container without water for 15 – 20 hours. The bled horseshoe crabs were not immediately put back in water in order to prevent them from absorbing water and regaining their blood volume

(Appendix 4). The time out of water was determined to be appropriate because it approximates the duration that horseshoe crabs may be out of water after bleeding at biomedical bleeding facilities. Temperatures to which horseshoe crabs were exposed were recorded using a temperature logger. The air temperature during this time was a stable 21°C. After this period of air exposure, the animals were returned to their holding tanks. Mortality then was monitored for the following two weeks.

Additionally, a subsample of 45 horseshoe crabs was used to monitor the recovery of blood volume in each of the bleeding treatments. Hemolymph samples of 0.5 ml were taken from the subsample of horseshoe crabs on days 0, 3, 8, and 12. Samples were centrifuged at 10,000 x g and the cell-free hemolymph was evaluated for total protein levels using a veterinary refractometer (VET360, Fisher Scientific).

Bleeding mortality study 2

The second bleeding mortality study used horseshoe crabs from the August 28th collection. Due to difficulty in obtaining the desired 200 study animals, 195 horseshoe crabs were available for this study. 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. The external stressors (i.e., air exposure, increased temperature, etc.) originated from simulated transport and holding procedures of the biomedical bleeding process. Since there is great variability in transport and holding procedures and conditions, we simulated an extreme protocol that would demonstrate horseshoe crab response under relatively poor conditions. These animals comprised the "stressed" group as they were exposed to additional stressors to which the first group was not.

During the acclimation week, the horseshoe crabs were held in the same conditions and handled in the same manner as the first group. Horseshoe crabs were assigned to one of five bleeding treatments in the same method as in the first bleeding mortality study. The bleeding process of this study 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. This outdoor phase lasted 6 hours and simulated the horseshoe crabs' time on the deck of a trawler. The morning air temperature of 21°C increased to 29°C by the end of this phase. Next, the horseshoe crabs were moved into a small, moving truck, which was not air-conditioned and was closed. The animals remained in this environment for 4 hours, which simulated transportation to the bleeding facility and holding time until placement into the coldroom of the laboratory. Over the course of this phase, the outdoor air temperature ranged between 29°C and 31°C. Temperatures inside the closed truck peaked at 36°C and declined to 32°C. After this, the animals were transferred to the HCRC's tank room where they remained for 16 hours in an air temperature of 21°C. This phase mimicked the holding time in a facility's coldroom prior to bleeding. Next, the animals were bled according to their assigned treatment group and in the same manner as in the first mortality study. 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. The overnight air temperature was 23 - 18°C. Temperature inside the truck ranged from 24 - 20°C. This phase simulated holding time in the truck and transport back to the boat. After this, the horseshoe crabs were returned to the environmentally-controlled recirculating aquaculture tanks. Mortality then was monitored over the following two weeks.

Statistical analyses

The data collected were processed with the aid of SAS (Statistical Analysis System, Version 8) using a significance level of $\alpha = 0.05$. Fisher's Exact Test was used to evaluate: (1) statistical significance of differences in mortality of unbled horseshoe crabs between the stressed and unstressed groups, and (2) significant differences in mortality of bled crabs between the unstressed and stressed groups. Logistic regression was used to examine the association between mortality and bleeding in the stressed group. Data were also assessed to see if the increase in bleeding amount was correlated with an increase in mortality by plotting the frequency of mortalities against bleeding treatment and applied a regression with a fitted quadratic curve. The total blood protein data were examined by repeated-measures ANOVA, which was used to detect the interactive effects of time and treatment on total protein levels. The total protein data for each bleeding treatment was averaged and then standardized to compare protein levels in the bled groups to the control group.

RESULTS

The initial hypothesis that substantial blood loss was the principle cause of mortality in horseshoe crabs was rejected. No mortalities occurred in either bled or unbled horseshoe crabs in the unstressed group. This indicated that post-bleeding mortality in *Limulus* did not arise solely from the effects of blood loss.

Comparing these results between the unstressed and stressed groups, mortality rates were not significantly different between unbled crabs ($N = 79$; $P = 0.4937$). In bled crabs, there was a mortality rate of 0% in the unstressed group and 8.3% in the stressed group, a significant difference in mortality rates between the two groups ($N = 316$; $P < 0.0001$).

Data from the stressed group was used to test for mortality associated with transport and holding stressors in combination with blood extraction at various amounts. There were a total of 14 mortalities distributed throughout the bleeding treatments (Table 3.1). All mortalities occurred within the first 7 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, indicating a relationship between mortality and bleeding under stressed conditions ($N = 195$; $P = 0.0088$) (Table 3.2). The bleeding variable was significant in the logistic regression model ($P = 0.0160$). There was no significant difference in mortalities between bled males and females ($N = 156$; $P = 0.6100$), with 7 deaths occurring in each group (Table 3.1). Mortality rates reached 13.6% for males in the 30% bled treatment and female mortality was as high as 29.4% in the 40% bled group (Table 3.2). The overall number of mortalities increased as the bleeding amount increased ($P = 0.006$; $r^2 = 0.994$) (Table 3.1)

The recovery of blood volume after blood extraction was examined. Repeated-measures ANOVA indicated an overall significant time-treatment interaction effect ($N = 45$; $P < 0.0001$). Total hemolymph protein levels showed no significant changes over time in the 0 % bled group (Figure 3.2). The 10, 20, 30, and 40% bled groups all demonstrated reduced protein levels between days 0 and 3 ($P < 0.0001$) (Figure 3.2). A significant decrease in protein levels was observed between days 3 and 8 in the 20% ($P = 0.0004$) and 30% ($P = 0.0042$) bled groups. Standardized total protein averages show the average decline in total blood protein in the bled groups relative to the control group (Figure 3.3).

DISCUSSION

Based on this study's use of 200 horseshoe crabs, no impact on mortality was observed with blood extraction up to 40% of the crab's blood volume in conditions excluding exposure to external stressors. This study suggested any mortality rate from this maximum level of bleeding was so low that it was not captured by our sample size of 40 individuals per bleeding treatment. Previous studies in *Octopus vulgaris* demonstrated that removal of a large amount of blood (up to 40%) was tolerated (Wells and Wells 1993). In contrast, mortality occurred from 27% blood loss in bluefish (*Pomatomus saltatrix*) and 14 % blood loss in the eel (*Anguilla* spp.), indicating that horseshoe crabs can survive acute volume depletion that is lethal to other aquatic animals. However, under different conditions, this response may be altered.

The results demonstrated that horseshoe crab mortality from blood loss was significant in the presence of external stressors (i.e., lengthy air exposure, elevated temperatures, etc.) as applied in our study. This finding contradicts those of Novitsky's (1991) report that up to 30% of blood volume can be safely extracted during a biomedical bleeding process, suggesting that low mortality may not always be the case. Findings from this study indicate that a combination of external effects and sub-lethal hemorrhage resulted in significantly increased mortalities, possibly from a synergistic interaction between the two types of stressors. Studies in other species exposed to various stressors also indicated that mortality might be affected by a synergistic combination of effects from multiple stressors (Schisler et al. 2000, Schulz and Dabrowske 2001, Hatch and Blaustein 2003). Such results emphasize the importance of considering the cumulative impact of multiple stressors.

Monitoring total blood protein after blood extraction provided insight into one particular physiological stressor, low blood volume. Observations revealed that total blood protein levels

stabilized within eight days after bleeding, with fluid uptake ceasing to be significant after that time. From this, we can infer that it took up to eight days for bled horseshoe crabs to recover their blood volume. These results concur with Novitsky's (1991) reported three-to-seven day period of blood volume recovery. Tracking how long it took for blood volume to be regained may indicate the duration of stress from hypovolemia. Low blood volume was a sub-lethal stressor in the first bleeding mortality study, and may have been the main source of stress resulting from blood extraction in that group. However, it may be this internal stressor in combination with external stressors that resulted in the synergistic effect on mortality observed in the second bleeding mortality study. It is interesting to note that it took up to eight days for blood volume to be regained, and it was within this time period that all mortalities took place. However, recovery from bleeding still continued after this eight-day period. Total hemolymph protein remained at decreased levels 12 days after bleeding. In addition, Novitsky (1991) reported that amebocyte regeneration required three to four months for cell counts to equal those obtained prior to bleeding. Recuperation therefore continues for an extended period of time, during which the animal must cope with numerous physiological stresses induced by blood loss.

The combination of multiple stressors would affect not only mortality, but could possibly affect the amount of blood extracted by the biomedical industry. It is likely that horseshoe crabs exposed to greater lengths of time to air and higher temperatures (i.e., mid-summer, heat-waves, etc.) would lose moisture from their exposed gills and become dehydrated. This would result in decreased blood volume with a smaller amount of blood available for extraction. Consequently, there may be fewer amebocytes harvested for the production of LAL. Dehydration rates in horseshoe crabs are unknown, but have been examined in a freshwater crayfish (Taylor et al. 1987). *Austropotamobius pallipes* is a facultative air breather that can survive about three days

out of water (Taylor et al. 1987). This study reported that when exposed to air (70 – 80% relative humidity) for 27 hours, crayfish 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. Perhaps horseshoe crabs exposed to water-saturated air would not become dehydrated, thereby decreasing the effects of that stress factor.

The results presented in this study provide insight into the combined effects of blood extraction and external stressors, from simulated transport and holding methods, on horseshoe crab mortality. There are a variety of ways to conduct the bleeding process. We simulated an extreme example of transport, holding, and bleeding methods that provided poor conditions, which had a significant effect on the horseshoe crabs. 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 would 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), periodically flushing them with seawater, 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. Since blood volume estimates now are known (Chapter 2), extraction amounts could be adjusted to suit the stress / condition level of the horseshoe crabs during a specific bleeding process. In addition, the blood volume extracted by biomedical companies could potentially be optimized by keeping horseshoe crabs hydrated and

bleeding a prescribed amount from individuals as opposed to gravitationally withdrawing a wide range of blood volumes. 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. This would decrease the biomedical industry's impact on the potentially declining horseshoe crab population and aid in conservation of this species.

ACKNOWLEDGEMENTS

This work is a result of research sponsored in part by the NOAA Office of Sea Grant, US Dept of Commerce, under Grant No. NA96RG0025 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. Cambrex Bio Science Walkersville, Inc. generously supplied us with horseshoe crabs for the study. I wish to thank Dr. Stephen Smith of the Virginia-Maryland College of Veterinary Medicine at Virginia Tech for his help in the planning of this study. I also thank Dr. Eric Hallerman of the Department of Fisheries and Wildlife Sciences of Virginia Tech for his helpful comments throughout this project. In addition, I thank Xin Zhong and Li Wang of the Department of Statistics at Virginia Polytechnic Institute and State University for statistical consulting. A special thanks is extended to numerous colleagues for their assistance in transporting, tagging, and/or bleeding horseshoe crabs for this study.

REFERENCES

ASMFC (Atlantic States Marine Fisheries Commission). 2001. Horseshoe Crab Fishery Management Plan, Addendum II, Section D. Atlantic States Marine Fisheries Commission, Washington, D.C.

---. 2002. Review of the fishery management plan for horseshoe crab (*Limulus polyphemus*). Horseshoe Crab Plan Review Team, Atlantic States Marine Fisheries Commission, Richmond, Virginia.

Berkson, J.B. and C.N. Shuster, Jr. 1999. The horseshoe crab: the battle for a true multiple-use resource. *Fisheries* 24(11):6-11.

Brown, G.G. and D. Clapper 1981. Procedures for maintaining adults, collecting gametes, and culturing embryos and juveniles of the horseshoe crab, *Limulus polyphemus*. pp. 268-290 *In:* (eds). Laboratory Animal Management: Marine Invertebrates. National Academy Press, Washington, D.C.

Clark, K. 1996. Horseshoe crabs and the shorebird connection. pp. 23-25 *In:* Farrell, J. and C. Martin, (eds). Proceedings of the horseshoe crab forum: status of the resource. University of Delaware Sea Grant College Program, Lewes, Delaware.

Ding, J.L. and B. Ho. 2001. A new era in pyrogen testing. *Trends in Biotechnology* 19:277-281.

Hata, D. and J.M. Berkson. 2003. Abundance of horseshoe crabs, *Limulus polyphemus*, in the Delaware Bay area. *Fishery Bulletin* 101(3):933-938.

Hatch, A.C. and A.R. Blaustein. 2003. Combined effects of UV-B radiation and nitrate fertilizer on larval amphibians. *Ecological Applications* 13:1083-1093.

HCTC. 1998. Status of the horseshoe crab (*Limulus polyphemus*) population off the Atlantic Coast. Horseshoe Crab Technical Committee. Atlantic States Marine Fisheries Commission, Washington, D.C.

Kurz, W. and M.J. James-Pirri. 2002. The impact of biomedical bleeding on horseshoe crab, *Limulus polyphemus*, movement patterns on Cape Cod, Massachusetts. *Marine and Freshwater Behaviour and Physiology* 35:261-268.

Lakshmanan, R. and K. Venkateshvaran. 1999. Biomedical potentials of the Indian horseshoe crab. *INFOFISH International* 1:46-48.

Manion, M., R. West and R. Unsworth. 2000. Economic assessment of the Atlantic Coast horseshoe crab fishery. Division of Economics, U.S. Fish and Wildlife Service, Arlington, VA.

Mikkelsen, T. 1988. The secret in the blue blood. Science Press, Beijing, China. 125.

Novitsky, T.J. 1991. Discovery to commercialization: the blood of the horseshoe crab. *Oceanus* 27(1):13-18.

Rudloe, A. 1983. The effect of heavy bleeding on mortality of the horseshoe crab, *Limulus polyphemus*, in the natural environment. *Journal of Invertebrate Pathology* 42:167-176.

Schisler, G.J., E.P. Bergersen and P.G. Walker. 2000. Effects of multiple stressors on morbidity and mortality of fingerling rainbow trout infected with *Myxobolus cerebralis*. *Transactions of the American Fisheries Society* 129:859-865.

Schrading, E., T.J. O'Connell, S. Michels and P. Perra. 1998. Interstate management plan for horseshoe crab. Atlantic States Marine Fisheries Commission, Washington, D.C.

Schulz, R. and J.M. Dabrowski. 2001. Combined effects of predatory fish and sublethal pesticide contamination on the behavior and mortality of mayfly nymphs. *Environmental Toxicology and Chemistry* 20:2537-2543.

Taylor, E.W., R. Tyler-Jones and M.G. Wheatly. 1987. The effects of aerial exposure on the distribution of body water and ions in the freshwater crayfish *Austropotamobius pallipes* (Lereboullet). *Journal of Experimental Biology* 128:307-322.

Thompson, M. 1998. Assessments of the population biology and critical habitat for the horseshoe crab, *Limulus polyphemus*, in the South Atlantic Bight. Master of Science. University of Charleston, Charleston, South Carolina.

Walls, B.A. 2001. A horseshoe crab (*Limulus polyphemus*) demographic study. M.S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Walls, B.A. and J.M. Berkson. 2003. Blood extraction effects on horseshoe crabs (*Limulus polyphemus*). *Fishery Bulletin* 101:457-459.

Walls, B.A., J.M. Berkson and S.A. Smith. 2002. The horseshoe crab, *Limulus polyphemus*: 200 million years of existence, 100 years of study. *Reviews in Fisheries Science* 10:39-73.

Wells, M.J. and J. Wells. 1993. Fluid uptake and the maintenance of blood volume in octopus. *Journal of Experimental Biology* 175:211-218.

Table 3.1. Comparison of the numbers of mortalities observed between bled and unbled horseshoe crabs at different bleeding levels in the stressed group. $N = 39$ for each group.

% Bled	Male mortality (No.)	Female mortality (No.)	Total mortality/ treatment (No.)
0%	1	0	1
10%	0	1	1
20%	2	0	2
30%	3	1	4
40%	1	5	6
Total	7	7	14

Table 3.2. Comparison of the percentage of mortalities observed between bled and unbled horseshoe crabs at different bleeding levels in the stressed group. $N = 39$ for each group.

% Bled	Male mortality (%)	Female mortality (%)	Total mortality per treatment (%)
0%	4.5	0	2.6
10%	0	5.9	2.6
20%	9.1	0	5.1
30%	13.6	5.9	10.3
40%	4.5	29.4	15.4
Total	6.4	8.24	7.2

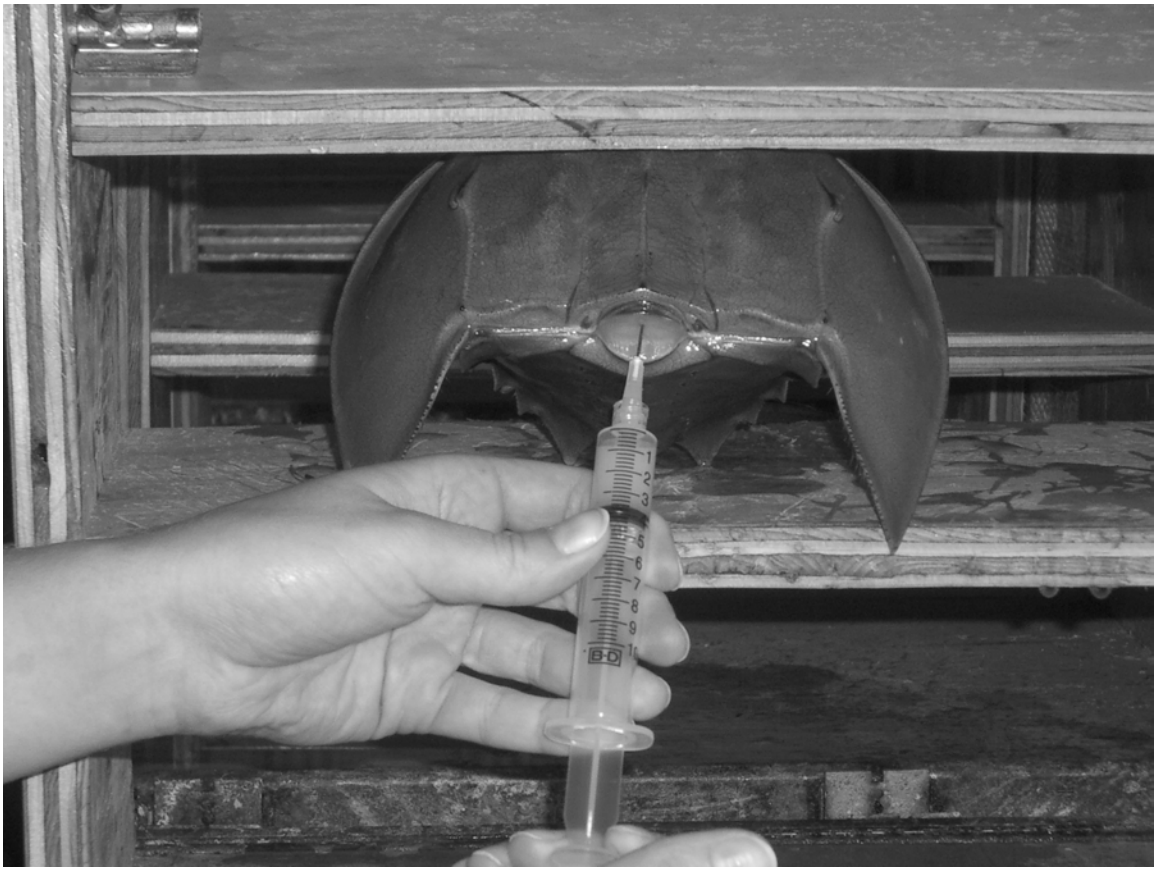


Figure 3.1. A horseshoe crab positioned in a bleeding rack to expose the arthodial membrane for blood extraction using an 18-gauge needle inserted through the membrane and into the cardiac sinus.

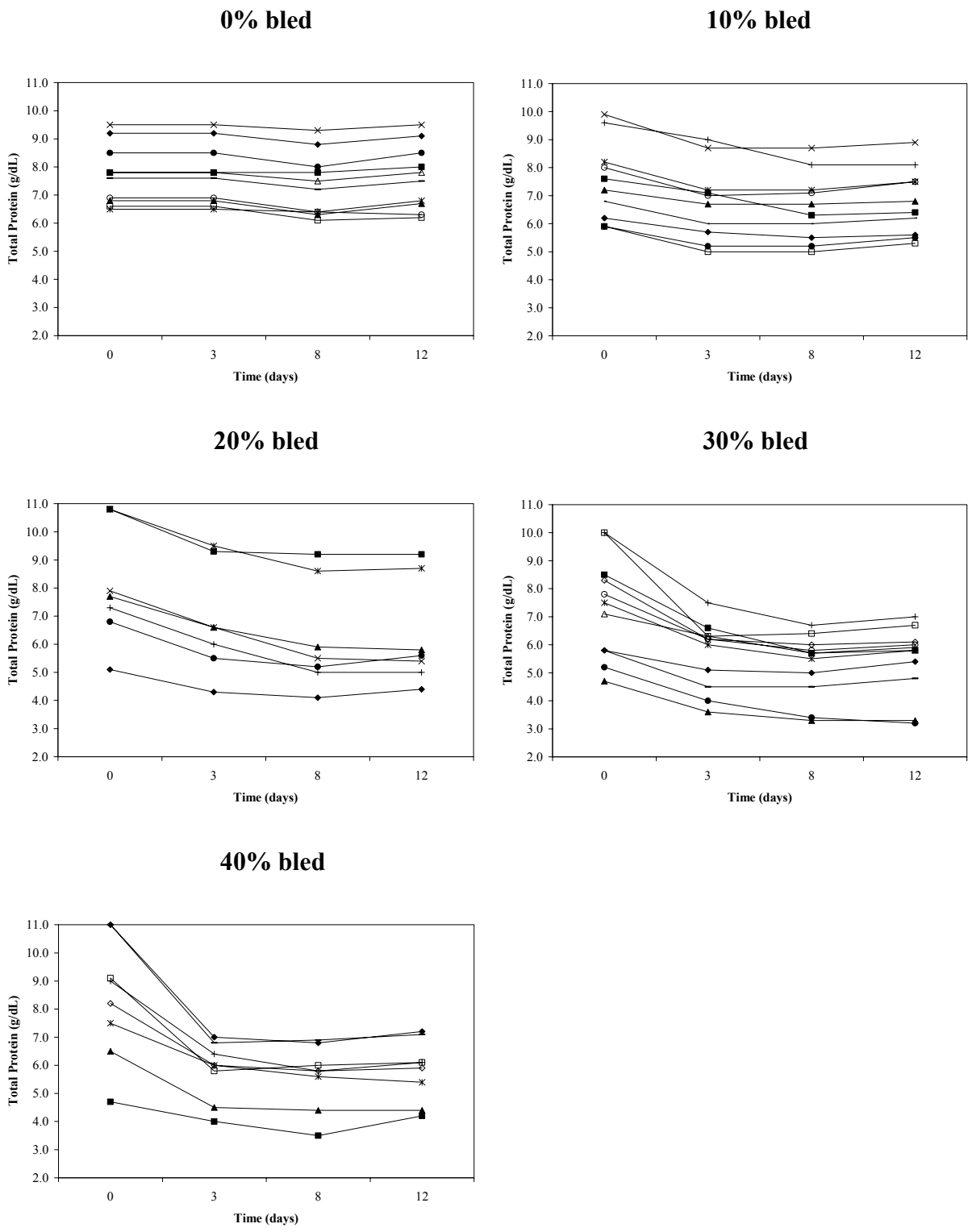


Figure 3.2. Comparison of total hemolymph protein levels over time after blood extraction in each bleeding treatment. In each graph, the symbols represent individuals of the subset tested (*N* = 45).

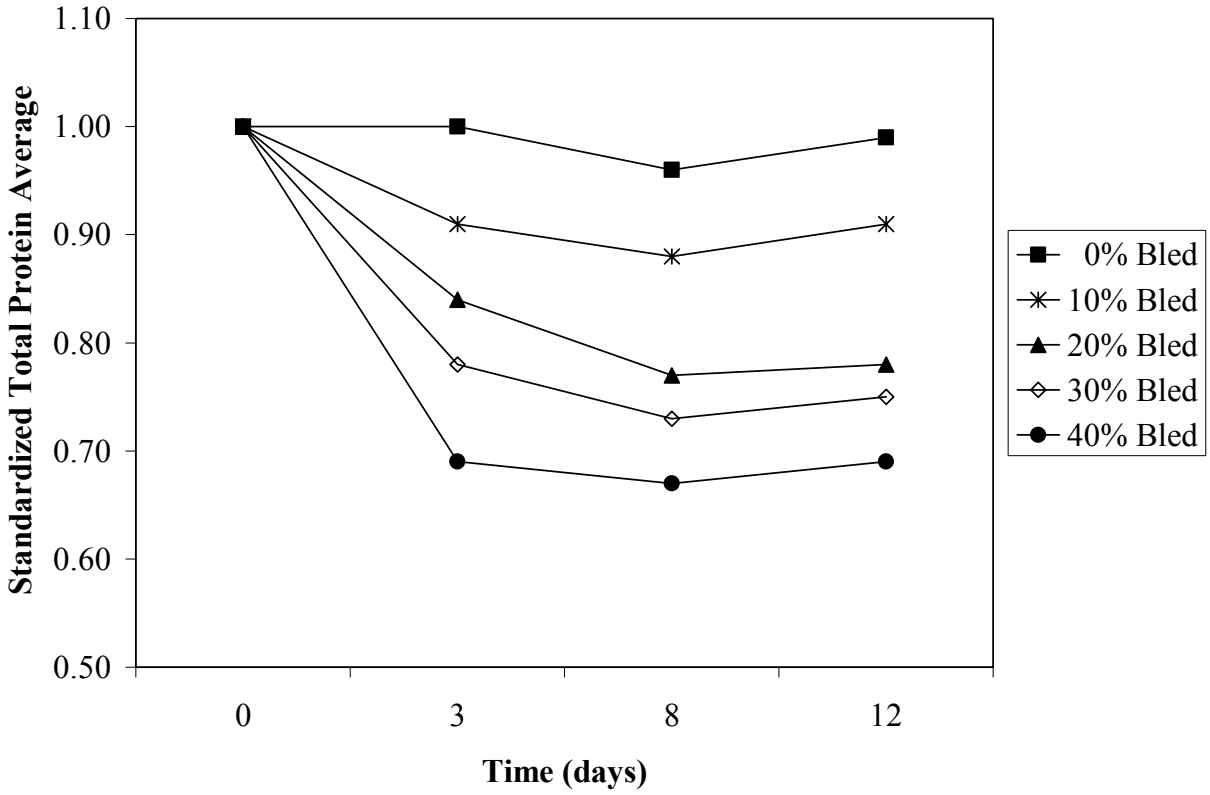


Figure 3.3. Comparison of standardized total protein averages, illustrating the average decline of total blood protein in the bled groups relative to the control group.

CHAPTER 4 - SELECTION OF A CELL CULTURE MEDIUM FOR *IN VITRO* MAINTENANCE OF *LIMULUS* AMEBOCYTES

ABSTRACT

This study provides information needed for future research aimed at producing *Limulus* Amebocyte Lysate (LAL) *in vitro*, which would have the potential to reduce the need to harvest and bleed horseshoe crabs for current methods of LAL production. To address the need for primary culture of horseshoe crab amebocytes, this study tested the effects of horseshoe crab serum supplementation and the effects of a variety of standard insect cell culture media on amebocyte morphology and viability after 7 days of maintenance. Amebocytes were less altered from *in vivo* form when cultures were supplemented with horseshoe crab serum than amebocytes in serum-free cultures. Serum-supplemented cultures supported significantly higher cell viability than serum-free cultures ($N = 6$; $P = 0.0147$). Amebocyte morphology was least altered in Grace's Insect Medium without serum, with no observed degranulation of cells, as compared to the other media tested. There were significant differences in amebocyte viability among the six insect cell culture media tested ($N = 36$; $P < 0.0001$). Grace's Insect Medium without serum sustained viability of 77.2 ± 5.1% (mean ± S.D.) of amebocytes, followed distantly by Grace's Modified Insect Medium with 35.1 ± 8.7% amebocyte viability. Results indicate that Grace's Insect Medium with horseshoe crab serum supplementation was the best candidate for future medium optimization for *Limulus* amebocyte requirements.

INTRODUCTION

Horseshoe crabs are collected for the biomedical industry for the production of *Limulus* Amebocyte Lysate (LAL). LAL is used for detecting the presence of endotoxins pathogenic to humans in pharmaceutical, medical, and dental products (Mikkelsen 1988, Novitsky 1991). While alternate tests exist for the detection of endotoxin, the LAL assay is the most sensitive, capable of detecting as 10^{-15} of a gram of endotoxin (Mikkelsen 1988, Novitsky 1991). The LAL test is used worldwide to protect public health, and horseshoe crabs are the primary source of this substance.

LAL is extracted from amebocytes, the predominant blood cell type circulating in *Limulus polyphemus* (Suhr-Jessen et al. 1989). These amebocytes contain intracellular granules, which are filled with clotting factors sensitive to nanogram quantities of endotoxin (Levin and Bang 1964). This clotting reaction of the granule proteins is triggered by the presence of Gram-negative bacterial endotoxin. This provides the basis for the LAL test, a clinically and commercially significant assay (Ding and Ho 2001).

The manufacture and application of LAL have become widespread, but there are a number of problems inherent in producing LAL from its natural source. The biomedical bleeding process is laborious, involving collection of horseshoe crabs, transport, holding, bleeding, and return of the crabs to the ocean or sometimes to bait fishermen. Mortality rates of up to 20% have reportedly resulted from this process (Rudloe 1983, Thompson 1998, Kurz and James-Pirri 2002, Walls and Berkson 2003). Furthermore, biomedical bleeding facilities must comply with various regulations regarding collection and use of horseshoe crabs (HCTC 1998, Schrading et al. 1998, ASMFC 2001). These regulations have arisen in response to concerns of overharvest of horseshoe crabs by the commercial fishery and a potential population decline. Despite these conditions, the biomedical industry has been able to distribute LAL to both

domestic and foreign markets. However, its current production rate cannot keep up with the demand for LAL, as there is an apparent shortage of lysate in the international markets (Lakshmanan and Venkateshvaran 1999). LAL sales also are limited by the seasonal production (Pearson 1980) resulting from the use of captured horseshoe crabs. Another important issue with naturally-derived LAL is the fluctuation of its quality. There is significant variation in the sensitivity and specificity of the lysate between manufacturers, batch, and even season (Pearson 1980, Frieberg et al. 1991, Ding and Ho 2001). This is likely due to variation among horseshoe crabs as well as in seasonal and environmental factors (Frieberg et al. 1991). Development of alternative LAL sources could potentially reduce or circumvent problems associated with producing LAL from captured horseshoe crabs.

Various efforts are underway to develop alternatives to LAL and to reduce the biomedical industry's dependence on horseshoe crabs. One alternative is to clone and express specific proteins that are present in LAL. Research has produced 'recombinant LAL' based on Factor C (rFC) found in LAL (Ding and Ho 2001). The rFC demonstrated greater sensitivity to endotoxin than naturally-derived LAL (Ding and Ho 2001), and could be produced continuously rather than seasonally. Patent applications for the compound have been filed in the United States, and rFC has been introduced to the market as PyroGene™ Recombinant Factor C Assay (Cambrex 2003b). Despite rFC's reported benefits over naturally-derived LAL, this assay advancement has its own drawbacks. PyroGene™ is not yet intended for clinical diagnosis of endotoxin (Cambrex 2003a), but there are numerous clinical tests dependant on LAL (Ding and Ho 2001). In addition, PyroGene™ has yet to be approved by the Food and Drug Administration. To replace LAL, rFC products must be developed for a variety of clinical uses, and consumer demand for rFC products must surpass the demand for LAL products. In the meantime, the bleeding of horseshoe crabs for the production of LAL will continue.

Another alternative to naturally-derived LAL is through the development of an *in vitro* culture system. Amebocyte culture also could be used to circumvent many of the problems associated with LAL produced from captured horseshoe crabs. Amebocyte culture potentially could produce a lysate that is not subject to the variability of the natural environment (Pearson and Woodland 1979) and which could be produced continuously year-round. With further research, this alternative could be scaled-up for commercial production.

It is unknown which of the alternatives would produce more of the LAL compounds, prove most sensitive to endotoxins, and be the cheapest to produce. Since, rFC is a product containing one of the many clotting factors of LAL, and amebocyte culture presumably would yield the lysate as a whole, each LAL alternative may prove useful for production of various endotoxin-detecting products. Thus, it would be advantageous to explore the potential of amebocyte culture.

Between 1979 and 1992, there were several attempts to culture amebocytes. Past studies (Pearson and Woodland 1979, Ding et al. 1988, Chen et al. 1989, Frieberg et al. 1991, Gibson and Hilly 1992) were either non-reproducible or yielded results conflicting with other studies. Pearson and Woodland (1979) reported to have optimized medium, pH, and temperature of culture conditions with resulting amebocyte growth. Pearson and Woodland (1979) worked with untransformed cells, and likely did not achieve any significant amebocyte replication because a mitotic index of less than 1 per 2000 amebocytes was reported by Sherman (1981), with other studies (Armstrong 1985, Copeland and Levin 1985) finding no mitosis present in circulating amebocytes of intermoult adults. Pearson (1980) estimated total DNA to ascertain growth. However, this method is not a direct measure of cell division, as DNA synthesis is not always indicative of mitosis (Mather and Roberts 1998). Pearson and Woodland (1979) also reported to have maintained amebocytes up to thirty days, but vacuolation and degranulation became

pronounced after ten days. Ding et al. (1988) attempted to reproduce Pearson and Woodland's methods, but Ding et al.'s (1988) culture did not remain viable for more than 2 weeks. Other amebocyte culture studies (Ding et al. 1988, Chen et al. 1989, Frieberg et al. 1991, Gibson and Hilly 1992) reported results from the use of various culture techniques using different media under different temperatures, pH levels, and osmolalities.

Over a decade later, Joshi et al. (2002) explored the potential of amebocyte culture using an organ culture technique. Based on information that suggested that the gill flaps of the horseshoe crab are the source of amebocytes (Gibson and Hilly 1992), Joshi et al. (2002) cultured gill lamellae in a standard insect cell culture medium, and reported amebocyte harvests for a period of 6 – 8 weeks (Joshi et al. 2002). Hence, there is renewed interest in this topic, fueled by difficulties associated with naturally-produced LAL that continue to challenge the biomedical industry. Additional information on improvements in amebocyte and gill flap culture media and techniques is still needed before commercial *in vitro* amebocyte production can occur.

With a variety of culture media and conditions used individually for previous amebocyte culture studies, an optimal medium for amebocyte culture has not been developed. This study's objectives were to test horseshoe crab serum supplementation and to test a variety of standard insect cell culture media for their effects on amebocyte morphology and viability; and to suggest the most appropriate medium a base medium for further optimization.

METHODS

Animals

Adult horseshoe crabs were obtained from Cambrex Bio Science Walkersville, Inc. (Cambrex), a commercial biomedical company, using a standard trawling procedure off the coast of Ocean City, Maryland (Hata and Berkson 2003). The animals were maintained in a

recirculating aquaculture system at the Horseshoe Crab Research Center at Virginia Tech University, Blacksburg, Virginia. The crabs were maintained under appropriate environmental conditions (Brown and Clapper 1981), with salinity at 34 ± 1‰ and temperature at 21 ± 1°C.

Collection of hemolymph

Hemolymph samples from two adult male intermoult horseshoe crabs were used. Hemolymph from one crab was used the serum effects culture and hemolymph from the other crab was used for the multiple media culture. The specimens were bled via cardiac sinus puncture. Their exposed arthrodial membranes were swabbed with a 70% ethanol-soaked cotton swab to disinfect the surface. An 18-gauge needle was inserted through the membrane into the cardiac sinus to extract the needed amount of hemolymph into a syringe. The syringe then was sprayed with 70% ethanol to disinfect the surface and moved into a laminar flow cabinet. The used needle was replaced with a new sterile needle, which was then ready for dispensing the whole hemolymph into the culture media and microcentrifuge tubes. Amebocytes were seeded into the culture media within 5 minutes after harvest.

Culture media

The following commercially-available cell culture media were tested for their ability to maintain viable *L. polyphemus* hemocytes *in vitro*: Leibovitz medium (L-15), L-15 double-strength (2x), Grace's modified insect medium – without lactalbumin hydrolysate, yeastolate, gentamicin, and serum (Grace's modified), Grace's insect medium – without serum (Grace's w/o serum), IPL-41 insect medium (IPL-41), and Insect-Xpress – protein-free culture medium. All culture media were obtained from Cambrex (Walkersville, MD). Of the six media tested, three had been used in previous amebocyte culture studies (Pearson and Woodland 1979, Ding et al. 1988, Gibson and Hilly 1992).

Amebocyte cultures

To test the effects of horseshoe crab serum on amebocytes *in vitro*, L-15 (2x) culture medium was selected, as it had been used previously for amebocyte culture (Joshi et al. 2002) and is not supplemented with any type of commercial sera. For this culture, six pyrogen-free microcentrifuge tubes were each filled with 100 µl of horseshoe crab hemolymph. These then were centrifuged at 150 x g for three minutes. With three of the tubes, the serum was removed and the cells were resuspended in 1000 µl of medium. These samples represented the serum-free cultures. In the remaining eppendorfs, the serum was not removed and 900 µl of medium was added to resuspend the pellet of cells in the culture medium now supplemented with horseshoe crab hemolymph. These samples underwent centrifugation in order to handle the amebocytes in a consistent manner. This lot of eppendorfs represented the 10% v/v hemolymph serum-supplemented cultures. The contents of these eppendorfs were transferred to individual wells in a pyrogen-free 12-well plate (B-D Falcon, Fisher Scientific). The plate was then incubated at 21°C in air and in the dark. Cultures were assessed using morphological observations and cell viability counts on day 7 after inoculation. This study duration was chosen to observe any significant short-term changes in amebocyte morphology and viability.

To test the effects of the different media on amebocyte maintenance *in vitro*, six culture media that were previously described were tested. Pyrogen-free 12-well culture plates (B-D Falcon, Fisher Scientific) were used to test each medium in triplicate. Wells were filled with 950 µl of the designated medium and inoculated with 50 µl of whole *Limulus* hemolymph. In this way, media were supplemented with 5% v/v horseshoe crab hemolymph. The plates were then incubated at 21°C in air and in the dark. Cultures were assessed using morphological observations and cell viability counts on day 7 after inoculation.

Morphology

Amebocytes were observed *in vitro* using an inverted microscope at 400x magnification. Cells were photographed in culture using an Olympus C-3000 digital camera fitted to the inverted microscope's camera port.

Amebocyte viability

Some of the cultures formed amebocyte monolayers, resulting in treatment with trypsin to dissociate the cells. Stock trypsin-EDTA (10x) was diluted to 1x using Phosphate Buffered Saline (without calcium or magnesium) (Mitsuhashi 2002). Culture medium was aspirated and replaced with the diluted 1x trypsin-EDTA solution. After 15 minutes of incubation, cells were suspended by pipetting. Viability of these cells was then assessed using the trypan blue dye-exclusion method (Mitsuhashi 2002). The cell suspension and 0.4% trypan blue solution were mixed in a volume ratio of 1:1. This mixture was loaded into a Neubauer type hemocytometer (Hausser Scientific, Horsham, PA) where stained and unstained amebocytes were counted.

Statistical analyses

Data were processed with the aid of SAS (Statistical Analysis System, Version 8) using a significance level of $\alpha = 0.05$. Amebocyte viability was compared between serum-free and 10% serum supplemented cultures using Wilcoxon rank sum test. To test for differences in amebocyte viability between the six selected culture media, normality of the data was checked using the Shapiro-Wilk Test. Subsequently, a square root transformation was applied to the data, which then were analyzed by performing an ANOVA for a completely randomized design. Tukey's Studentized Range Test was used to make pairwise comparisons among the six population means.

RESULTS AND DISCUSSION

In the artificial environment of *in vitro* culture systems, most cells will be rounded when grown in suspension and flattened when grown on a surface such as tissue culture plastic (Mather and Roberts 1998). In mammalian cell culture, the detachment and rounding-up of cells is indicative of morphological deterioration (Freshney 1987). However, some cells may show increased function when they are allowed to maintain a rounded shape (Mather and Roberts 1998). For example, cultured hemocytes from the hard clam *Meretrix lusoria* and the colonial protochordate *Botryllus schlosseri* did not adhere to the substrate and even multiplied in suspension (Mitsuhashi 2002).

In my study, three main forms of amebocytes were observed:

1. contracted (C) – relatively spherical cells with little attachment to the surface,
2. granular flattened (GF) – partially flattened cells attached to the surface with refractive granules present, and
3. degranulated flattened (DGF) – completely flattened cells attached to the surface without granules present.

It was also noted that cultures with greater proportions of observed contracted cells also had greater viability than cultures filled with primarily degranulated flattened cells, suggesting that the contracted amebocyte morphology is the most desired form *in vitro*. This inference regarding cell morphology is supported by Chen et al. (1986), who documented a progression of amebocyte morphology *in vitro*. They observed amebocytes initially in the contracted form, which after a period of hours or days in culture flattened on the substratum. The flattening of the cells was reversible, but the cells flattened irreversibly once degranulation occurred. In addition, there were many vacuoles, instead of granules, in cells after spontaneous degranulation (Chen et al. 1986). In other cell types, vacuolation is often a sign of morphological deterioration

(Freshney 1987). These observations provided the criteria used in assessing the success of amebocyte cultures on a morphological basis. However, morphological evaluation of amebocytes was coupled with cell viability results for an overall assessment of how successful a culture was.

In testing the effects of horseshoe crab serum on amebocyte morphology *in vitro*, distinct differences between serum-free and 10% serum-supplemented cultures were observed. Amebocytes in the serum-free culture appeared to be smaller than cells in the serum-supplemented culture, with few granulated cells in the field of view (Figure 4.1*a*). Few contracted cell forms were observed, with the majority of cells either displaying granular flattened or degranulated flattened forms (Figure 4.1*a*). Amebocytes in the serum-supplemented culture appeared to be mostly in the contracted or granular flattened forms, with granules visible in those cells (Figure 4.1*b*).

Analysis of amebocyte viability between serum-free and horseshoe crab serum-supplemented media indicated a significant difference between the two treatments ($N = 6$; $P = 0.0147$). After 7 days, cell viability was 4.0 ± 4.6% (mean ± S.D.) in serum-free L-15 (2x) and 35.8 ± 9.3% in 10% v/v serum-supplemented L-15 (2x) medium (Figure 4.2). These observations, supported by those of Chen et al. (1986), indicate that supplementation with horseshoe crab serum is necessary for maintaining the amebocyte's contracted form and cell viability (Figure 4.1*b*; Figure 4.2).

In testing the effects of the six different media on amebocyte maintenance *in vitro,* different frequencies of amebocyte morphology were observed among the media by day 7. The L-15 medium contained very few contracted cells, with the majority of cells in the degranulated flattened form (Figure 4.3*a*). More contracted and granular flattened cells were observed in the L-15 (2x) medium. However, there were cells that were vacuolated, and the majority of cells in

the field of view were in the degranulated, flattened form (Figure 4.3*b*). Grace's modified medium had a number of cells in either the contracted or granular flattened states, but vacuolated and degranulated flattened cells were observed (Figure 4.3*c*). Grace's medium without serum had a large number of cells in the contracted form, with no observed degranulation by day 7 (Figure 4.3*d*). The IPL-41 medium contained few contracted and granular flattened cells, with the rest of the amebocytes in degranulated flattened form (Figure 4.3*e*). No contracted cells were observed in the Insect-Xpress medium. In this medium, some granular flattened cells were seen; however, the majority of amebocytes were in degranulated, flattened form (Figure 4.3*f*).

In the six culture media, amebocyte viability was 77.2 ± 5.1% in Grace's (w/o serum), 35.1 ± 8.7% in Grace's modified, 15.2 ± 5.7% in L-15 (2x), 13.3 ± 4.9% in IPL-41, 10.3 ± 2.4% in L-15, and 1.1 ± 1.9% in Insect-Xpress (Figure 4.4). Cell viability data from the six insect cell culture media deviated significantly from a normal distribution ($P = 0.001$). The square root-transformed data met the normality criteria. The ANOVA results indicated that at least one of the treatment means was different from the others ($N = 36$; $P < 0.0001$). Tukey's Studentized Range Test showed significant differences in cell viability between Grace's (w/o serum), Grace's modified, and Insect-Xpress. The L-15, L-15 (2x), and IPL-41 media did not exhibit significant differences in cell viability, but were grouped together as differing significantly from the other three media.

Among several standard insect cell culture media, one medium in particular provided results distinctive from those of all other media tested. Grace's (w/o serum) maintained amebocytes that retained their contracted form and did not appear to spontaneously degranulate for the duration of the culture (Figure 4.3*d*). Compared to all other cultures tested, Grace's (w/o serum) medium contained amebocytes that exhibited very light attachment to the substrate, which in other cell types is a sign of morphological deterioration. Yet, cell viability in this

culture was considerably higher than that in other culture media (Figure 4.4), lending support to our morphological observations. With Grace's (w/o serum) medium outperforming all other media tested, it is recommend that this medium, supplemented with horseshoe crab serum, be used for future amebocyte culture and further media optimization.

Cells in both of the Grace's insect media surpassed those in other media in morphological quality and viability, thereby warranting further work with media composition. These two media have higher levels of calcium, potassium, and magnesium salts relative to the other media tested (Table 4.1), bringing the medium closer to being isotonic to horseshoe crab hemolymph, which has 12.5 mEq/L of potassium, 39.0 mg/dL of calcium and 96 mg/dL of magnesium (S. Smith, Virginia Polytechnic Institute and State University, pers. comm.). The dramatic improvement in amebocyte morphology and viability in Grace's (w/o serum) relative to Grace's modified suggests a critical component is present in Grace's (w/o serum). The difference may be due to the presence of lactalbumin hydrolysate, yeastolate, and/or an increased level of magnesium salts (Table 4.1). This is contradictory to the results of Pearson and Woodland's (1979) study, where there were no improvements with lactalbumin hydrolysate and inhibitory effects from yeastolate. These observations provide a starting point for medium modification, which generally renders the medium more isotonic to the cell's *in vivo* environment. Even without much modification, Grace's (w/o serum) supplemented with horseshoe crab serum could improve amebocyte culture and harvest duration with Joshi et al.'s (2002) culture technique. Optimization of the culture medium would likely further enhance the results they obtained.

An optimized amebocyte culture medium would contribute to further exploration and improvement of amebocyte culture techniques. Future research can progress in two main directions. Transforming the amebocytes would be an approach to establish a permanent amebocyte cell line for lysate production. Some positive results have been reported on this

technical option (Ding et al. 1988). This would allow relatively easy scale-up for commercial production. The other direction of research is to culture amebocytes from hematopoietic tissue. The gills were originally identified as a center for amebocyte production (Gibson and Hilly 1992). More recently, hemocytopoiesis was reported to occur in connective tissues adjacent to areas of gametogenesis (Hong et al. 2000).

Hence, there are numerous avenues of approach to culturing *Limulus* amebocytes for LAL. This study recommended a culture medium suitable for optimization to *Limulus* amebocyte requirements, contributing to the progress of amebocyte culture. The appropriate medium and culture methods likely will be established with continued efforts on this topic. Amebocyte culture has potential for commercial production of LAL. In this way, *in vitro* production of LAL would drastically reduce the need to harvest horseshoe crabs.

ACKNOWLEDGEMENTS

This work is a result of research sponsored in part by the NOAA Office of Sea Grant, US Dept of Commerce, under Grant No. NA96RG0025 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. Cambrex Bio Science Walkersville, Inc. generously supplied me with horseshoe crabs and cell culture media for the study. I wish to thank Dr. Stephen Smith of the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech for the use of his laboratory. I also thank Dr. Eric Hallerman of the Department of Fisheries and Wildlife Sciences of Virginia Tech for his helpful comments throughout this project. In addition, I thank Xin Zhong and Li Wang of the Department of Statistics at Virginia Polytechnic Institute and State University for their statistical consulting.

REFERENCES

Armstrong, P.B. 1985. Adhesion and motility of the blood cells of *Limulus*. pp. 77-124 *In:* Cohen, E., (ed). Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology. Alan R. Liss, Inc., New York.

ASMFC. 2001. Fishery management report No. 32b - Addendum II to the interstate fishery management plan for horseshoe crab, 5 p. Atlantic States Marine Fisheries Commission, Washington, D.C.

Brown, G.G. and D. Clapper. 1981. Procedures for maintaining adults, collecting gametes, and culturing embryos and juveniles of the horseshoe crab, *Limulus polyphemus*. *In:* Laboratory Animal Management: Marine Invertebrates p. 268-290. National Academy Press, Washington, D.C.

Cambrex. 2003a. BioWhittaker™ PyroGene™ Recombinant Factor C Endotoxin Detection System. Available online at http://www.cambrex.com/catalognews/PyroG_insert_final_Mar18.pdf. Accessed 12/14/03.

Cambrex. 2003b. Introducing PyroGene™ Recombinant Factor C Assay. Available online at http://www.cambrex.com/catalognews/PyroGene%20Flyer%20.pdf. Accessed 12/14/03.

Chen, I.-J., Y.-M. Chen, S.-J. Hong and L.-C. Chiang. 1986. Morphological changes of horseshoe crab amebocytes during *in vitro* cultivation. *Kaohsiung Journal of Medical Sciences* 2:769-773.

Chen, I.-J., S.-J. Hong, Y.-M. Chen and Y.-C. Yang. 1989. Cultivation of horseshoe crab amebocytes. *Kaohsiung Journal of Medical Sciences* 5:515-521.

Copeland, E. and J. Levin. 1985. The fine structure of the amebocyte in the blood of *Limulus polyphemus*. I. Morphology of the normal cell. *Biological Bulletin* 169:449-457.

Ding, J.L. and B. Ho. 2001. A new era in pyrogen testing. *Trends in Biotechnology* 19(8):277-281.

Ding, J.L., J.C. Kim and B. Ho. 1988. Pokeweed mitogen stimulates DNA synthesis in cultured amoebocytes of *Carcinoscorpius rotundicauda*. *Cytobios* 55:147-154.

Freshney, I.R. 1987. Culture of animal cells: a manual of basic technique. Alan R. Liss, Inc., New York. 397.

Frieberg, J.A., P.J. Weathers and D.G. Gibson, III. 1991. Culture of amebocytes in a nutrient mist bioreactor. *In Vitro Cell. Dev. Biol.* 28A:215-217.

Gibson, D.G., III and J.B. Hilly. 1992. Patent No. 5,082,782: Production of horseshoe crab amebocytes *in vitro*. US Patent & Trademark Office, Washington, D.C.

Hata, D. and J.M. Berkson. 2003. Abundance of horseshoe crabs, *Limulus polyphemus*, in the Delaware Bay area. *Fishery Bulletin* 101:933-938.

HCTC. 1998. Status of the Horseshoe crab (*Limulus polyphemus*) population off the Atlantic coast, 48 p. Horseshoe Crab Technical Committee. Atlantic States Marine Fisheries Commission, Washington, D.C.

Hong, S.-G., Q. Huang and Z.-M. Ni. 2000. Ultrastructural observations on haemocytopoiesis in adult horseshoe crab (*Tachypleus tridentatus*). *Acta Zoologica Sinica* 46(1):1-7.

Joshi, B., A. Chatterji and R. Bhonde. 2002. Long-term *in vitro* generation of amoebocytes from the Indian horseshoe crab *Tachypleus gigas* (Müller). *In Vitro Cell. Dev. Biol. - Animal* 38:255-257.

Kurz, W. and M.J. James-Pirri. 2002. The impact of biomedical bleeding on horseshoe crab, *Limulus polyphemus*, movement patterns on Cape Cod, Massachusetts. *Marine and Freshwater Behaviour and Physiology* 35:261-268.

Lakshmanan, R. and K. Venkateshvaran. 1999. Biomedical potentials of the Indian horseshoe crab. *INFOFISH International* 1:46-48.

Levin, J. and F.B. Bang. 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns Hopkins Hosp.* 115:265.

Mather, J.P. and P.E. Roberts. 1998. Introduction to cell and tissue culture. Plenum Publishing, New York. 241p.

Mikkelsen, T. 1988. The secret in the blue blood. Science Press, Beijing, China. 125p.

Mitsuhashi, J. 2002. Invertebrate cell culture methods. Springer, New York. 446p.

Novitsky, T.J. 1991. Discovery to commercialization: the blood of the horseshoe crab. *Oceanus* 27(1):13-18.

Pearson, F.C. 1980. Patent No. 4,229,541: *In vitro* cultivation of horseshoe crab amebocytes. US Patent & Trademark Office, Washington, D.C.

Pearson, F.C. and E. Woodland. 1979. The *in vitro* cultivation of *Limulus* amebocytes. p. 93-102 *In*: Cohen, E. (ed). Biomedical Applications of the Horseshoe Crab (Limulidae). Alan R. Liss, Inc., New York.

Rudloe, A. 1983. The effect of heavy bleeding on mortality of the horseshoe crab, *Limulus polyphemus*, in the natural environment. *Journal of Invertebrate Pathology* 42:167-176.

Schrading, E., T.J. O'Connell, S. Michels and P. Perra. 1998. Interstate management plan for horseshoe crab. Atlantic States Marine Fisheries Commission, Washington, D.C.

Sherman, R.G. 1981. Chelicerates. pp. 355-384 *In:* Ratcliffe, N.A. and A.F. Rowley, (eds). Invertebrate Blood Cells. Academic Press, New York.

Suhr-Jessen, P.L. Baek and P.P. Jakobsen. 1989. Microscopical, biochemical, and immunological studies of the immune defense system of the horseshoe crab, *Limulus polyphemus*. *Biological Bulletin* 176:290-300.

Thompson, M. 1998. Assessments of the population biology and critical habitat for the horseshoe crab, *Limulus polyphemus*, in the South Atlantic Bight. M.S. Thesis. University of Charleston, Charleston, South Carolina.

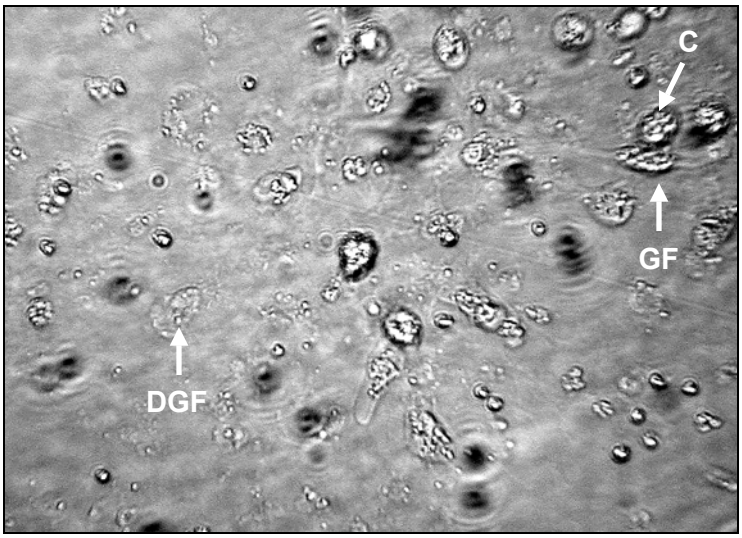
Walls, B.A. and J.M. Berkson. 2003. Blood extraction effects on horseshoe crabs (*Limulus polyphemus*). *Fishery Bulletin* 101:457-459.

Table 4.1. Comparison of media components and chemistries. Note the differences in components present in substantially higher or lower quantities in Grace's (w/o serum) than in the other tested culture media.

Media Component/Chemistry	Media					
	L-15 [1]	L-15 (2x) [1]	Grace's Modified [1]	Grace's (w/o serum) [1]	IPL-41 [1]	Insect-Xpress [1]
Inorganic Salts (mg/L)						
$CaCl_2$	0	0	0	0	500	n/a
$CaCl_2 \cdot 2H_2O$	140	280	993	993	0	n/a
KCl	400	800	4,100	4,100	1,200	n/a
Mg based salts	191	383	3,638	5,060	918	n/a
NaCl	8,000	16,000	0	0	1,160	n/a
$NaH_2PO_4 \cdot H_2O$	0	0	1,008	1,008	0	n/a
Other Components (mg/L)						
Lactalbumin Hydrolysate	0	0	0	3,330	0	n/a
Sucrose	0	0	26,680	26,680	1,650	n/a
Yeastolate	0	0	0	3,330	0	n/a
Chemistries						
pH	7.67	7.52	6.00	5.17	6.15	6.20
Osmolality (mOsm)	324	644	353	407	325	364

[1] Cambrex (Walkersville, MD)

4.1(*a*)



4.1(*b*)

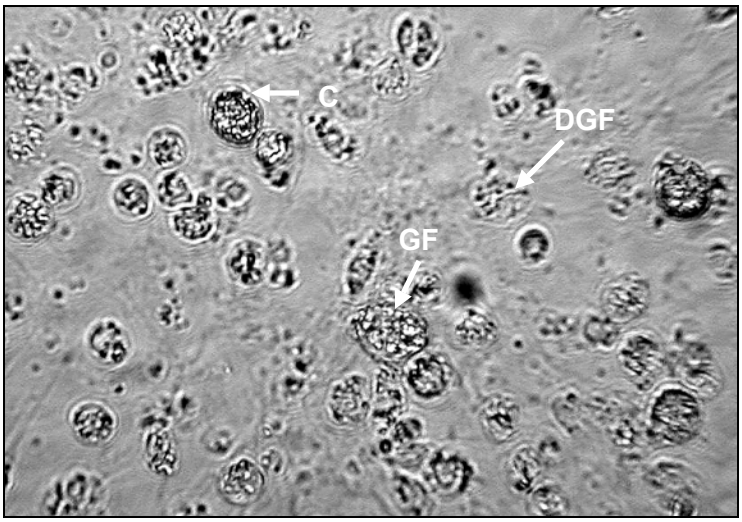


Figure 4.1 (*a-b*). Comparison of *Limulus* amebocytes maintained for seven days in the presence or absence of horseshoe crab serum. x400. Figure 4.1*a*. Cells maintained in L-15 (2x) medium. Figure 4.1*b*. Cells maintained in L-15 (2x) medium supplemented with 10% v/v horseshoe crab serum. C: contracted form, GF: granular flattened form, DGF: degranulated flattened form.

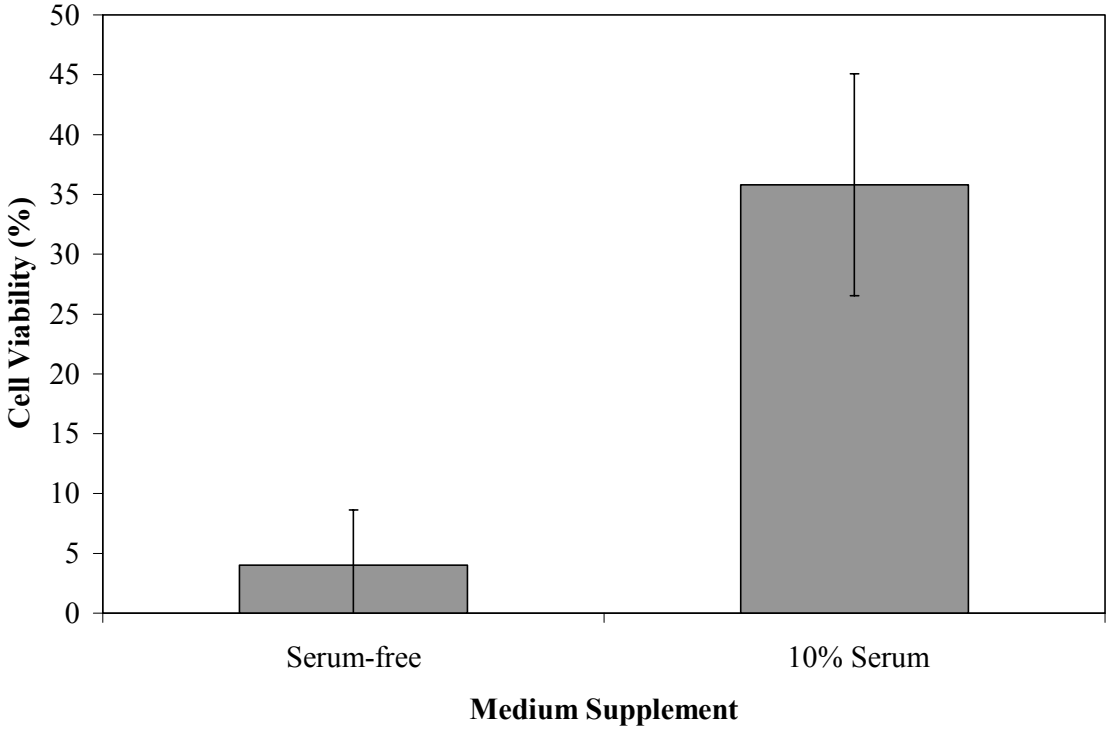


Figure 4.2. Effects of horseshoe crab serum supplementation in L-15 (2x) medium on amebocyte viability. Viability was quantified on day 7 of culture. Values are means ± S.D.

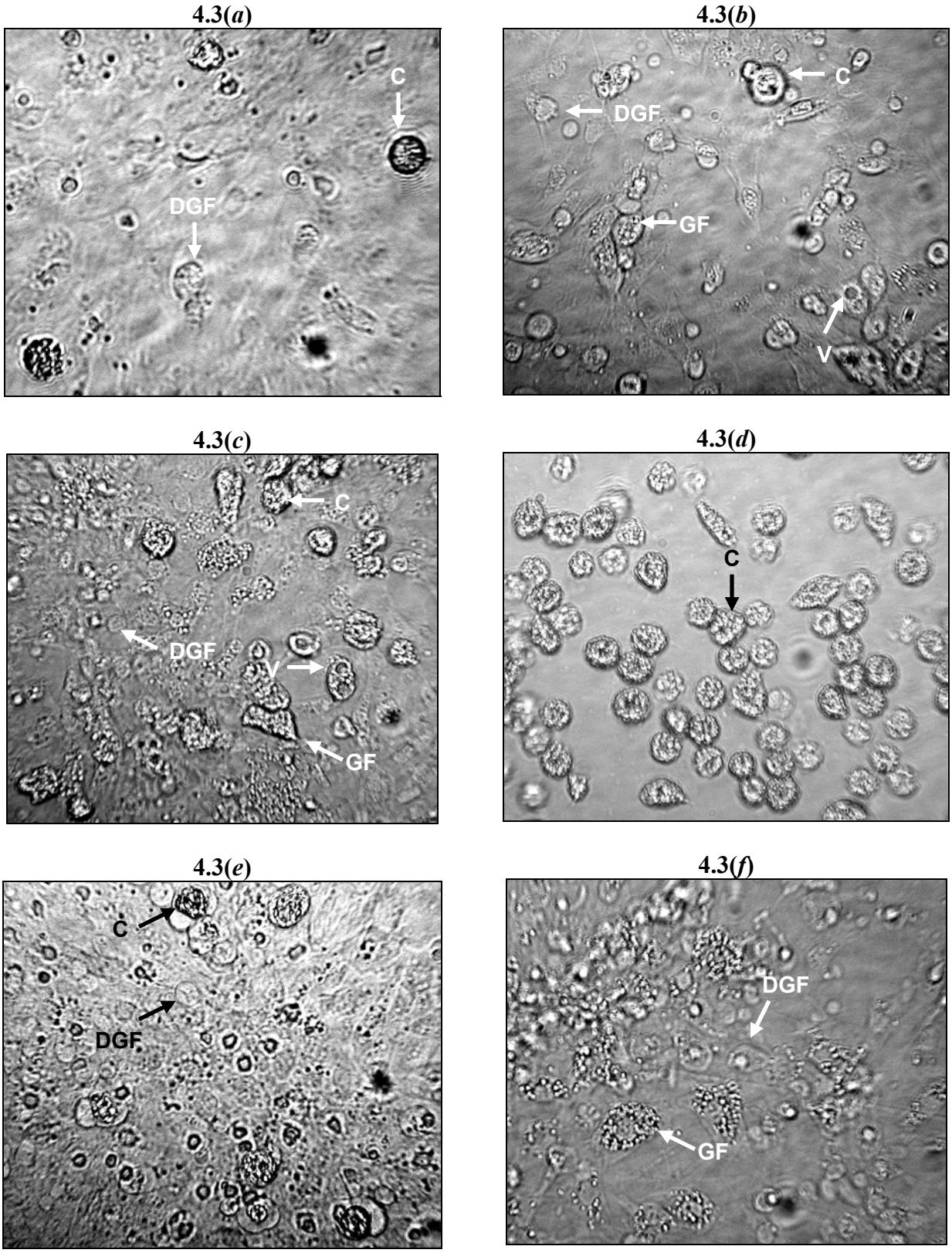


Figure 4.3 (*a-f*). Comparison of *Limulus* amebocytes maintained for seven days in various media supplemented with 5% v/v horseshoe crab serum. x360. Figure 4.3*a*. L-15. Figure 4.3*b*. L-15 (2x). Figure 4.3*c*. Grace's modified. Figure 4.3*d*. Grace's (w/o serum). Figure 4.3*e*. IPL-41. Figure 4.3*f*. Insect-Xpress. C: contracted form, GF: granular flattened form, DGF: degranulated flattened form, V: vacuole.

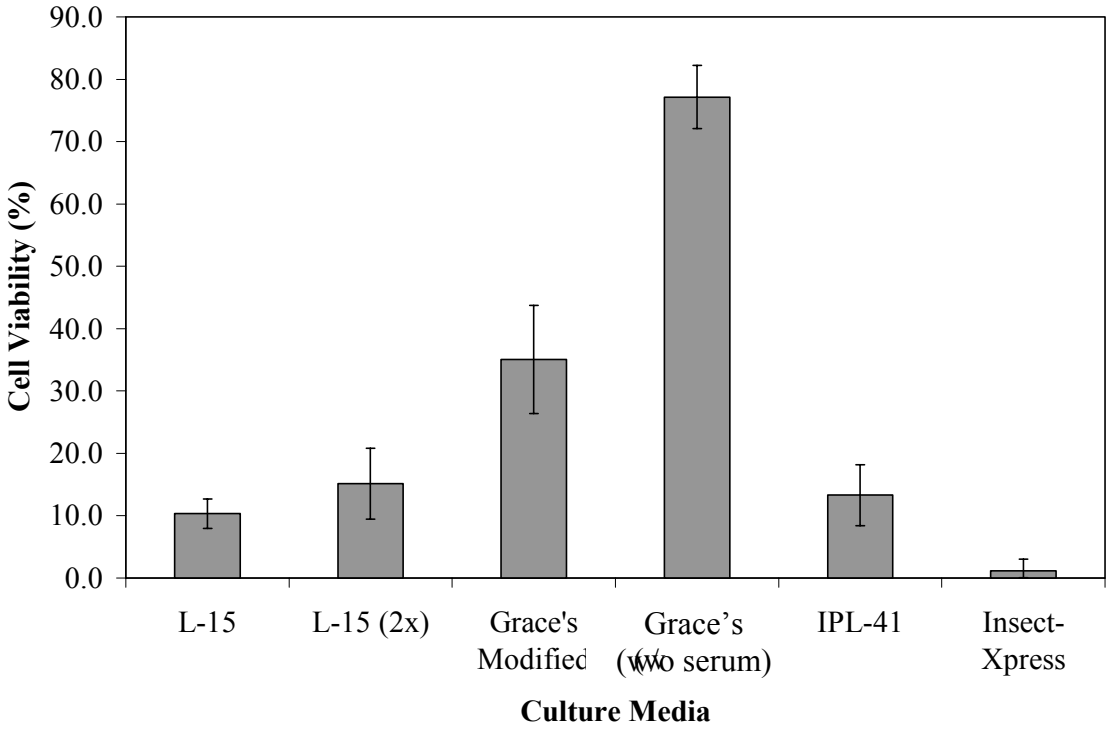


Figure 4.4. Effects of various cell culture media on amebocyte viability. Cell viability was determined on day 7 of culture. Values are means ± S.D.

APPENDICES

APPENDIX 1 – PRELIMINARY INULIN DOSAGE STUDY

I conducted a preliminary investigation of inulin dosage in adult horseshoe crabs. My goal was to identify an inulin dosage that would yield a detectable absorbance value once hemolymph samples were processed, but would also have a manageable injection volume. Robertson (1970) injected inulin into horseshoe crabs at a dosage of 88 mg inulin per 100 g of wet body weight for larger juveniles. However, using a recommended working solution of 30 mg/ml of inulin (J. Shields, Virginia Institute of Marine Science, pers. comm.), the injection volume needed for an adult would be very large at Robertson's (1970) dosage (i.e., 29 ml inulin solution for a 1 kg adult).

Six horseshoe crabs, 2 males and 4 females, were selected and equally divided into either a 100 or 200 mg/ kg wet body weight dosage group. The particular inulin dosage was administered to the animals and allowed to circulate for one hour. Hemolymph samples were processed using Shields' (Virginia Institute of Marine Science, pers. comm.) protocol and the absorbance at 520 nm was read.

The 200 mg/kg dosage yielded absorbance values nearly double those of the 100 mg/kg dosage, and had a manageable injection volume of 6.7 ml per 1 kg of wet body weight. Hence, a dosage of 200 mg inulin per 1 kg wet body weight was concluded to be appropriate for use in this study.

APPENDIX 2 – PRELIMINARY INULIN DISTRIBUTION STUDY

It was necessary to identify an appropriate circulation time for the inulin in order to allow complete mixing with the hemolymph. Five horseshoe crabs were arbitrarily selected from the water where they were held at temperature of 21.5°C. Using a 30 mg/ml working solution, the animals were injected with inulin solution based on a 200 mg/ml per 1 kg wet body weight dosage. Hemolymph samples were collected at 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 hours after the injection. Samples were placed in the refrigerator until they were processed using the methods of Roe et al. (1949). Absorbance was read at 520 nm, and time dilution curves of hemolymph inulin concentrations were constructed (Figure A2.1). Absorbance readings leveled off by hour 6, indicating that inulin distribution had taken place by this time. Absorbance readings after this time indicated a very slow decline over the following 6 hours. This was attributed to excretion of inulin. Therefore, it was concluded that an appropriate inulin circulation time was at least 6 hours at a holding temperature of 21.5°C.

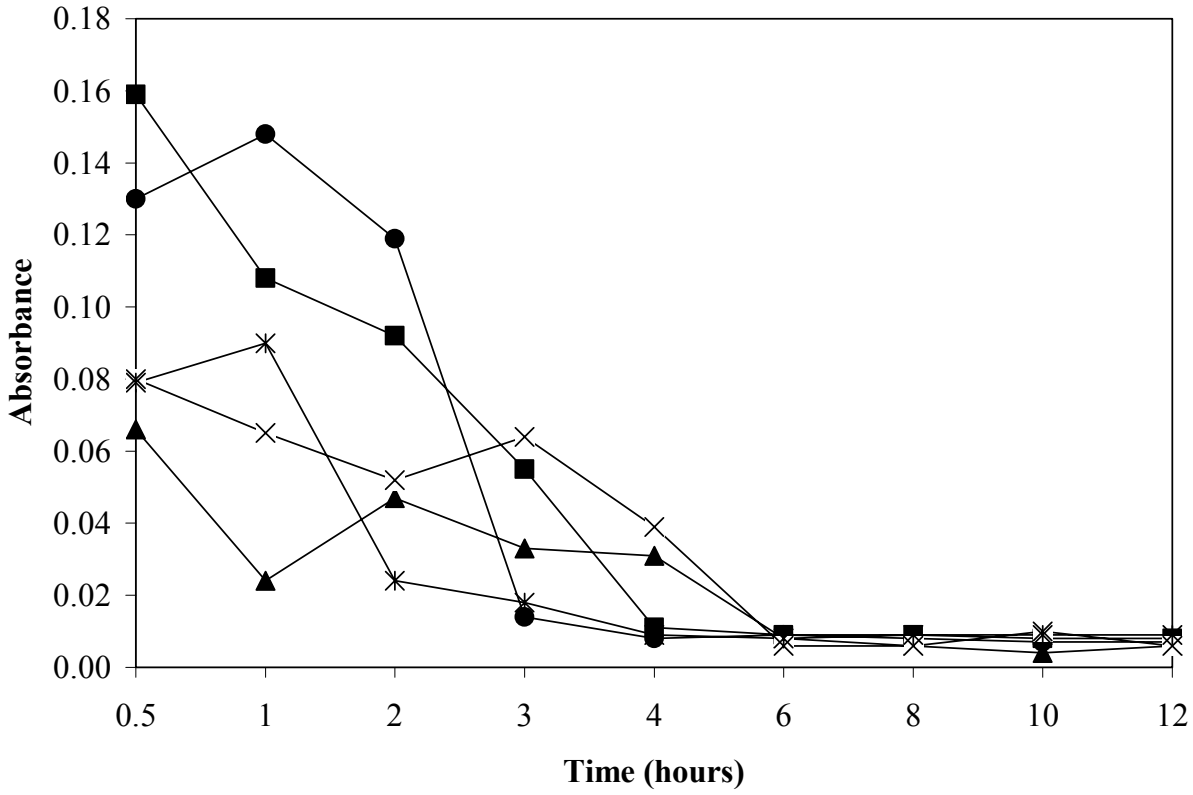


Figure A2.1. Time dilution curves of hemolymph inulin concentrations in five horseshoe crabs.

APPENDIX 3 – STANDARD CURVE EVALUATION

Evaluation of samples with the spectrophotometer took approximately one hour. During this time the blanks, standard, and standard curve dilutions were read every 15 minutes (i.e., 0, 15, 30, 45, and 60 minutes) to monitor any color development or fading. Therefore, five standard curves were constructed corresponding to the five times at which the standard curve dilutions were read. Inulin concentrations from the 60 hemolymph samples were derived from each of the five standard curves. These concentrations were evaluated for significant changes over time using Repeated Measures testing with an autoregressive covariance structure (Ott and Longnecker 2001). This test determined whether it was necessary for absorbance values from the hemolymph samples to correspond to a specific standard curve based on the time the hemolymph sample was read.

A change in color intensity was observed in the standard curve during the one-hour time period in which all samples were processed through the spectrophotometer. The Repeated Measures test showed significant differences in inulin concentrations over time, or between the five standard curves ($N = 60$; $F = 427.08$; $P<0.001$). Because of this, it was necessary to match the hemolymph absorbance values to the standard curve representing the closest time at which the hemolymph sample was read, allowing for the most accurate inulin concentration determination.

APPENDIX 4 – MAXIMUM BLOOD VOLUME AVAILABLE FOR EXTRACTION

The hemolymph volume equations established in Chapter 2 estimate total hemolymph volume in *Limulus*. However, horseshoe crabs have an open circulatory system, enabling a significant amount of blood to remain in the body within the muscle, tissue spaces, and sinuses. Therefore, it is not possible for 100% of the predicted blood volume to be extracted. To establish the bleeding mortality study's bleeding treatments, it was necessary to estimate the maximum amount of blood that could be withdrawn.

For this preliminary study, 3 male and 3 female horseshoe crabs were selected. Each animal was taken from the holding tank and placed in a bleeding rack. An 18-gauge needle was inserted through the membrane into the cardiac sinus to extract as much blood as possible. Once no more blood could be withdrawn, a 30-minute rest period was given to allow the cardiac sinus to refill with blood. After this, a second blood extraction took place until no more blood was withdrawn and then the animals were put back in their holding tanks.

The maximum amount of blood extracted was 50 ± 13% (mean ± S.D.) of the predicted blood volume, and ranged from 39 – 67% (Table A4.1). This variability could be due to the amount of muscle and tissue space in the animal as it would retain hemolymph within extra-cellular spaces. In females, it may also be due to gravidity. It was necessary to set bleeding levels that would have a high likelihood of being withdrawn from the bleeding mortality study subjects. Therefore, 40% was selected as the maximum bleeding level.

In addition, it was observed that no mortality occurred within one week of the bleeding. This most likely was due to the recuperative effects of being placed back in the water immediately after blood withdrawal. Exposure to their aquatic environment allowed the animals to uptake fluid and begin recovering blood volume they had lost. Returning them immediately to the water likely counteracted physiological effects from the blood extraction. As a result, it was deemed necessary to keep the test animals out of water for a period of time after bleeding during the mortality study.

Table A4.1. Maximum amounts of blood extracted from horseshoe crabs compared with the horseshoe crab's predicted blood volumes calculated using the inter-ocular width equation (Chapter 2).

Sex	IO (cm)	Max Blood Extracted (ml)	Predicted Blood $Volume_{IO}$ (ml)	% Blood Volume Extracted
F	15.5	205	510	40
F	16.0	378	562	67
F	17.0	296	681	43
M	11.5	91	236	39
M	12.0	116	260	45
M	12.0	174	260	67

VITA

Lenka Hurton was born in Victoria, British Columbia, Canada in 1978. She attended high school at Windsor Secondary School in North Vancouver, British Columbia. She was awarded a full athletic scholarship to attended college at Fairfield University in Fairfield, Connecticut. There, she received a B.S. in Biology with minors in Environmental Science and Marine Science in May of 2000. After graduation, she worked on Long Island for a year at Cold Spring Harbor Laboratory in a proteomics lab. She then began work on her thesis research at the Horseshoe Crab Research Center in June 2001. In December of 2003, she completed her M.S. in Fisheries and Wildlife Sciences in the Department of Fisheries and Wildlife Sciences at Virginia Polytechnic Institute and State University in Blacksburg, Virginia.

Lenka V. Hurton