

Estimation of total hemolymph volume in the horseshoe crab *Limulus polyphemus*

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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 for this species is not known. The hemolymph volume of 60 adult horseshoe crabs was estimated using an inulin dilution technique. Blood volume of the horseshoe crab represented as a percentage of wet body weight was $25 \pm 2.2\%$ for males and $25 \pm 5.1\%$ (mean \pm SD) for females. Relationships between hemolymph volume and weight ($p = 0.0026$, $r^2 = 0.8762$), hemolymph volume and prosomal width ($p < 0.0001$), and hemolymph volume and inter-ocular width ($p < 0.0001$) were observed. No significant differences were observed between males and females. The relationship of animal size and hemolymph volume can be used to predict how much blood can be drawn from horseshoe crabs used by the biomedical industry, and can be of further use in future bleeding mortality studies.

Keywords: Extracellular fluid volume, hemolymph volume, horseshoe crab, inulin, Limulus

Introduction

The horseshoe crab *Limulus polyphemus* is a multiple-use living marine resource that has become the center of controversy among user groups (Berkson & 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 to the ocean within 72 h of capture (HCTC 1998). Mortality rates resulting from the bleeding process are reported to be as high as 20% (Kurz & James-Pirri 2002; Rudloe 1983; Thompson 1998; Walls & Berkson 2003). Amounts of blood extracted from an individual animal ranged from 100 to 300 mL (Rudloe 1983; Walls 2001), while the maximum available blood volume was believed to be 200–300 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 (Roe et al. 1949; Ruhl et al. 1995; Steinitz 1938). Inulin has been used in numerous blood volume studies on other species, such as rock lobster (*Panulirus longpipes*) (Dall 1974), crab (*Carcinus maenas*) (Harris & Andrews 1982) and snail (*Littorina littorea*) (Jones & Kamel 1984).

To date, two estimates of blood volume exist for juvenile horseshoe crabs (Robertson 1970; Shuster 1978), however Robertson's work (1970) was the only study using inulin estimated extracellular volume, or total hemolymph volume, in horseshoe crabs. One other report on hemolymph volume estimated the total blood volume of one 3 kg female from Delaware Bay to be 300 mL (Shuster 1982). As there is relatively little information on blood volume of horseshoe crabs, a survey across a representative size range of adult *Limulus* was examined to estimate the relationship of total hemolymph volume and body size.

Methods

Specimens 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 and 30‰ and water temperature between 21 and 23°C. We chose salinities and temperatures that are within the normal range of tolerance of the horseshoe crab and are acceptable for maintaining test animals in good condition (Brown & Clapper 1981; Bullis 1981). They were allowed to acclimate for two weeks, during which they were tagged, weighed (kg), sexed, and measured. Measurement consisted of recording the inter-ocular (*IO*) width (cm), which is the distance between eye-slits of the horseshoe crabs' compound eyes, and the prosomal (*P*) width (cm), which is the distance across the crab's carapace.

Sixty specimens, 30 males and 30 females, ranging from 0.90 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, personal communication, 2002). 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 (Hurton 2003). Based on this dosage, a portion of the working solution was injected through the arthroal membrane into the cardiac sinus. Preliminary experiments also showed that full mixing was achieved within 6 h at a 21.5°C holding temperature (Hurton 2003). After 7 h, 1 mL of hemolymph was withdrawn from the cardiac sinus to provide enough hemolymph for one sample per test animal ($n=60$). The samples were centrifuged at 10,000 *g* to isolate the cell-free hemolymph. Samples then underwent a colorimetric assay (Roe et al. 1949; J. Shields, Virginia Institute of Marine Science, personal communication, 2002) and the absorbance, at 520 nm wavelength, was measured. The spectrophotometer was calibrated before analysis using a blank and checked thereafter with the blank every 15 samples to ensure the machine's absorbance readings did not drift.

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

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

where V = blood volume, d = injected volume, c_1 = injected concentration of inulin, and c_2 = final concentration of inulin. This equation is based on the standard equation for dilution with one additional term ($-d$), which corrects for the additional fluid volume added to the animal's hemolymph when injecting the inulin solution.

Data were analyzed with the aid of SAS (Statistical Analysis System, Version 8, 1999) 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 (males: 0; females: 1) 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* and *IO* widths were each regressed with the blood volume data by a non-linear function and 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:

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

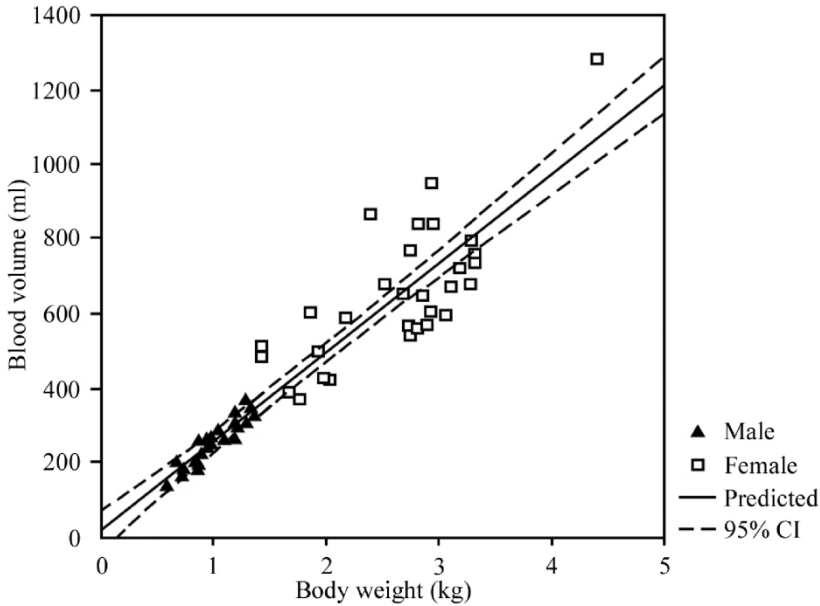


Figure 1. The linear regression of wet body weight against estimated hemolymph volume ($n=60$, $r^2=0.8762$, $p=0.0026$) is characterized by the equation $H=-5.7+92s+257.2w-41ws$, where H =hemolymph volume (mL), s =sex (males: 0; females: 1), and w =wet body weight (kg). The population mean is enclosed by a 95% confidence interval of the mean.

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 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 1). Blood volume of the horseshoe crabs represented as a percentage of wet body weight was $25 \pm 2.2\%$ (mean \pm SD) for males and $25 \pm 5.1\%$ (mean \pm SD) for females.

In the field, it was 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. Females within the same size classes demonstrated greater variability in blood volume than males (Figure 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 3. Once again, females within the same size classes demonstrated greater variability in blood volume than males (Figure 3).

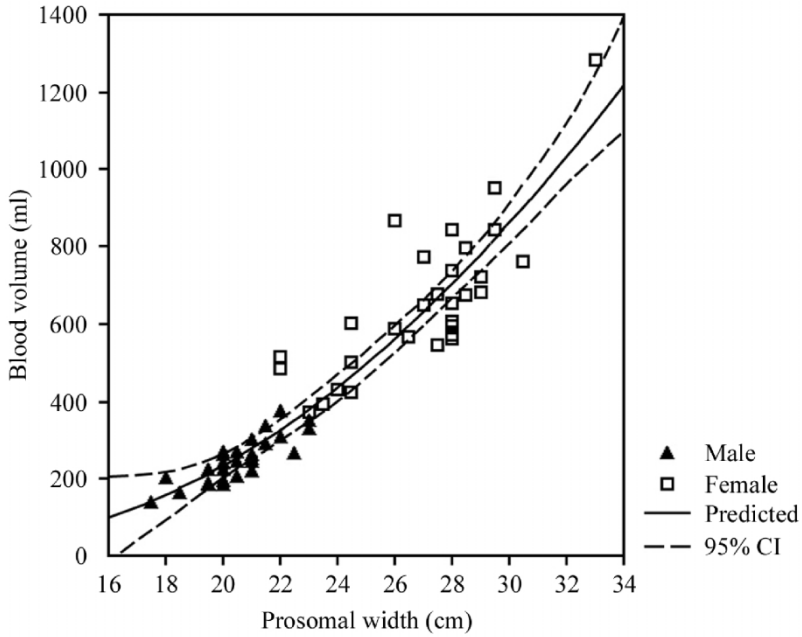


Figure 2. The non-linear regression of P width against estimated hemolymph volume with an exponential fit ($n = 60, p < 0.0001$) is represented by the equation $H = 21.6 e^{0.1234(P)}$, where H = hemolymph volume (mL) and P = prosomal width (cm). The population mean is enclosed by a 95% confidence interval of the mean.

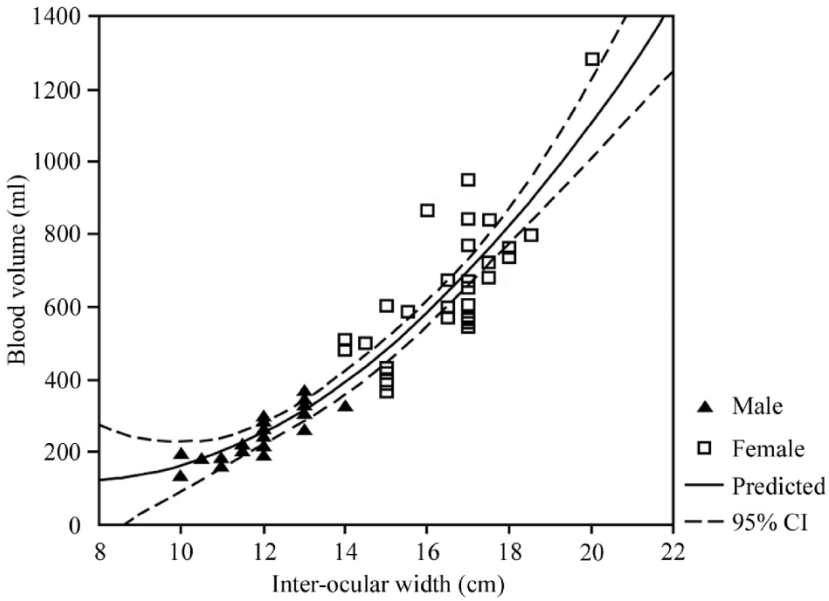


Figure 3. Non-linear regression of IO width against estimated hemolymph volume with an exponential fit ($n = 60, p < 0.0001$) is expressed by the equation $H = 25.7 e^{0.1928(IO)}$, where H = hemolymph volume (mL) and IO = inter-ocular width (cm). The population mean is enclosed by a 95% confidence interval of the mean.

Discussion

We 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 & Zubkoff 1977) as compared to the crab *Carcinus maenas* with a blood volume of 33.0% of wet body weight (Harris & 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 & Weinstein 1950), and scorpion *Heterometrus fulvipes* with 33.4% (Kumari & 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. Our 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 our 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 arthroïdial 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 injected inulin 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 (Foglietta & Herrera 1996; McIver & Macknight 1974). These observations stem from work on several mammalian species, as well as the toad *Bufo marinus* and the holothurian *Isostichopus badionotus* Selenka (Foglietta & Herrera 1996; McIver & Macknight 1974). Despite this, inulin is regarded as a suitable extracellular marker (Foglietta & Herrera 1996; McIver & Macknight 1974). 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 (Foglietta & Herrera 1996; McIver & Macknight 1974). 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 (Harris & Andrews 1982; Martin et al. 1958). 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 h 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 min. 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 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 and incorporating this variable into a multi-measure estimate would likely 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 & Luču 1995), salinity in the gastropod *Littorina littorea* (Taylor & Andrews 1988), nutritional state in various decapod crustaceans (Dall 1974; Depledge & Bjerregaard 1989), and parasitism in *Littorina littorea* (Jones & Kamel 1984). Therefore, our 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.

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