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The Effect of Heavy Bleeding on Mortality of the Horseshoe Crab, *Limulus polyphemus*, in the Natural Environment

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The goal of this study was to ascertain the impact of bleeding such as is done to produce *Limulus* lysate (LAL) on a horseshoe crab population after animals are returned to the natural environment. Techniques used to evaluate the impact of bleeding on subsequent survival included a field tagging experiment and an analysis of survival in the laboratory after bleeding. Approximately 10,000 mature *Limulus polyphemus* were collected, described, and individually tagged. Half were bled and half were handled as controls. All were released into the field. An analysis of the rate of tag recovery for the two groups indicates that bleeding increases mortality by 10% during the first year after bleeding. Crabs rebled after 4 weeks at large had recovered their blood volume. Animals recovered during the second year showed an 11% increase in mortality of bled over control animals. Animals held in the laboratory showed no significant differences in activity after bleeding as compared to unbled controls. Lysate bleeding followed by release of the animals does not appear to constitute a threat to current population levels of *L. polyphemus*.

KEY WORDS: *Limulus polyphemus*, horseshoe crab; *Limulus* lysate test; bleeding, impact: survival, field conditions.

INTRODUCTION

Since the original description of the *Limulus* lysate test (Levin and Bang, 1968), it has proved to be of value not only for the detection of endotoxins associated with Gram-negative sepsis in the blood, (Levin et al., 1970a,b, 1972; Reinhold and Fine, 1971; Jacob et al., 1977), but also in the diagnosis of endotoxemia in conjunction with cirrhosis (Tarac et al., 1977: Scevola et al., 1979), cancer (Platica and Hollander, 1978), meningitis (Jorgensen and Lee, 1978), eye disease (Avallone et al., 1978), dental problems (Fine et al., 1978; Dahlen and Bergenholtz, 1980), gonorrhea (Spagna and Prior, 1981; Prior and Spagna, 1981), boutonneuse fever (Loschiavo et al., 1980), and bacteriuria (Nachum and Shanbrom, 1981). It is also being utilized as an assay for lipopolysaccharides (Hollingdale et al., 1980), and in water quality research (Evans et al., 1978).

The *Limulus* amebocyte lysate (LAL) test is superior to prior methods of detecting endotoxemia (Cohen, 1979; Cooper et al., 1971; Van Noordwijk and deJong, 1976; Steere et al., 1978). The mechanism of the reaction is now being elucidated (Young et al., 1972; Newsome, 1977; Gaffin, 1976; Tai and Liu, 1977; Scully et al., 1980) and further advances in the sensitivity of the test have been made (Coates, 1977; Nandan et al., 1977: Nandan and Brown, 1977; Nakamura, 1977).

Clearly this test has become a valuable and versatile research tool. However, if the LAL test is to become a standard procedure, populations of horseshoe crabs must be protected from careless overexploitation and managed as a valuable marine resource.

Continuing and widespread interest in the LAL test ensures long range and increasing pressure on populations of horseshoe crabs, both *Limulus polyphemus* in America and *Tachypleus tridentatus* in Japanese waters. Animals are often removed from breeding beaches for bleeding, with preference given to large females. Estimates of the current lysate related demand for horseshoe crabs are 30,000 animals per

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year (Pearson, pers. commun.). Currently, many American operations involve bleeding animals at the coast and then releasing the more or less drained animals to the water. Amounts of blood taken range from 100 to 300 ml per animal (maximum available volume is 200 *v* 300 ml) and the animal may or may not be returned to an area similar to its own habitat. Previous studies on *Limulus* have been concerned with the behavioral ecology of the species (Rudloe, 1979a,b, 1980, 1981; Rudloe and Herrnkind, 1976, 1980).

The horseshoe crab can withstand massive physical injury and blood loss. In addition, some animals bled and held in captivity have recovered their blood volume in 3 months. As a result, it has been widely assumed that simply returning the bled animals to the water constitutes adequate conservation procedures. However, prior to this study, nothing was in fact known about survival of bled animals under field conditions. Their ability to resist predators, disease, and other stresses encountered in the wild was unknown, and delayed mortality of bled animals could conceivably be a significant problem.

A twofold approach to the problem of field survival of bled crabs was therefore implemented. The first phase consisted of a field tagging experiment conducted in a defined basin with limited population, and heavy collecting pressure. Both bled and unbled tagged crabs were released so that a comparison of recovery rates was possible.

In addition to the tagging effort, activity and survival of bled and unbled animals held under seminatural conditions in the laboratory was monitored using event recorders.

MATERIALS AND METHODS—FIELD STUDY

The field tagging/survival experiment was initiated in St. Joe Bay, Gulf County, Florida (Fig. 1), on April 27, 1980, following a month of surveying for collecting sites and preparing the necessary facilities in the field. This rectangular basin is approximately 15 miles long by 5 miles wide (24 by 8 km) and is landlocked on three sides. Depths range from 1 to 2 m on *Thalassia* flats to 5–7 m in the center of the basin. Bottoms are composed of sand and shell hash. Very large populations of *Limulus* occur in this area and can readily be collected either on sand bars or from trawlers away from shore.

A total of 25 people were involved in collecting, tagging, bleeding, and releasing the animals, most of whom were volunteers from Earthwatch, Inc., of Belmont, Mass.

Crabs were collected from seven sites in St. Joe Bay (Fig. 1) and one site in Panacea, Florida, within 2 hr of high tide. Animals were picked up by hand and loaded into boats or canoes until return to the field camp, by boat or truck. There they were either tagged immediately or placed in one or more of eight holding pens built of plasticcoated 1½-in. (3.8 cm) mesh wire with dimensions of 12 × 6 × 3 ft (3.7 × 1.8 × 0.9 m). These pens were located on grass flats where the tidal range was 0.5 to 1 m of water. A maximum loading of 200 crabs per pen was used and animals were never held for more than 96 hr from time of capture to time of release. Animals were not bled immediately if they had been out of water more than 3 hr by the time they reached the bleeding station. Such animals were placed in pens for a minimum of 3 hr prior to bleeding. Animals collected at a given site on a given date were generally treated as a group and kept separate from other groups throughout their processing.

Approximately 2500 animals were obtained from trawlers in the deeper central areas of the bay, the remainder being collected from intertidal flats as described above. Animals from trawlers were collected during the night and picked up at the docks each morning at 0800 hr. Subsequent handling was as described above. Animals from trawlers were generally released in 5–6 m of water. Others were returned to the site of collection as much as possible. In

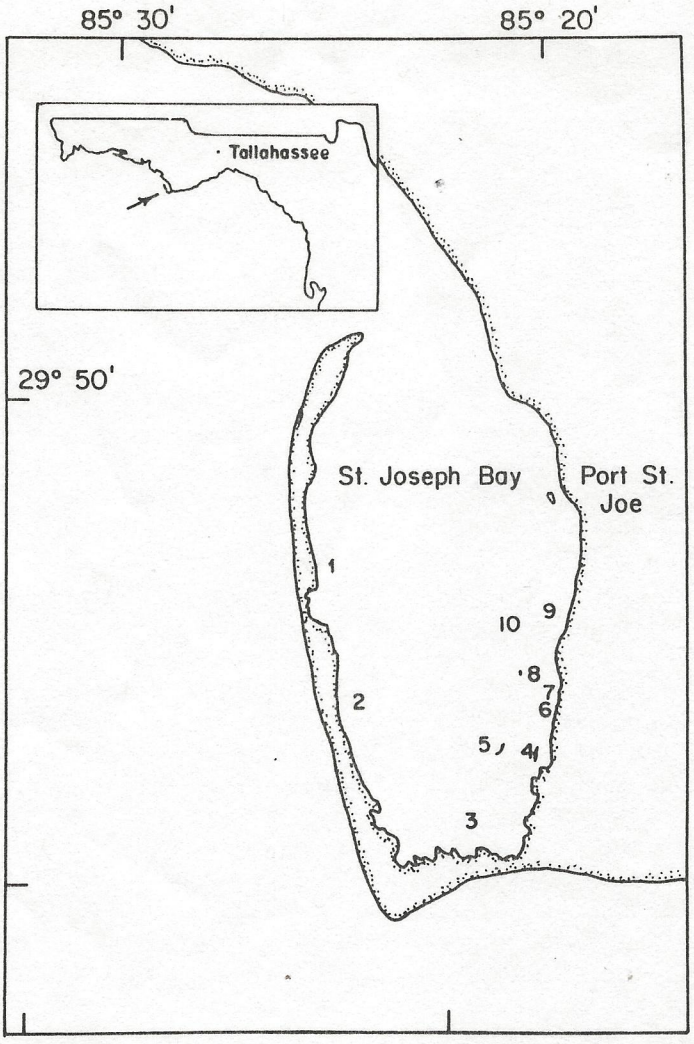

FIG. 1. Release and recovery sites: (1) Eagle Harbor, (2) Tapper, (3) Head of Bay, (4) Conch Island, (5) Black Island, (6) Houseboat, (7) Presnell, (8) Grassy Island, (9) N. of Presnell, (10) Drop off.

some cases, animals were released in other collecting locations, due to tide, weather, etc.

Animals were tagged using plastic carapace dart tags (manufactured by Floy Tag Co.) inserted at the left margin of the prosoma. With its shaft, the tag resembles a thumbtack with barbs. Each animal was described in terms of sex, prosomal width, and condition of shell and lateral eyes—i.e., fouled, scratched, or clean shell and clear, opaque, or semiopaque lateral eyes. As they were tagged, animals were divided into odd and even tag numbers, with even crabs being taken to the bleeding areas and odds being held in shaded areas. Thus, both classes of crabs were subject to comparable handling and exposure to air.

We originally attempted to hold groups of crabs for at least 12 hr after the evens had been bled in order to observe any immediate deaths. However, this procedure was dropped after approximately half the sample had been completed, due to pen-related tag loss. Of the approximately 5400 control and bled animals held in pens after bleed-

ing, the death rate was 1.3% and the deaths were almost equally divided between controls and bleds, suggesting that crowding and stresses other than bleeding were primarily responsible. Animals that appeared weak or freshly wounded were not used.

Even-numbered animals were taken to an open air shed where four bleeding racks were set up, each holding ten animals. Animals were dipped briefly in tap water and sand and other debris washed off. They were then placed in the racks, flexed with the dorsal surface toward the front. The muscle above the heart was thereby exposed and swabbed with 70% isopropyl alcohol. A sterile, disposable, pyrogen-free 16-gauge 1½-in. (3.8 cm) hypodermic needle was opened and inserted into the heart so that blood flowed into a 200 *v* ml plastic cup. The crab was bled until the flow slowed to an intermittent drip, whereupon the needle was removed and the blood volume measured and recorded. The animal was left in the rack until all animals in the rack had been bled and then removed into holding buckets and placed with the control crabs for release. The next batch of ten animals was placed in the rack and the process repeated until all animals being processed had been bled. Then the entire load, both bled and control, was taken by boat to the release point. Animals released in the shallows were flipped into an upright position and allowed to disperse before being left. Approximately 400 animals were processed in each working day until the quota of 10,000 was achieved.

The blood generated by this project was sent to various biochemical laboratories.

MATERIALS AND METHODS—LABORATORY ACTIVITIES PATTERNS

In addition to the tagging studies, it was desirable to directly observe possible sublethal behavioral changes that might ensue after bleeding. Therefore, patterns of locomotor activity of bled and unbled horseshoe crabs were evaluated in a laboratory setting.

Four circular pools, each 2 m in diameter and approximately 0.5 m deep were set up at the Florida State University Marine Laboratory with running sea water and a sand substrate. Each pool was supplied with two plexiglass rods suspended into the water near the edge of the pool. As animals moved around the pools, they tended to maintain physical contact with the pool walls and deflect the rods in passing. The rods were attached to mercury switches which tilted as the rods were deflected, closing a circuit and recording the event on an Esterline–Angus event recorder. This record was used as a quantitative indication of timing and levels of locomotory activity exhibited by animals in the pools.

Each pool was supplied with four mature female *Limulus* for a total of 16 animals per each trial except the fourth trial when three per pool were used, for a total of 60 animals. Each animal was individually described and tagged as in the field experiment. Two pools contained animals bled as in the field experiment while the other two contained unbled control animals. Water temperatures, salinities, and light dark cycles were ambient. Trials were run for a minimum of 28 days and control and experimental pools were alternated each time a new trial began. Four such trials were done between August and December 1980.

RESULTS—FIELD TAGGING STUDY

A total of 10,259 adult horseshoe crabs were collected between April 28, 1980, and May 30, 1980, with a sex ratio of 0.81 males per female. Of these animals, 47% were bled while 53% were control animals (Table 1).

Of the 10,259 crabs, 75 died in the pens prior to release and 122 lost their tags in the pens, leaving 10,062 that were released, constituting the sample for the survival experiment. Pen-related loss was due to tags being caught by the wire mesh and pulled out of the shell as the crab broke free within the crowded pen. Nothing comparable to this would be encountered once the animals

TABLE 1
TOTAL CRAB SAMPLE

	Male	Female	Total
No. of animals	4595	5664	10259
Mean blood volume	63 ml ± 25 ml	137 ml ± 64 ml	
Mean prosomal width	16.4 ± 1.4 cm	20.7 ± 3.1	
No. of experimental crabs	2101	2728	4829 (47%)
No. of control crabs	2494	2936	5430 (53%)
Percentage black shell			18
Percentage clean shell			16.9
Percentage fouled shell			19.8
Percentage clear eye			42.6
Percentage opaque eye			21.7

were released and no evidence of tag loss was ever seen outside of the pens. Animals were held during processing for periods ranging from 12 hr to 4 days prior to release.

A total of 1415 first-year recoveries were compiled through March 1, 1981, with recovery rates from the various release sites ranging from 4 to 28%. Ninety-four percent of these recoveries were alive when recovered, while 85, or 6%, were dead upon recovery (Table 2). Of the 1330 live recoveries, 402 animals had relocated between release and recapture, while the remainder were recovered at the release point.

The total recoveries, live recoveries, and dead recoveries for the first year are compared to the total sample population in Table 3. The bled control ratio of live recoveries is 44%/56%. For dead recoveries it is 55%/45%. Sex ratio, mean prosomal width, mean blood volume, and shell conditions are compared for these groups. A comparison is also made of live recoveries that moved prior to recapture versus those that were recaptured at the point of release.

During the last week of the field tagging phase of the study, 26 tagged crabs (11 male, 16 female) that had been bled more than 2 weeks prior to recapture were returned to the field station and rebled to evaluate recovery of blood volume. Mean blood volumes on second bleeding were 49 ml for males and 125 ml for females, only slightly below the original blood volumes for the entire sample (63 ml for males and 137 ml for females). Twelve animals gave a higher yield on second bleeding. The dura-

TABLE 2
RELEASE/RECOVERY SUMMARY

Release site	No. Released	No. Recovered	Percentage recovered	No. and % of recovered crabs that moved		No. and % of recovered crabs dead	
Eagle Harbor	1,081	99	9	35	35	11	11
Tapper	87	14	16	11	79	2	14
Head of Bay	1,366	113	8	63	56	9	8
Conch Island	3,054	822	27	52	6	35	4
Black Island	598	44	7	24	55	5	11
Houseboat	217	61	28	18	30	1	2
Presnell	732	117	16	75	64	11	9
Grassy Island	613	54	9	51	94	8	15
N. of Presnell	69	7	10	6	86	1	14
Drop off	2,245	84	4	67	80	2	2
Total	10,062	1415	14	402	28	85	6

TABLE 3
COMPARISON OF VARIOUS TAG RECOVERY SUBSAMPLES TO THE TOTAL POPULATION

	Total animals	Live recoveries	Dead recoveries	All recoveries	Live recoveries stationary	Live recoveries relocated
No. of animals	10,259	1330	85	1415	928	402
Sex ratio (M/F)	0.81	0.75	0.60	0.74	0.81	0.72
Bled/control ratio	0.89	0.79	1.22	0.75	0.78	0.77
Male mean prosomal width (cm)	16.4	16.1	16.1	16.1	16.0	16.1
Female mean prosomal width (cm)	20.5	20.2	20.2	20.2	20.0	20.3
Male mean blood volume (ml)	63	52	56	52	55	50
Female mean blood volume (ml)	137	135	144	135	130	138
Percentage clean shell	47	10	8	10	8	12
Percentage black shell	18	34	27	33	36	32
Percentage fouled shell	20	32	26	31	27	35
Percentage clear eye	43	24	21	24	25	24
Percentage opaque eye	22	31	27	31	31	30

tion from first release to recapture for these animals varied from 13 to 36 days, with a mean of 26 days.

A χ^2 test was performed comparing the bled/unbled ratio of the total sample to the total first year recoveries, based on 1415 tag returns. The ratio was 47% bled/53% control for the total sample versus 43% bled/57% control for the recoveries. χ^2 equaled 3.89 with 1 *df* and $5\% > P$ ($\chi^2 \geq 3.89$) $> 2.5\%$.

These data were then further analyzed using a method developed by Paulik and Robson (1969) to evaluate changes in ratios within a population.

This test estimates the survival ratio, θ, of bled to not bled animals as indicated by differential recaptures within the two groups.

If X_1 = tagged, bled at time$_1$, time$_1$ = tagging time, Y_1 = tagged, not bled at time$_1$, time$_2$ = recapture time, $P_1 = X_1/N_1$, $N_1 = X_1 + Y_1$, then: $\theta = (Y_1 X_1)/(Y_2 X_2)$ and: $V[\theta] = [P_1(1 - P_2]^{-4} (1 - P_2)^2 V[P_1] + (1 - P_1)^{2P2} V[P2]$.

For this experiment, $X_1 = 4822$, $X_2 = 628$, $Y_1 = 5437$, $Y_2 = 787$, $N_1 = 10{,}259$, $N_2 = 1415$, $P_1 = 0.470$, $P_2 = 0.44$, $\theta = (5437/4822)/(787/628) = 0.90$, and $V(\theta) = 0.00522$. Thus, the standard deviation of $\theta = 0.90$ is 0.09. That is, if the probability of recovering an unbled animal is 1, then probability of recovering a bled animal is 0.90, more than 1 standard deviation of difference.

Mean blood volume of males and females for recaptures was compared to the mean blood volume of the total sample for males and females. $\chi^2 = 1.66$ with 1 *df* indicating no significant difference in blood volumes removed from the recaptures relative to the total sample. Similarly, live recaptures

were compared to the total sample and $\chi^2 = 1.99$ with 1 *df*, not a significant difference.

A paired *t* test was done on the two blood volumes obtained from the 26 animals that were recaptured and rebled. $t = 0.97$ with 25 *df* indicating no significant difference in the first and second blood volumes following regeneration periods of from 13 to 36 days in the field. This blood was inadvertently discarded so that no further examination of it was possible.

During the second summer after tagging was completed, a total of 167 recoveries were made between March 1 and October 1, 1981. Of these 17 or 10% were dead and 90% were alive. The bled/control ratio was 42% bled/58% control, or 0.72 as compared to 0.75 for the first season; $\theta = 0.89$. Eight animals were recovered outside St. Joseph Bay, seven of which had traveled approximately 20 miles west to the next bay system along the coast, St. Andrew Bay at Panama City, Florida.

RESULTS—LABORATORY ACTIVITY

The animals used in these experiments are described in Table 4. Chart deflections were counted for each pool between 0200 and 0400, 0800 and 1000, 1400 and 1600, and 2000 and 2200 hr each day. Since the animals were primarily active at night, the activity levels between 0200 and 0400 hr were used for the comparisons. Activity in each pool of bled crabs was compared to that for each pool of control crabs in each of the four experiments, for 4 comparisons per trial or a total of 16 comparisons.

Of the 16 comparisons of activity, there was no significant difference between bled and control groups in 6 cases. In 6 cases the bled crabs were significantly more active, while in 4 cases the control crabs were significantly more active. No given pool consistently reported more or less activity than any other pool, i.e., as a result of possible differences in probe sensitivity.

An additional sample of 80 adult animals was collected and tagged. Half the animals were bled, and all were placed in a large (4000 gallons) tank that is routinely used for holding horseshoe crabs for periods of up to 6 months. Postbleeding mortality was then recorded for 30 days. During that period three animals died, two of which were control animals, and one of which, a female, had been bled of 81 ml. All three animals had not molted recently as indicated by poor eye and shell condition.

DISCUSSION

The χ^2 comparison of bled/control ratios for the total sample versus total recaptures indicates a slight difference in probability of recovery of bled versus unbled horseshoe crabs, with bled crabs less likely to be re-

TABLE 4
ANIMALS USED IN LABORATORY ACTIVITY TESTS

Trial No.	Source of animals	Total animals	Dates of trial	Water temperature range, °C	Prosomal width (cm)	Mean blood volume of bled crabs (ml)	Mortality
1	St. Joseph Bay, Fla.	16	8/16/80 – 9/14/80	30–34	19.4 ± 1.24	106 ± 22	2 Bled
2	Panacea, Fla.	16	9/22/80 – 10/27/80	28–20	22.2 ± 1.5	106 ± 41	0
3	Gulf Specimen Co., Inc.	16	10/27/80 – 11/26/80	20–17	22.5 ± 1.6	141 ± 53	1 Control
4	Gulf Specimen Co., Inc.	12	11/29/80 – 12/27/80	17–9	24.1 ± 2.4	133 ± 44	0

covered. The Paulik and Robson change-in-ratio test indicates that the difference is relatively little, 0.9 to 1 for the first year. That is, a 10% increase in mortality among bled crabs can be expected above the rate of natural mortality which is currently unknown. This increased to 11% for second year recoveries.

However, the good recovery of blood volumes in the animals that were recaptured and bled a second time, the survival of animals held in a large tank, and the lack of any consistent differences in activity in the laboratory all suggest that any deleterious effect of bleeding may be limited to a few animals, while the vast majority that recover do so completely and in a relatively short time. Furthermore, the bled/control ratio for live recoveries that moved versus live recoveries that did not move is nearly identical, indicating that bleeding does not affect the probability of significant postbleeding movement in the field.

For this study to provide data useful for a fisheries management effort, the blood volumes removed must be comparable to those taken in a commercial bleeding laboratory. The bleeding done during this study differed from commercial lysate bleeding in several ways, two of which could affect the blood volumes obtained. First, the animals used in this experiment, located in the Gulf of Mexico, were significantly smaller than those normally collected on the Atlantic coast for lysate purposes. Thus one would expect lower blood volumes due to animal size. This should not bias survival. Second, however, the bleeding was done by a series of 22 volunteer workers, none of whom had prior experience in bleeding horseshoe crabs. Their initial inexperience might have affected the blood volume obtained as well, presumably resulting in lower volumes. This second point would constitute an artifact that could affect survival positively because a smaller proportion of total blood volume was removed. Conversely, inexperienced bleeders might have affected survival negatively due to damage inflicted while inserting the needle.

In an effort to separate the relative impacts of animal size and bleeder experience on the blood volumes, 240 animals were bled at the field site by a technician from Limulus Laboratories, Inc., of Horseshoe Beach, Florida, using his own equipment. It was assumed that the volumes obtained by an experienced worker who had routinely bled Gulf of Mexico crabs would be valid for animals of this size.

Total mean blood volume for that sample was 98 ml as compared to 105 ml for the entire sample. Thus, the use of volunteer bleeders did not seriously reduce the blood volume taken from the crabs as compared to a professional lysate bleeder. The low death rate of animals held in pens subsequent to bleeding also suggests that use of volunteers was not a major problem.

In order to wisely manage the horseshoe crab resource, the significance of the 10–11% increase in mortality among bled crabs must be determined. Additional information on the natural mortality rate and the proportion of the population being exploited is necessary to establish the overall increase in mortality that could be expected.

Once those parameters were estimated, data on survival, sex ratio, age of sexual maturity, and fecundity would also be needed to project future population levels at various degrees of exploitation (O'Neill et al., 1981).

Although this information is not available at present, comparisons with fisheries for other species can provide some insight into the significance of this level of increased mortality. For instance, the spiny dogfish *Squalus acanthias* differs from most other commercially exploited fish and resembles *Limulus* in terms of its longevity, slow growth, and relatively low fecundity. Estimates of maximum substainable yield for this species suggest an annual fishing mortality of 45% in excess of natural mortality (Wood et al., 1979).

Similarly, the Dungeness crab, *Cancer magister,* of the Pacific northwest is estimated to sustain a 85% fishing mortality

among male crabs, with no harvest of females so that overall exploitation is approximately 40% as well (McKelvey et al., 1980). In light of these rates, it would not appear that lysate bleeding presently constitutes a serious threat to existing populations of *Limulus* in the United States.

In addition to its exploitation by the pharmaceutical and biological supply industries, this species is subject to rapidly increasing harvest by commercial fisherman who use them by the thousands as eel bait. This may well be a far greater source of mortality than lysate bleeding but the magnitude of the harvest is unknown at present. Conversations with eel fisherman, however, suggest that it is significantly reducing many local populations. The earlier fishery in Delaware Bay for *Limulus* as fertilizer did greatly reduce that vast population over a number of years (Shuster, pers. commun.) although it now appears to be increasing again. Data on this point are needed in order to more completely evaluate the status of *Limulus* populations.

This research provides the first test of one current hypothesis—that large scale commercial bleeding of *Limulus* does not impact existing populations. Thus far, the hypothesis seems to be a valid one.

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