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Horseshoe crab gonad, NMJ, and CNS structure/ immunology

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GONADS, SIZE, AND SEX DISTRIBUTION IN JUVENILE HORSESHOE CRABS

A Major Qualifying Project Report

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By

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I would like to thank Dr. Daniel Gibson III for all of his help and guidance with this project.

ABSTRACT

Adult horseshoe crab females are larger than males; we hypothesized that this may be due to differential growth from an early age. When 139 synchronous molts from a discrete population were sized and sexed we found no significant difference in size. Immature gonads were sought in males and females using light and electron microscopy; gonads run alongside digestive diverticulae. Developing gonads were found down to 3 years in females but only in mature males.

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Background

The Atlantic Horseshoe crab, *Limulus polyphemus*, is an ancient arthropod that has evolved little in the last 200 million years. It is sometimes referred to as a "living fossil" and is an organism of much interest to scientists because it has managed to live so long and evolve so little despite the fact that their levels of polymorphism and heterozygosity of allozyme loci are similar to more rapidly evolving organisms (Selander et all, 1970). The horseshoe crab is also an organism of great interest to scientists because its blood is used to make *Limulus* amebocyte lysate (LAL) which is used to test to for endotoxin contamination in drugs (Shuster, 2004).

The horseshoe crab is composed of three main parts: the prosoma (cephalothorax), the opithosoma (abdomen), and the telson (tail). The prosoma contains the intestinal tract, nervous system, and five paired legs. The opithosoma contains muscle groups for operation of the book gills, the book gills, and the tail. The first flap of the book gills is the genital operculum and the other five contain sensory branches and gill leaflets (Ecological Research and Development Group, 2009).

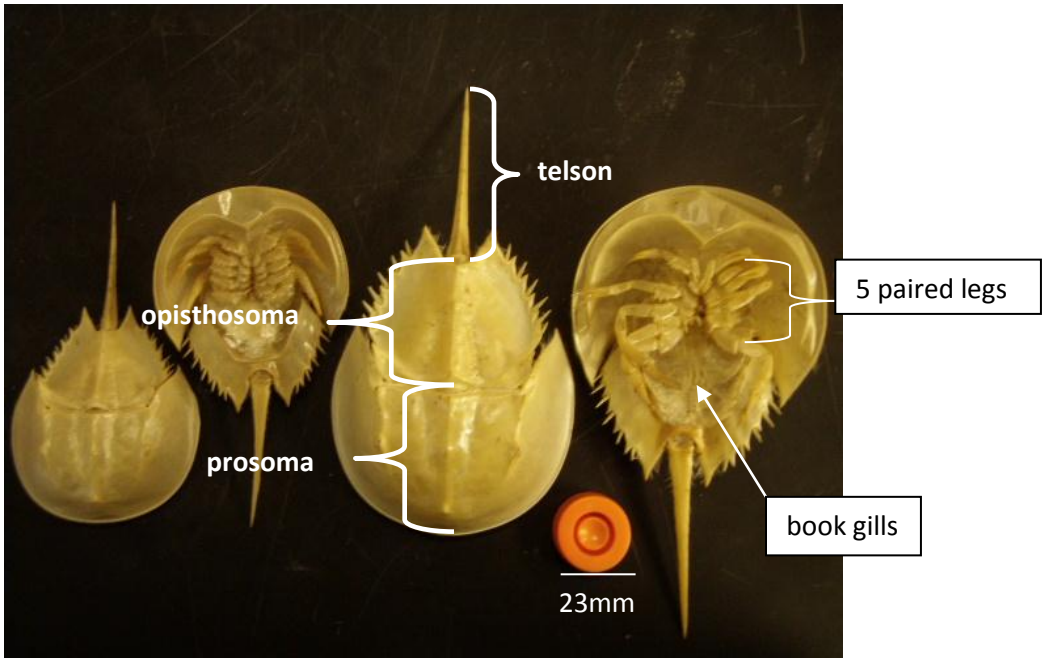

Figure 1: Basic horseshoe crab anatomy.

Horseshoe crabs make use of three different habitats throughout their lifetime. They spawn on sandy beach areas inside bays and coves in order to protect the eggs from rough waters. They tend to prefer porous, well oxygenated sediments and areas near large intertidal sand flats. After hatching juveniles spend their first two years in intertidal sand flats. Older juveniles head out into deeper bay waters but will not leave until they are adults. Adults are bottom dwellers and can survive in either estuarine or continental shelf habitats. Horseshoe crabs are able to survive in a wide range of salinities, oxygen levels and temperatures. Salinities of less than four parts per thousand are lethal and they prefer an optimal salinity range of 20 to 30 parts per thousand (ERGD, 2009).

Development and Molting

After horseshoe crab eggs have been fertilized they undergo four molts before hatching, which occurs 1-3 days after the fourth molt. At hatching they are about 4mm in prosomal width. They molt by crawling forward out of the old shell which splits starting at the front of the rim along the prosoma. The new shell is soft from growing under the old shell and hardens with time. The time it takes to molt increases with the size of the shell. The time in between molts and the difference in molt sizes also increases with each molt (Shuster, 2004). Horseshoe crabs molt 5-6 times during the first year of life and 3 or 4 times for the first several years of life. As they get older and become sub-adults (5-7 years) they molt only about once a year. Males reach maturity after about nine years and the sixteenth molt. Females do not reach maturity until their tenth year around the seventeenth or eighteenth molt (ERGD, 2009). Horseshoe crabs exhibit stepwise growth, abrupt changes in physical size. A graph found in The American Horseshoe Crab shows the step-wise growth of laboratory raised *Limulus polyphemus* and *Techypheleus tridentatus* and can been seen in Figure 2(Shuster, 2004).

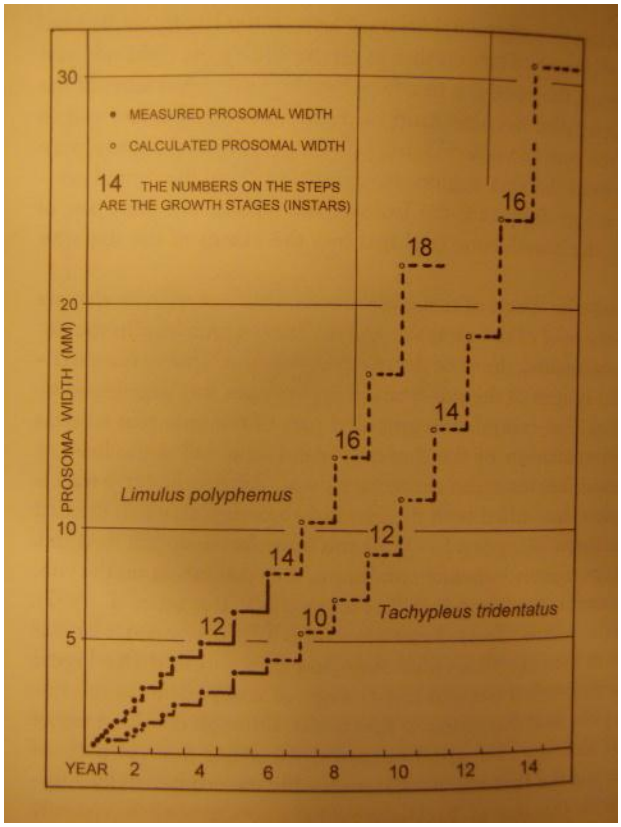

Figure 2: "The two steps contrast the ages and incremental increases in the size of each molt for Limulus polyphemus and Tachypleus tridentatus. The solid circles represent actual prosomal measurements (in centimeters) and the open circles represent widths based on the rate of growth calculations."[From pg.122 (Shuster, 2004)].

Sexual Morphology

There are several ways morphologically that male and female horseshoe crabs differ. One of the most obvious ways mature horseshoe crabs can be sexed is by their front pedipalps. After the terminal molt males have modified front pedipalps that resemble boxing gloves used for hanging on to the female during mating. Females' front pedipalps remain unaltered after the terminal molt. Figure 3 shows the differences between male and female front pedipalps (Shuster, 2004).

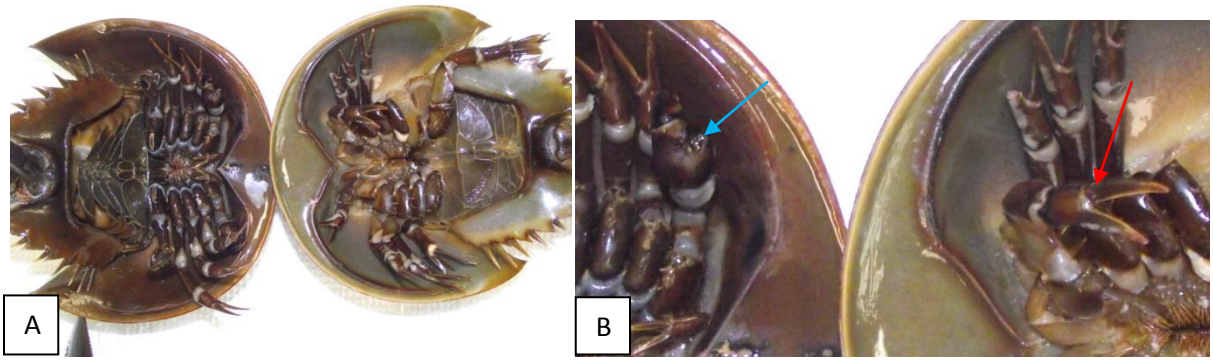

Figure 3: A: Male and female horseshoe crab side by side. B: Mature male distributing modifies front pedipalps (blue arrow) and mature female exhibiting unmodified front pedipalp (red arrow).

The sex of the horseshoe crab can also be determined by observing the genital operculum. This method of sexing can e used on both mature and immature horseshoe crabs and is the method that will be used in this project. Males exhibit genital papillae or hard raised bumps on the underside of the genital operculum while females have gonopores that appear to be softer bumps with slits in them (Shuster, 2004). These differences in genital operculum can be seen in Figure 4.

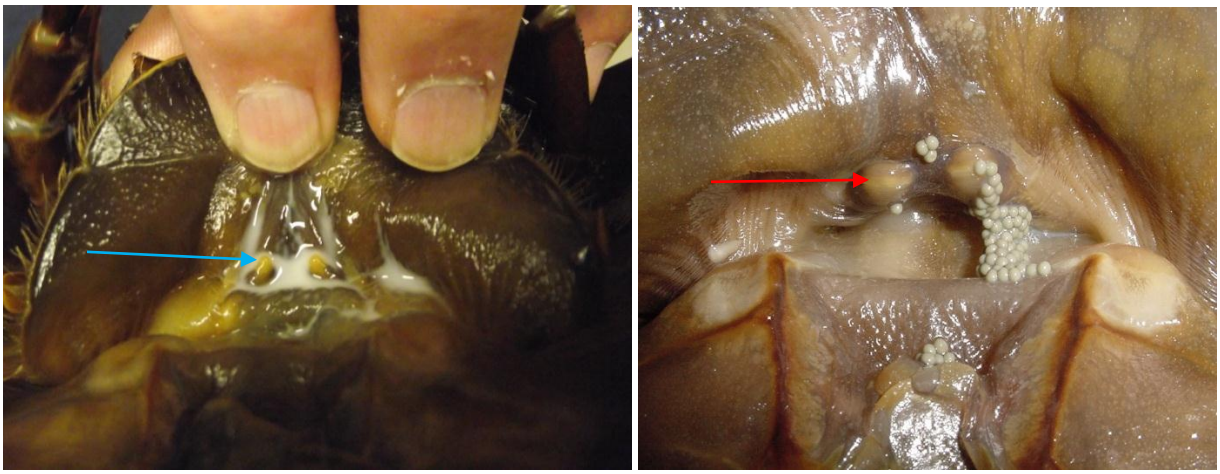

Figure 4: Gonopores (red arrow) on female genital operculum on right and papillae (blue arrow) on male genital operculum on left.

Both males and females have similar placement of ovarian and testicular networks. Both sexes have lateral and anterior branches that subdivide into large ovarian and testicular networks that ramify throughout the prosoma in their respective sexes. A third smaller third branch of the system descends into the opisthosoma and connects to the genital operculum (Fahrenbach, 1998). It is known that the tubules of the testes are intermingled with the tissue mass of the digestive gland and the ovarian network mainly covers the dorsal surface of the digestive gland (Shuster, 2004).

Fahrenbach (1998) states that oogonia are present in young juvenile females and maturing oocytes in adult females develop in groups that are covered by a thin layer of fibroblasts that later disappear. After oocytes grow greater than 50 µm they rupture out of the epithelium and muscle in the ovarian ductules but remain attached by a solid cellular stock. When the eggs are mature they return to the inside of the ovarian tubules (Fahrenbach, 1998).

In males seminiferous tubules are about 30-500 µm in diameter and are continuously lined with spermatogenic cells as opposed to having them concentrated at one end of the tubules as in many other arthropods. The globular sperm sacs are found in the lumen of the seminiferous tubule and are about 100 µm in diameter. All of the sperm developing within the sacs are in the same stage of development as spermatogenesis proceeds in a synchronous fashion (Fahrenbach, 1998).

Considering this information when juveniles are looked at to determine whether developing gonads or gametes are present tissue samples will be taken from the prosoma where the digestive, ovarian, and testicular networks are known to be found. Developing spermatogonia, sperm, oocytes, and eggs will be searched for.

Size and Sex Distribution

It has been found in studies done by Shuster (1985) that the sex ratio of mature horseshoe crabs on spawning beaches in New Jersey and Delaware varies between 5 and 3 males for every 1 female. In a study done by Rudlow (1980) on the sex distribution on the spawning beaches of Florida the mean sex distribution was 3.6 males for every 1 female. In 1998 the Maryland Department of Natural Resources reported a sex ratio of 4 males to every one female. This difference must have some cause as sex chromosome segregation would suggest a ratio of 1:1. These differences in sex ratios may be due to several factors. A previous project completed by Uzma Ali (2000) hypothesized that the differential sex ratio in adults is due to males turning into females after they reach a particular size or molt. In her project, molts from pre-adult and juvenile populations in Sippewissett and West Dennis were examined for size and sex. The results were inconclusive due to poor molt conditions , however some male characteristics such were found in female crabs such as dips in the anterior rims and slightly more rounded front pedipalps. However, sufficient evidence to support the males turning into females hypothesis was not found. No, molt with male pedipalps has ever been found, and that argues for the pedipalp molt being the terminal molt. It has also been hypothesized that the sex ratio may be 1:1 and that males and females may simply just be present in differing sex ratios in different locations. This theory is supported by Rudloe's (1980) research findings that offshore trawl collections showed inverse sex ratios of three females to every one male and two females to every one male. Swan's research (1998) also supported this theory, finding a sex ratio of 1.17 females to every one male. In conclusion there is no conclusive data determining why there is a sex ratio difference. This project will explore the sex distribution in a cohort of three year old *Limulus* in order to determine if the sex ratio difference is apparent in juveniles.

Adult females have been found to be 25-30% larger than adult males on average (Shuster, 2004). It is unknown whether this difference in size is due to differential growth from the juvenile stages or attained in the one additional molt females undergo as compared to males to reach maturity. In a study done by Sekiguchi et al (1998) a one-step difference in the instar stages between males and females was found in laboratory raised *Limulus polyphemus*. However, the authors themselves state in the discussion that they are not sure if this discrepancy is real or not. For this project it was hypothesized that size differences were due to differential growth from an early age.

Materials and Methods

Size and Sexing

In order to determine whether there was a difference in the size of male and female juvenile horseshoe crabs 200 synchronously-shed molts from a cohort of three year old *Limulus polyphemus* in Little Sippewissett Salt Marsh in West Falmouth, Massachusetts were collected for analysis. A sample of theses molts can be seen in Figure 5 below.

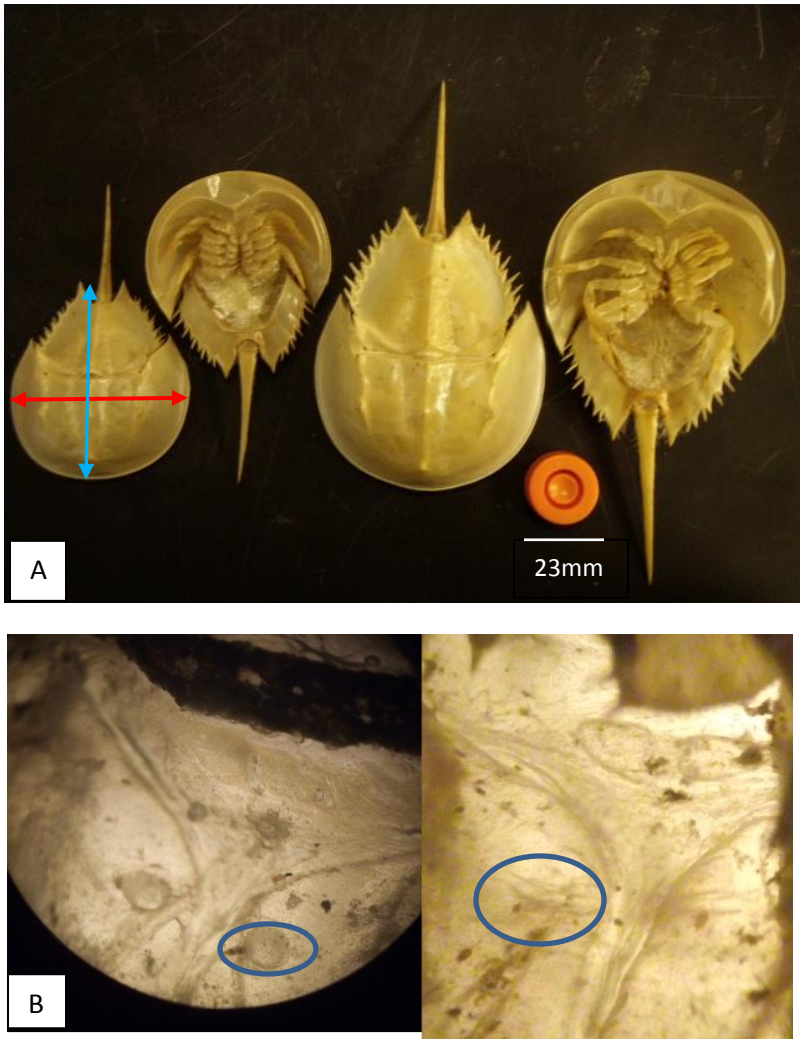

Figure 5: A: Molts gathered from Little Sippewissett Salt Marsh in West Falmouth, Massachusetts. Red line represents width measurements; blue line represents length measurements. B: Backlit genital operculum of molts male (left) and female (right).

Once the molts were collected they were measured for size from the top of the prosoma to the bottom of the opisthosoma (blue line, Figure 5) and across the widest part of the prosoma (red line, Figure 5). The molts were sexed by viewing the backlit genital operculum to determine whether they had papillae or gonopores.

Tissue Collection

In order to determine if gonads or developing gametes were present in juvenile horseshoe crabs tissue sample were taken from adults and young juveniles. Tissue was taken from a mature male measuring 159mm across and 197mm lengthwise, a mature female measuring 241mm across and 315mm lengthwise, and an immature female measuring 152mm across and 203mm lengthwise via biopsies for comparison to juveniles. The mature male, mature female, and immature female can be seen in Figure 6.

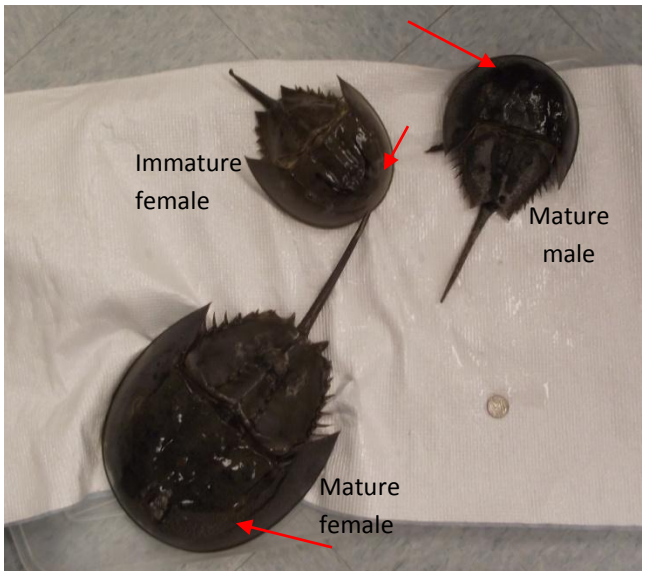

Figure 6: Mature female, mature male, and immature female. Red arrows point to biopsy site.

Biopsies were also taken from juveniles of three years of age; a female measuring 45mm across and 53mm lengthwise and a male measuring 51mm across and 63mm lengthwise(Figure 7).

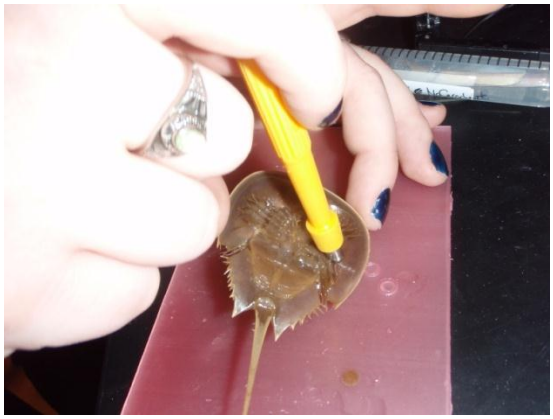

Figure 7: Biopsy being taken from 3 year old *Limulus polyphemus.*

All biopsies were taken from the left lateral prosoma in the area where the digestive tissue can be found using a 5mm biopsy punch. According Shuster (2004) the tubules of the testes are found intermingled with the tissue of the digestive gland and the ovarian network is found on the dorsal surface of the digestive gland, therefore in order to isolate developing gonads one should sample the tissue intermingled with and on the dorsal surface of the digestive gland. This location of the gonads is also supported by research conducted by Fahrenbach (1998) that found that the testes and ovaries ramify throughout the prosoma, with ducts leading to the genital operculum.

Horseshoe crabs of 5mm, 8mm, 10mm, 12mm, and 15mm were looked at to determine whether gonads or developing gametes were present however they were too small to take biopsies from. In these smaller crabs dissection was used to in order to reveal the digestive tissue. The crab was cut into 5 or 6 longitudinal slices and a sample was taken from the area where gonads would develop.

Tissue Fixation and Embedding

Tissue was fixed using glutaraldehyde and osmium tetroxide as it is known to yield good preservation of fine structures (Hyat, 2000). The tissue samples obtained through biopsies and dissections were fixed in 2% glutaraldehyde in 100 mM NaCacodylate buffer with pH 7.4, for one hour at room temperature. Glutaraldehyde is known as an excellent cross-linker of proteins. After a buffer rinse, tissue was post fixed in 1% osmium tetroxide for one hour at room temperature. Osmium

tetroxide is primarily used to fix lipids, especially unsaturated fats. The tissue was then rinsed in dH_20 and dehydrated for ten minutes per solution in an ethanol series of 70%, 100%, 100%, 100%. Tissue was sometimes additionally dehydrated using propylene oxide. When this occurred, two periods of ten minutes each in 100% propylene oxide were added after dehydration in the ethanol series. After dehydration was complete the tissue was embedded in either Spurrs low viscosity resin or Epon Araldite (epoxy resins). This was accomplished with a series of mixtures of the resin and ethanol or the resin and propylene oxide. The tissue was put through hour long wait periods in each mixture of resin and dehydrating compound. The mixtures of resin and dehydrating compound the tissue was put through were 1:2, 2:1, and 100% resin respectively. The tissue was then placed in fresh 100% resin, put in molds, and cured at 70°C for at least 12 hours.

Sectioning and Staining the Embedded Tissue

After the resin was cured it was removed from the mold and the tissue was exposed. A face containing tissue was exposed using a saw. The face was then smoothed down using a series of finer grade sandpapers. Next, a razor blade was used to fashion a trapezoidal face containing the tissue sample that was raised above the remainder of the resin. Sections were cut using a diamond knife on a Porter-Blum Ultramicrotome. Thick sections of 0.5 µm were cut for analysis via light microscopy and thinner gold sections of 60-90 nm were cut and collected on various copper grids (200+ mesh) for analysis via transmission electron microscopy

After sections were cut contrast needed to be enhanced before the structures could be viewed and analyzed to determine if they contained gonads or developing gametes. Staining for light microscopy was done by placing 0.5 µm sections of the embedded tissue on a glass slide and placing it on a hot plate. Toluidine blue was then applied and allowed to sit for 1-2 minutes before gently being rinsed off the slide with H_20. The slide was allowed to dry completely on the hotplate before being viewed on the light microscope.

Contrast of sections to be used for transmission electron microscopy was enhanced with heavy metal stains. The 60-90 nm sections on copper grids were inserted into drops of uranyl acetate from the side (completely immersed) and left for 5 minutes. The sections were then rinsed gently with dH_20 before being inserted into drops of lead citrate from the side (completely immersed) for 30 seconds, rinsed with dH_20 again, and allowed to dry completely. After staining, the tissue samples were observed using transmission electron microscopy.

Results

In order to determine if the sex and size differences seen in adult horseshoe crabs were due to differential growth and sex distribution from an early age 200 synchronously shed molts from a cohort of three year old *Limulus polyphemus* were analyzed. Of the 200 molts that were collected and analyzed only 139 molts were able to be sexed due to poor molt condition. Some molts were missing the genital operculum completely and in some tears and rips were present making it impossible to sex. Of the 139 that were able to be sexed 71 were males and 68 were females (Table 1). The sex and size distribution for males and females can be seen in Figure 8. The sex ratio for this cohort of three year olds is 1:1. There is no size difference between males and females. A two tailed T-test was run on the sizes of males and females and a p-value of 0.72 was obtained indicating there 70% probability that they are not different.

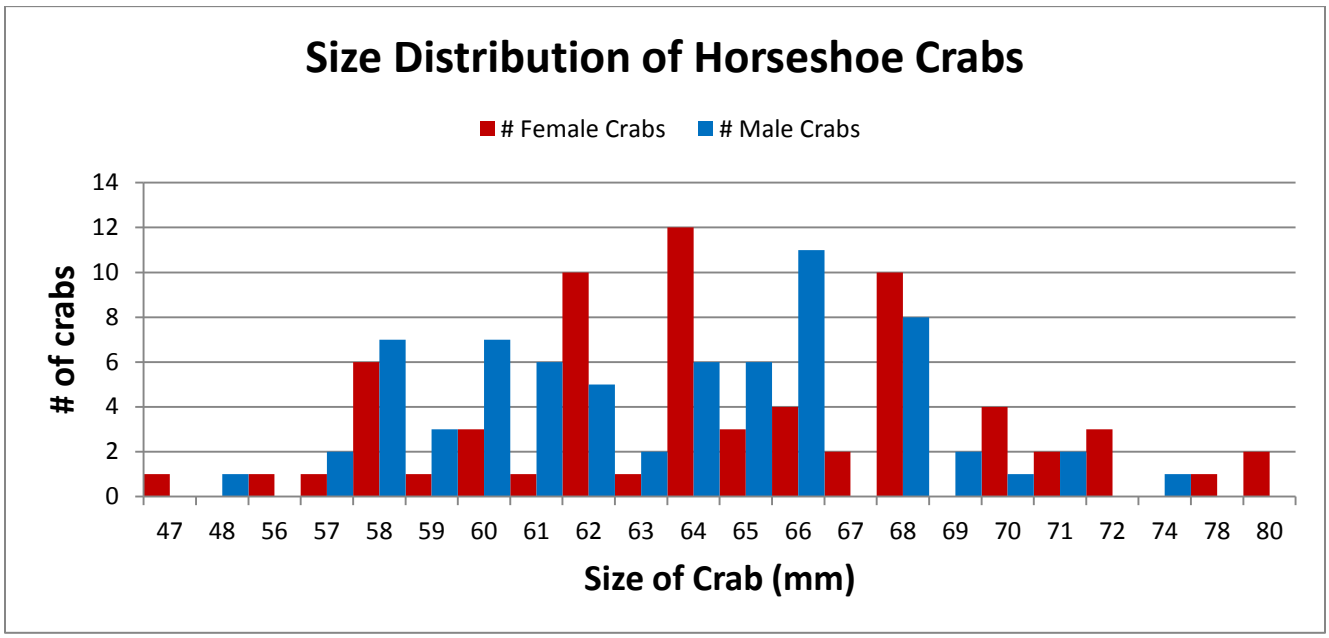

Figure 8: Size distribution by sex of 139 synchronously-shed molts from a cohort of three year old *Limulus polyphemus* from Little Sippewissett Salt Marsh in West Falmouth, MA.

# of males	# of females
71	68

Table 1: Sex distribution of 139 synchronously-shed molts from a cohort of three year old *Limulus polyphemus* from Little Sippewissett Salt Marsh in West Falmouth, MA.

In order to determine if gonads or developing gametes were present in juvenile crabs a mature female of 241mm prosomal width and a mature male of 159mm prosomal width were analyzed in order to view gonads and developing gametes for comparison to juveniles. In the mature female (Figure 9) clusters of oocytes can be seen (red arrows) as well as several eggs in various stages of vitellogenesis (green arrow). Figure 10 also shows eggs in the mature female in varying stages of vitellogenesis.

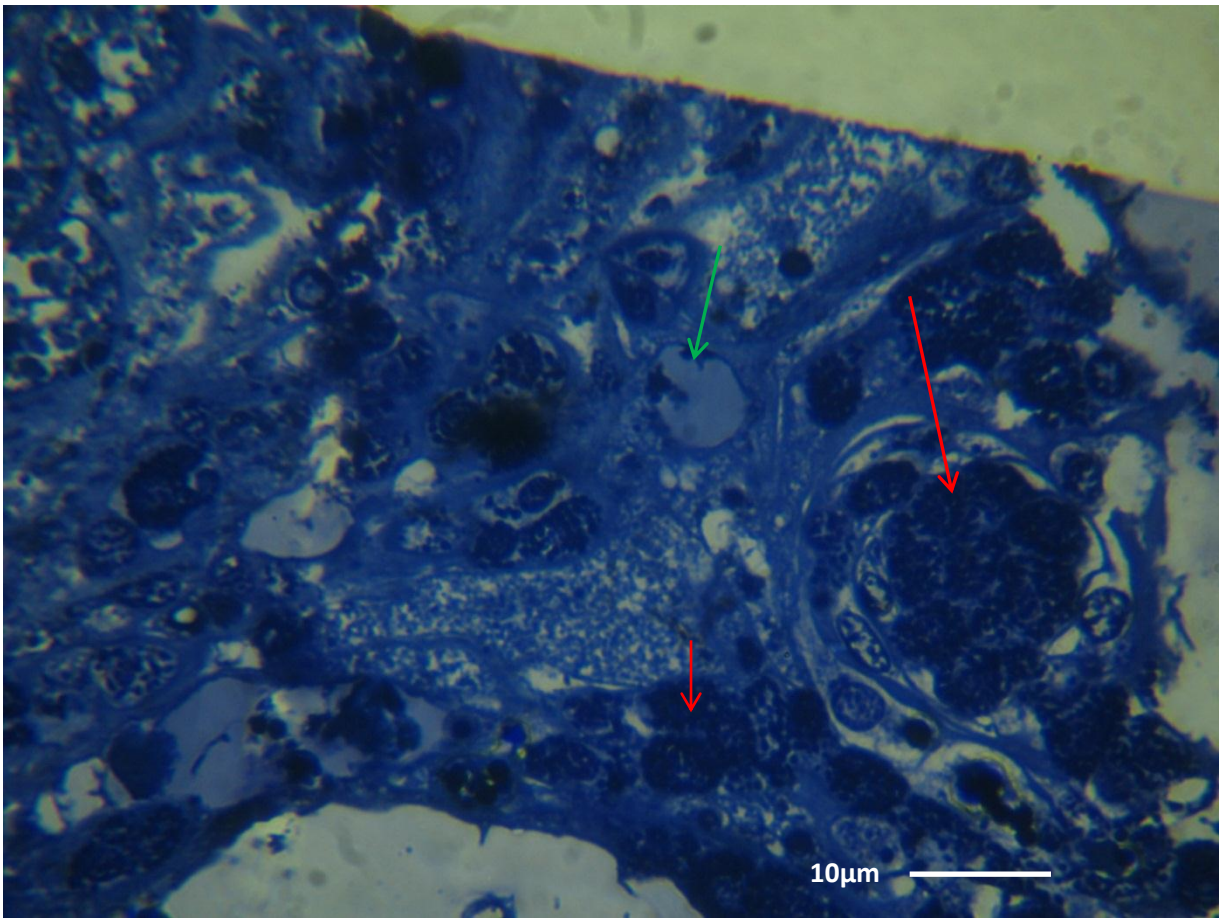

Figure 9: Mature female of 241mm prosomal width at 1000X magnification. The red arrows point at clusters of developing oocytes and the green arrow points at vitellogenesis.

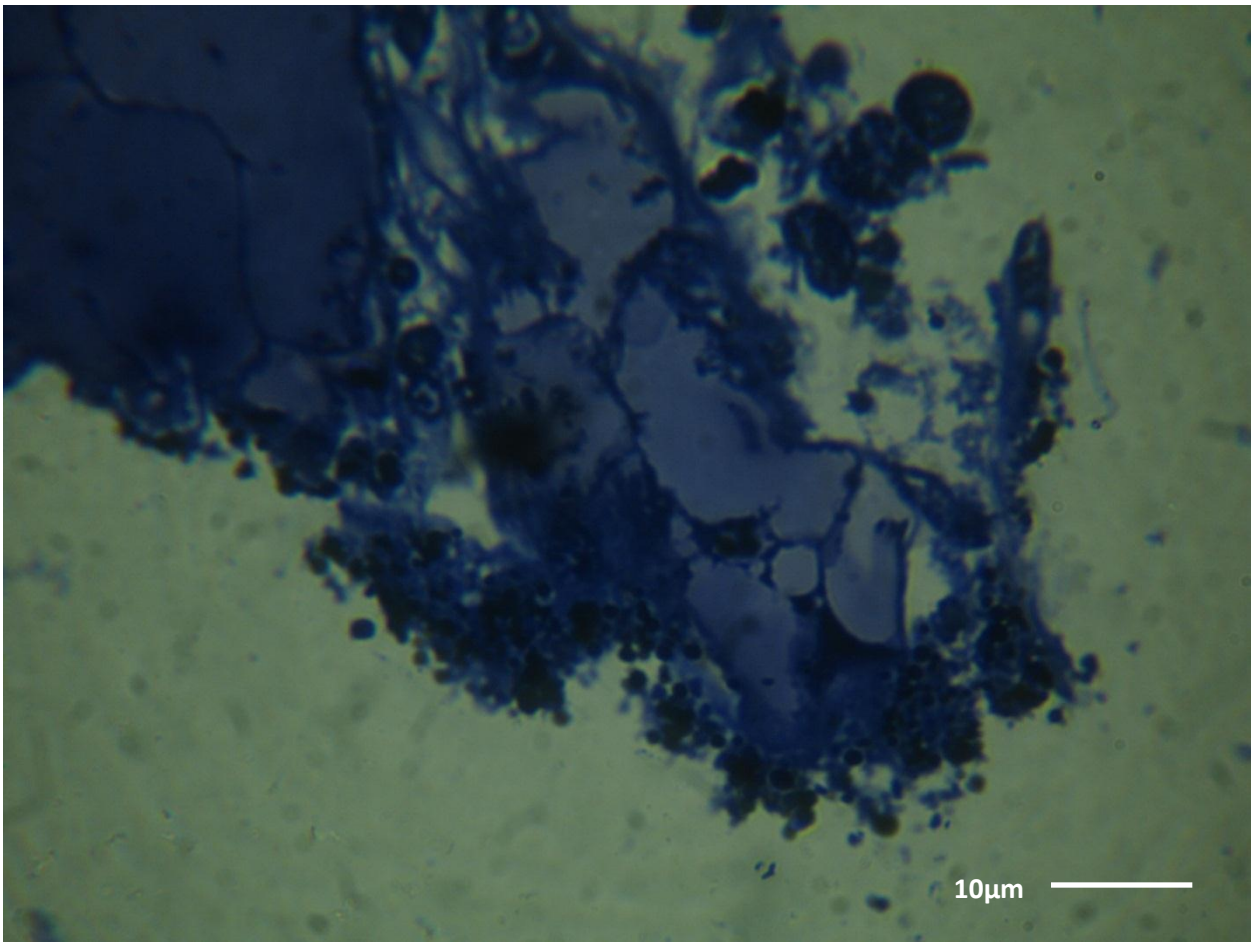

Figure 10: Mature female of 241mm prosomal width at 1000X magnification showing eggs in various stages of vitellogenesis.

In Figure 11, mature male tissue, two light microscopy pictures taken at 400X magnification can be seen showing a wide view of the seminiferous tubules and sperm sacs.

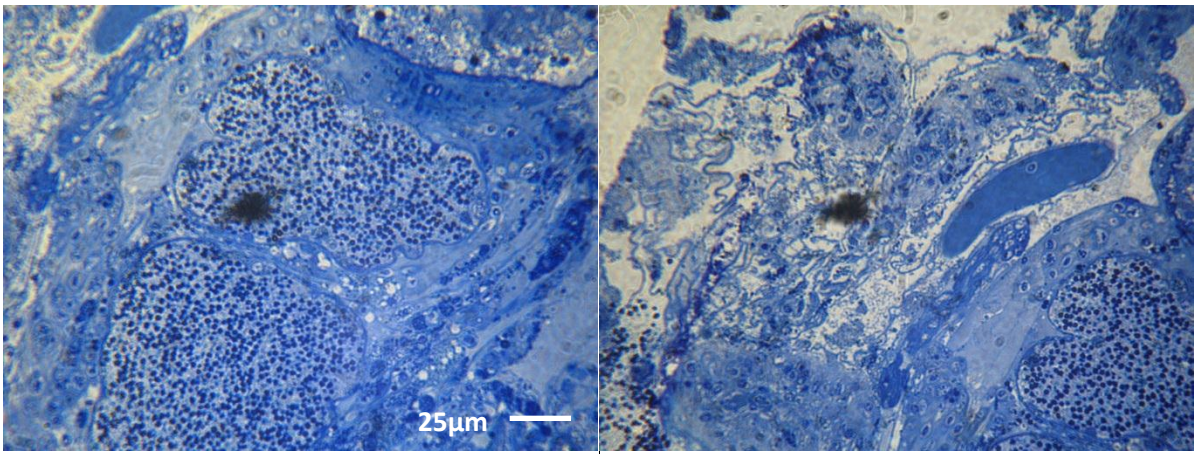

Figure 11: Mature male of 159mm prosomal width at 400X magnification.

In the mature male tissue, Figure 12, many sperm can be seen inside the sperm sacs (red arrow) and developing spermatogonia can also be seen along the sides of the tubule (green arrows).

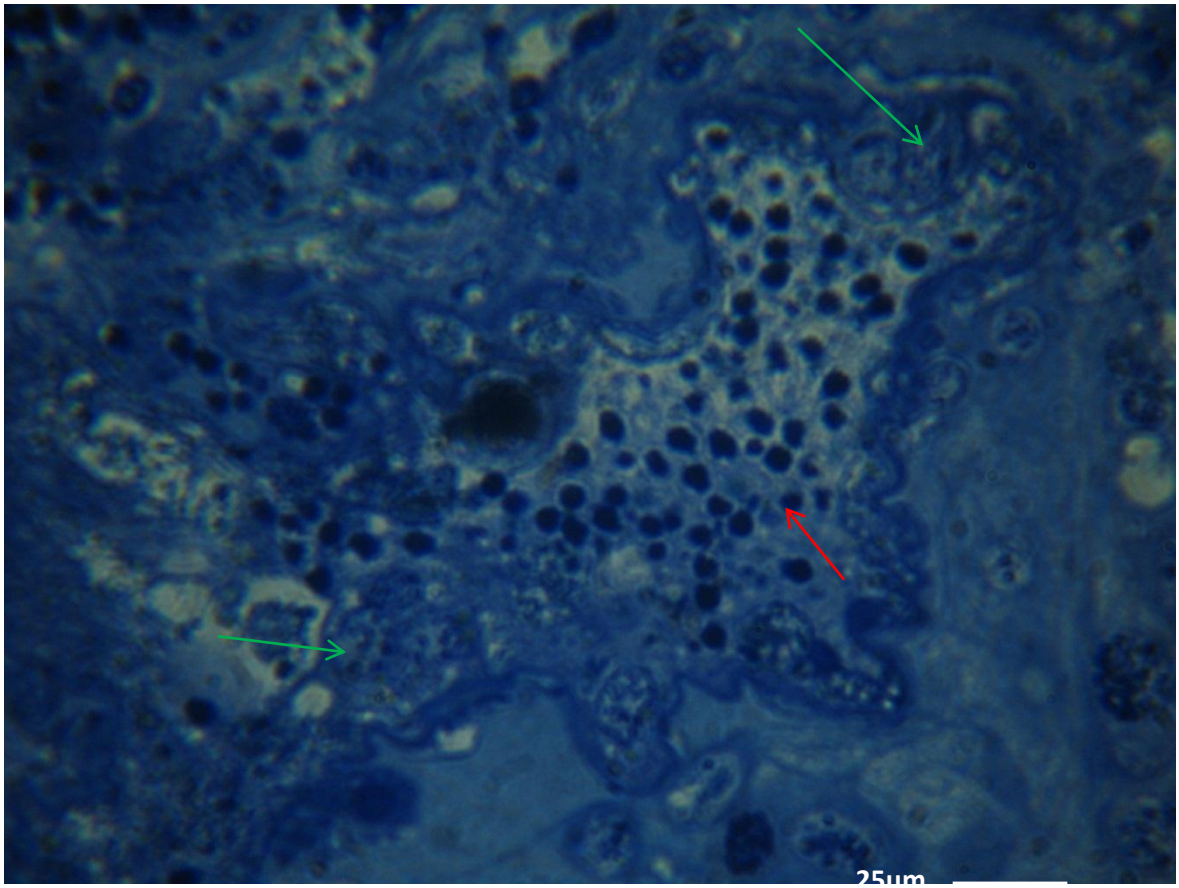

Figure 12: Mature male of 159mm prosomal width at 400X magnification, green arrow shows spermatogonia and red arrow shows sperm.

An immature sub-adult female of 152mm prosomal width (Figure 13) displayed developing oocytes (red arrows) that look similar to the developing oocytes in the mature female (Figure 9) on a smaller less developed scale. No developing or mature eggs were seen in this female.

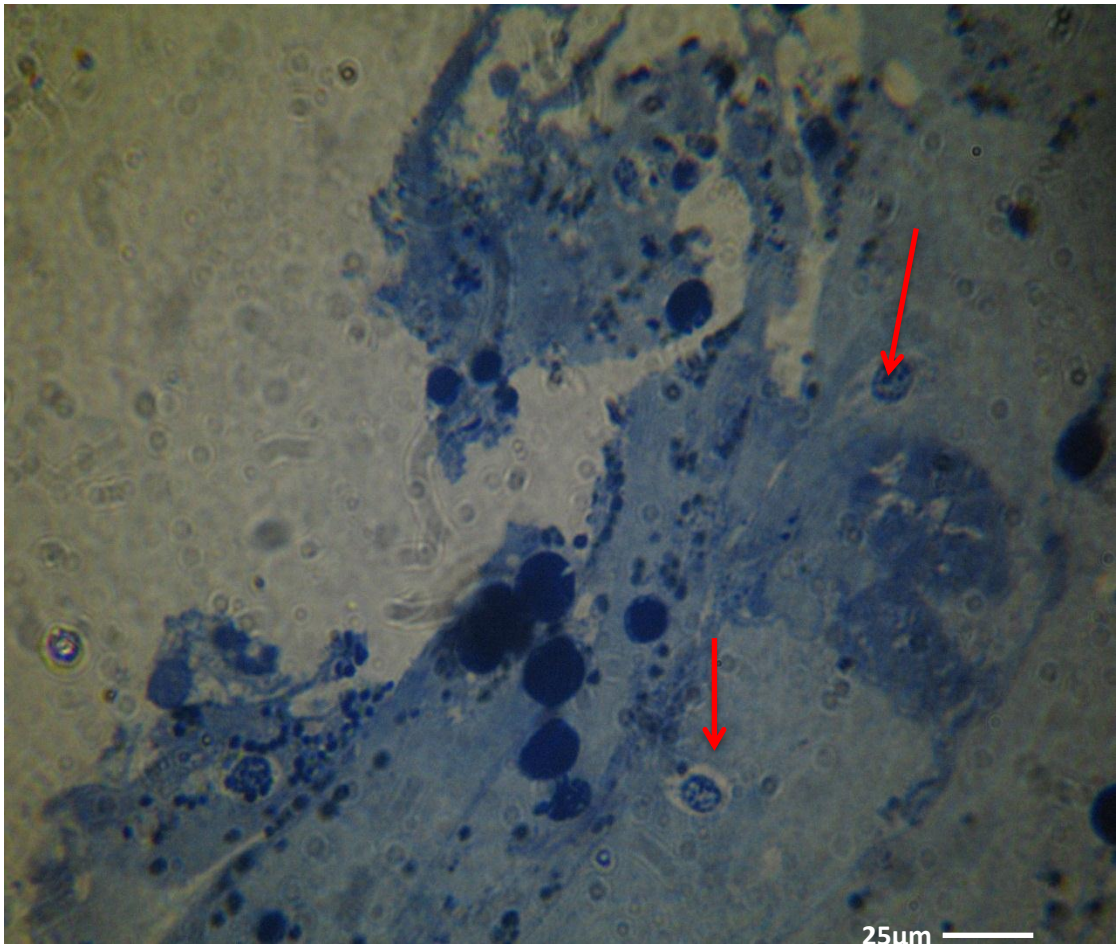

Figure 13: Immature female of 152mm prosomal width at 400X magnification.

When a three year old juvenile male of 63mm prosomal width (Figure 14) was examined no developing gametes were found. There were no seminiferous tubules or sperm sacs seen. This is tissue from around the digestive gland as the curvy outer membrane of the gut can be clearly seen in the photographs; therefore, the right location in the crab was being searched.

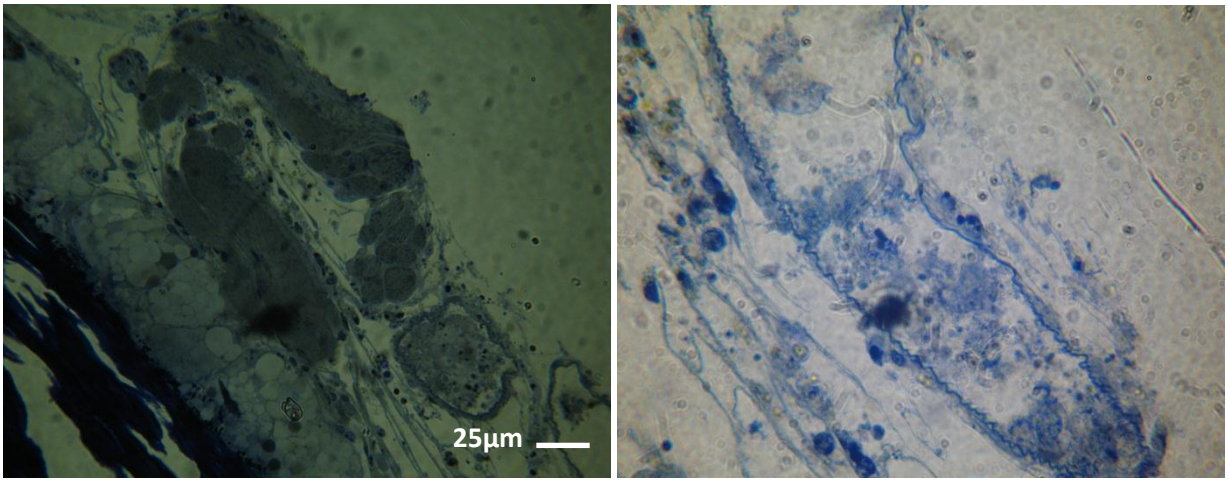

Figure 14: Immature male, 63mm at 400X magnification.

When a three year old juvenile female of 53mm prosomal width (Figure 15) was examined the curvy outer membrane of the gut was apparent placing the search in the right location. No oocytes or developing eggs were discovered, however, possible germ cells were seen (red arrow).

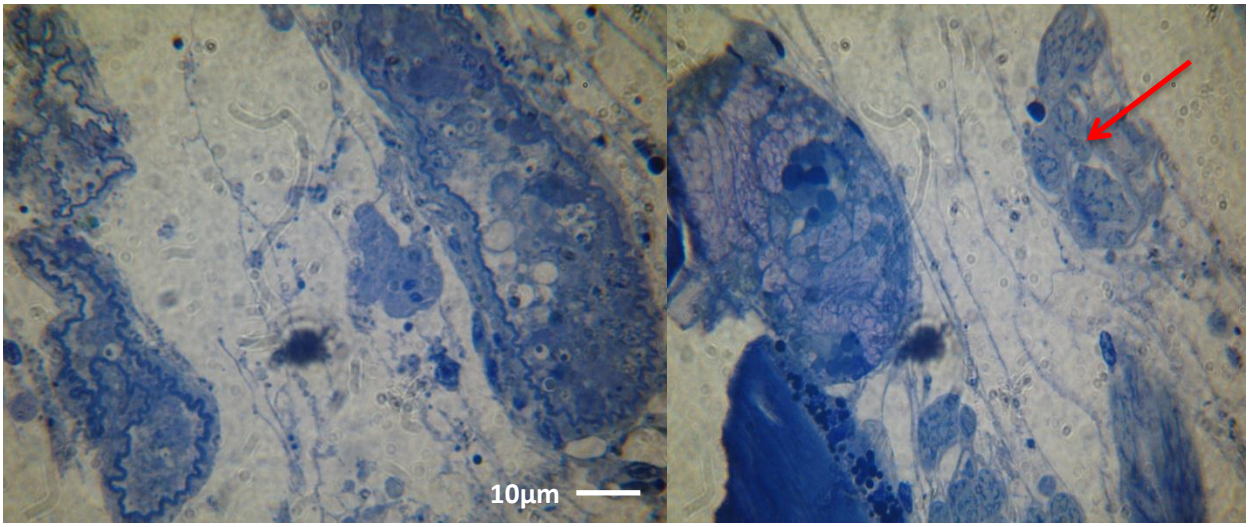

Figure 15: Immature female, 53mm, 1000X magnification; red arrow points to possible germ cells.

In the crabs of 15mm, 12mm, 10mm, 8mm, and 5mm no developing gonads, gametes, or possible germ cells were found in either sex. Tissue samples were found to be near the gut as digestive

tissue could be identified in all of the slides (Figure 16) meaning they should have been in the correct area to see developing gonads, gametes, or germ cells although none were identified.

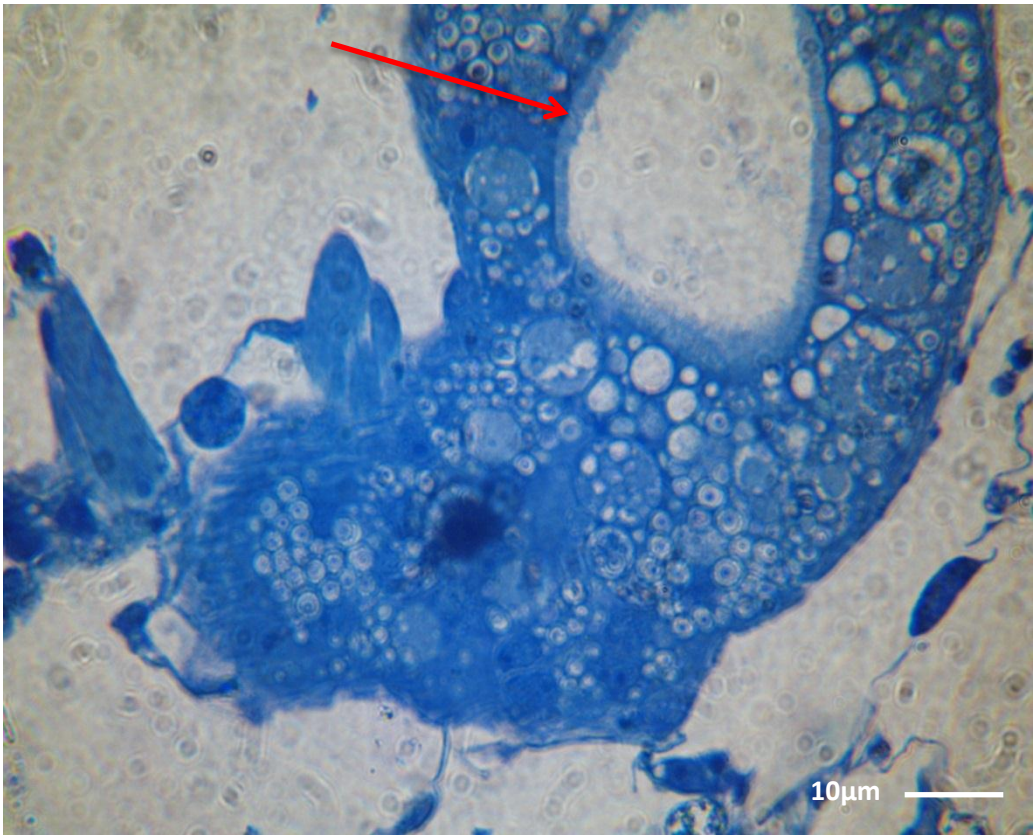

Figure 16: 10mm crab, 1000X magnification. Red arrow: microvilli in gut.

No gonad or gamete tissue was identified through transmission electron microscopy although components of digestive tissue were, meaning it was searched for in the correct location. In Figure 17, a TEM photo at 6,700 magnification, a spherite found in the acidophils of the hepatopancreas used for digestion can be seen. Identifications made by comparison to the work of Fahrenbach (1988).

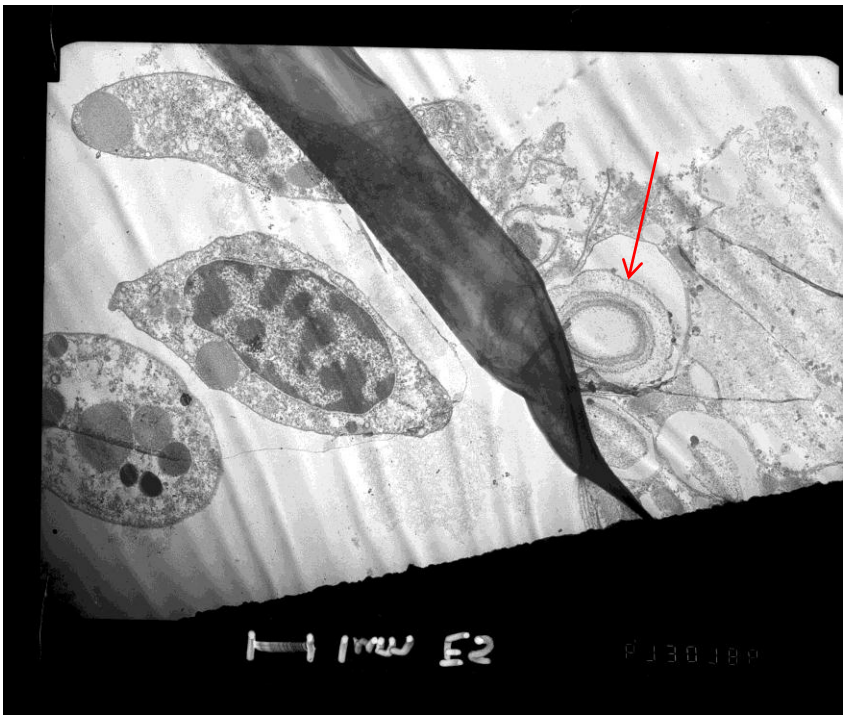

Figure 17: TEM photo at 6,700X magnification of first year juvenile; red arrow: spherite in acidophils of hepatopancreas used for digestion

Discussion

Size and Sex distribution in 3-year old Little Sippewissett Salt Marsh cohort

In the three year old cohort of *Limulus* gathered from Little Sippewissett Salt Marsh in West Falmouth, MA (Figure 9) there was no discernable difference in size distribution by sex. A T-test obtained a p-value of 0.72 indicating a 70% probability that the sizes in female and male crabs are not different. This data indicates that the size difference in mature females and males is not due to differential growth in crabs of three years of age or less, refuting the original hypothesis of this experiment. It lends support to the hypothesis that female crabs are larger as a function of the extra molt they undergo as compared to males before reaching maturity. However, it is a possibility that crabs size may begin to diverge after three years of age. Further studies must be done on crabs of age 3-9 years in order to rule out the possibility that there is in fact differential growth in juvenile stages after three years of age. Furthermore, the source of information about the additional molt cycle in females that could result in their larger size needs to be verified.

It was also found for this cohort that there were 71 sexable males and 68 sexable females. This is a female to male ratio of 1:1, disproving the hypothesis that sex ratio differences seen in adults are displayed in juvenile horseshoe crabs. It lends support to the research of Rudloe (1980) and Swan (1998) that theorized that the sex ratio in adult horseshoe crabs is in fact 1:1 and the difference in ratios found in other research is due to the fact that differing numbers of males and females are found in different locations such as shoreline mating areas as compared to deeper waters. In this study the molts were collected form a contained area, a pool in a salt marsh, before the crabs move out to deeper waters and may redistribute themselves by sex. Therefore, it should be an accurate measurement of the sex ratio at this age and it can be said with confidence the sex ratio for 3 year old *Limulus* is 1:1. Further research needs to be completed on horseshoe crabs of age 3-9 years in order to rule out the possibility that there is a difference in sex ratio after three years of age.

Sexing by genital operculum

It was found that crabs can only be sexed with confidence down to 10mm by use of the genital operculum method. Even when the genital operculum is backlit, in crabs of fewer than 10mm, gonorpores and papillae are indistinguishable. Removing the genital operculum and observing it under a light microscope did not enhance gonopore and papillae visibility.

Spurrs resin vs. Epon Araldite

Spurr's resin was found to be better to embed the tissue with as compared to Epon Araldite. It produced less chatter when sliced on the microtome allowing for better visualization of tissue structure under the light microscope. Epon Araldite was also found to be more difficult to section on the microtome in general as it was a bit softer and if the tissue was not dehydrated, embedded, and cured in an exact and specific manner it was often unable to be cut on the microtome.

Developing gonads and gametes in juvenile horseshoe crabs

In the juvenile horseshoe crab tissue examined comparisons were made to photographs taken in research conducted by Fahrenbach (1988) and to the gonad and gamete tissue of the mature male and female that were biopsied and analyzed via light and electron microscopy to determine whether developing gonad or gamete tissue was present. Developing oogonia and vitellogenesis were seen in an immature female of 203mm (Figure 13) and possible germ cells were found in three year old female crabs of 50+mm (Figure 15). No developing gonad or gamete tissue was found in any male juvenile horseshoe crabs or in female juveniles less than three years of age. The 5mm, 8mm, 10mm, 12mm, and 15mm horseshoe crab tissues all displayed no reproductive tissue even though gut tissue was present in the section. Gut tissue was found in all crabs examined indicating that we were searching in the correct location for developing reproductive tissue. The fact that no developing gonads and gametes were found in juvenile males of any age or in females of less than three years of age does not mean it is not present in this age group at all, it simply means it was not found in our research. It may be possible

because the research is conducted on such a tiny scale, in sections of at most 0.5 µm thicknesses, that reproductive tissue was simply missed. It is also possible that reproductive tissue that was present may not have been seen due to the orientation of the tissue in the resin. It can be said with a reasonable measure of confidence that reproductive tissue is not found in horseshoe crabs in their first year of life (5-15mm) as that is where much of this project's research was focused. Many crabs in that age group were dissected and analyzed and no reproductive tissue was found in any of them. In crabs beyond the first year of life it cannot be said that they do not have reproductive tissue, as only a few juveniles above this age were analyzed. In order to state with confidence that they lack reproductive tissue further research would need to be conducted on a larger number of crabs.

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Relationship of ACh and AChR in Horseshoe Crab Muscle

A Major Qualifying Project Report

Submitted to the faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

_____

Natalie Eaton

April 28, 2011

Approved by:

_____

Doctor Daniel Gibson, Advisor

Abstract

Previous MQPs have indicated that ACh is a neurotransmitter in horseshoe crab peripheral nerves near muscle. However ultrastructural confirmation of its presence in neuromuscular synapses has been elusive. Antibodies against acetylcholine and against acetylcholine receptors were used with appropriate secondary antibodies for fluorescence microscopy, laser-scanning confocal microscopy, and transmission electron microscopy. Immunofluorescence and fluorescence light microscopy results placed ACh and AChR in the vicinity; TEM showed nerves in position hypothesized by Fourtner and Sherman.

Acknowledgements

I would like to thank Dr. Daniel Gibson for being my MQP advisor and providing me his knowledge, patience, and guidance throughout this project. I would additionally like to thank Dr.Michael Buckholt and Abbie white for providing required materials and equipment and Vicky Huntress for her assistance with the confocal microscope.

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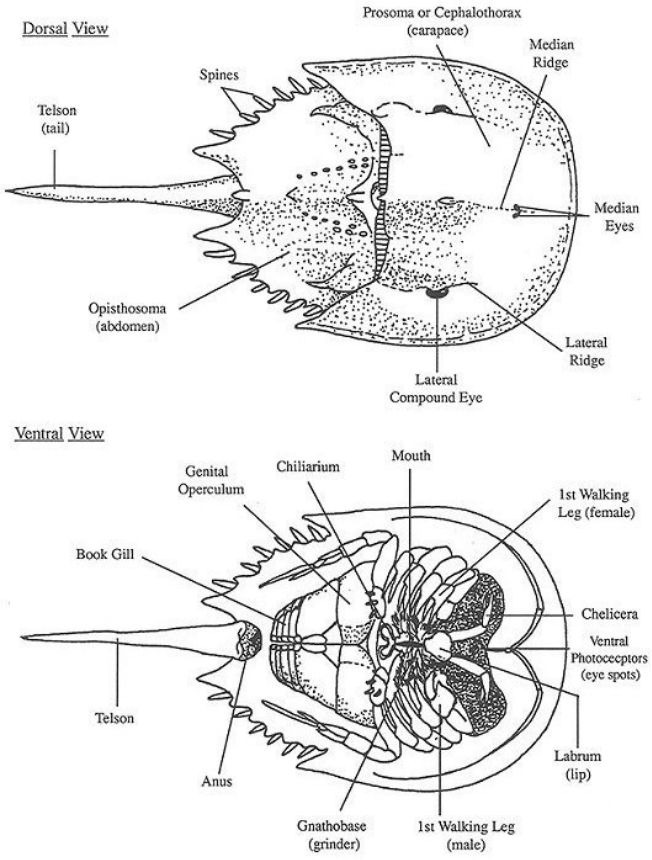
Introduction

The evolutionary divergence between vertebrates and horseshoe crabs was well established 500 million years ago, but both have the same physiological necessity when it comes to making muscles contract: nerves must release a stimulating chemical and the muscle must respond to it. This project explored neuromuscular transmission in the horseshoe crab, examining its chemical nature and its ultrastructure.

The Horseshoe Crab

The horseshoe crab is a member of the Arthropod phylum which means articulated body and limbs. There are three major classes of Arthropods: Insects, Arachnids, and Crustaceans. The horseshoe crab is part of its own class called Merostomata meaning "legs attached to the mouth". Even though horseshoe crabs are called "crabs", their taxonomy shows otherwise. They are closely related to spiders and have the same arrangement of paired pedipalps, four pairs of walking legs, jawless mouths, and pre-oral *chelicerae* to assist with feeding (Figure 1). There are currently four species of horseshoe crabs, and in this project the species *Limulus polyphemus* was used. *Limulus polyphemus* can be found along the eastern coast of North and Central America. The other three species are Asian: *Tachypleus gigas, Tachypleus tridentatus,* and *Carcinoscorpius rotudicauda*. All of these species are similar in morphology, ecology, and serology. The basic anatomy of *Limulus* has not been through significant change in the past 360 millions years and it is possible that its physiology has stayed unchanged as well (Horseshoe Crabs, Ecological Research & Development Group; Shuster et al, 2003).

Figure One. Top and Bottom drawing of Horseshoe Crab



(http://njscuba.net/zzz_biology/anatomy_horseshoe_crab_ext.jpg)

Horseshoe crabs have played an important role in the pharmaceutical and medical device industries by guaranteeing that intravenous drugs, vaccines, and medical devices are free from bacterial contamination. The Horseshoe crab blood is used in the production of the Limulus Ameobocyte Lystate (LAL) assay. The assay works by adding solutions of components of lysed Limulus blood cells that coagulate in the presence of bacterial endotoxins. This assay as a result is very reliable and quick in determining bacterial contamination (Horseshoe Crabs, Ecological Research & Development Group).

Horseshoe crabs have also played an important role as models for vision research. In 1926 H. Keffer Hartline studied electrical impulses from the optic nerve of horseshoe crab eyes

and the results from these studies helped in the discovery of certain functions of the human eye. This research won a Nobel Prize in medicine in 1967 (Horseshoe Crabs, Ecological Research & Development Group).

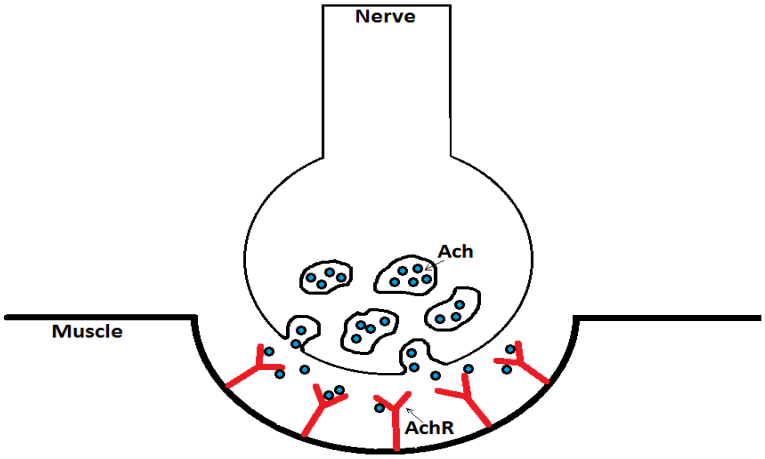
Horseshoe crab muscles and nerves have been researched (Rane and Wyse, 1987; Sherman and Fourtner, 1972; Lasovsky, 1998), but some unanswered questions remain about muscle excitation and neuromuscular transmission in *Limulus*: What is the transmitter? What is the pattern of innervation? Most of the reported findings are still short of definitive.

Neuromuscular Junctions (NMJ)

Vertebrates: Muscle cells are stimulated by motor neurons that regulate body movements. The axons divide when entering the muscle and every axon terminal has short, curling branches that form a neuromuscular junction with a single muscle fiber. Each muscle fiber has at least one neuromuscular junction that is located in the middle of the fiber's length. There remains a space called the synaptic cleft between the nerve and the muscle fiber. At the axon terminal there are synaptic vesicles which are small membrane bound sacs that contain neurotransmitters such as acetylcholine (ACh). The motor end plate, which is the "trough like" region of the muscle fiber's sarcolemma, is highly folded. These folds increase the surface area where the neurotransmitter receptors (example, Acetylcholine receptors (AChR)) are located. When an impulse travels through a nerve and reaches the end, voltage-gated calcium channels in the nerves membrane open, allowing the flow of calcium into the nerve. The presence of calcium results in the fusion of the synaptic vesicles containing neurotransmitters with the terminal end of the axon and the neurotransmitters are released into the synaptic cleft via exocytosis. The released neurotransmitters then diffuse across the synaptic cleft and bind to the

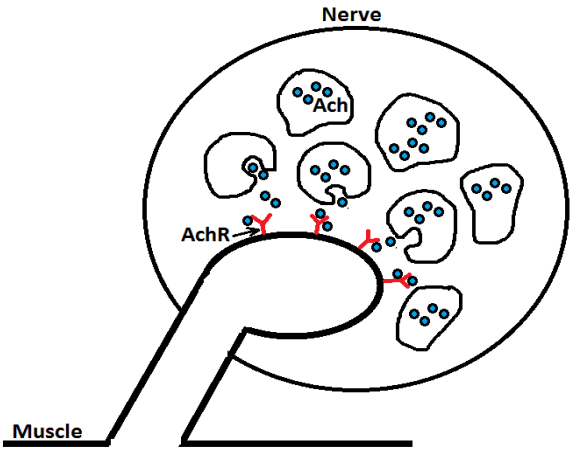
receptors on the sarcolemma. At the conclusion of this event, the neurotransmitters are broken down by the enzyme Acetyl Cholinesterase to prevent the continued muscle fiber contraction (Marieb et. al., 2007).

Figure Two. Typical Neuromuscular Junction



Horseshoe Crab NMJs: The previously mentioned neuromuscular junction is a typical model in vertebrates; however Fourtner and Sherman (1972) formulated a different structure of the neuromuscular junctions in *Limulus polyphemus*. They discovered that the synapses of the walking leg muscles of *Limulus* consisted of sarcoplasmic evaginations that are penetrated by numerous axon branches. Since the nerves occur on evaginatinos of muscle fibers, it makes them more difficult to locate (Sherman and Fourtner, 1972).

Figure Three. Neuromuscular Junction Proposed by Sherman and Fourtner



Neurotransmitters

Glutamate has been determined to be the main excitatory neurotransmitter in the PNS of arthropods (Atwood and Cooper, 1996). Shupliakov and colleagues explored what the main neurotransmitter is in the peripheral nervous system (PNS) of arthropods by treating abdominal and leg motor neurons of crayfish with anti-glutamate antibody and secondary antibody with colloidal gold balls. The gold balls were found to collect on the synaptic vesicles of the nerves which provided evidence that glutamate was the neurotransmitter in the NMJ of arthropods (Shupliakov, et. al., 1995)

Studies on neurotransmitter actions in *Limulus* abound; there is agreement on most of the findings about CNS and sensory neurons, but the question about which transmitter is the natural one at neuromuscular junctions has not been answered to our satisfaction. A summary of neurotransmitter research in *Limulus* follows.

The abdominal ganglia of *Limulus* were stimulated with possible neurotransmitters by James and Walker (1979). They discovered that carbachol, ACh, nicotine, and octopamine were possible candidates since they excited the nerves; however, the nerves were inhibited by GABA, noradrenaline, and dopamine. Additionally glutamate, 5-HT, and histamine either

excited/inhibited some cells or caused no effect. They concluded that ACh and GABA were important neurotransmitters in the central nervous system of *Limulus*. As for the sensory neurotransmitter they concluded that ACh was the main neurotransmitter. (James and Walker, 1979).

Rane in 1987 investigated the neurotransmitter in the NMJ of *Limulus* and hypothesized that it didn't use the main neurotransmitter of a majority of arthropods, glutamate. They found that the release of glutamate was inhibited by pentobarbital, which blocks postsynaptic muscle excitation, when the motor nerve was stimulated electrically, but aspartic acid was not inhibited. This indicated that glutamate was released postsynaptically and asparagine was released presynaptically. Asparagine being presynaptically released supports that it could be the main excitatory neurotransmitter in *Limulus* neuromuscular junctions (Rane, 1987). Even though Rane and Wyse concluded that Asparagine was the primary neurotransmitter in *Limulus* neuromuscular junctions, Kin Futamachi in 1972 provided the groundwork for the suggestion that ACh is instead the primary neurotransmitter.

Futamachi proposed that ACH was the primary neurotransmitter in crayfish muscles. She observed that when tonic flexor muscles of the crayfish abdomen were treated with ACh via iontophoresis, the muscle experienced depolarization. When an abundance of ACh was added to the muscle, desensitization of the NMJ was observed. Futamachi concluded that crustaceans could use two independent transmitter systems for the excitation of skeletal muscle and could utilize glutamate and ACh as excitatory neuromuscular transmitters (Futamachi, 1972).

In 1982, Rane and Wyse found evidence of ACh in the peripheral system of *Limulus*. They discovered that the treatment with ACh resulted in claw closure and impulses in the leg nerve in a dose-dependent approach. When KCl was applied, the impulses in the leg nerve were

blocked, but claw closure was not affected. Additionally a low calcium concentration solution blocked contractions of the muscle but not the impulses in the nerves. This evidences results in the conclusion that ACh stimulates the nerves in the leg (Rane and Wyse, 1982). So, although Rane and Wyse place ACh at the scene, they do not consider it a candidate for stimulating muscle, only for stimulating nerves that stimulate muscle, holding fast to Rane's (1987) contention that the NMJ transmitter is aspartic acid.

Previous MQPs at Worcester Polytechnic Institute have explored the possible primary neurotransmitter in the NMJ of horseshoe crabs and provided evidence that ACh is the primary neurotransmitter.

Yip Wong used a histochemical approach to demonstrate the presence of ACh and AChR at the NMJ. Sections and whole mounts were treated with fluorescent, enzymatic, or electron-opaque enzymes. She was able to demonstrate through fluorescence the presence of ACh and AChR inside the nerves but not near muscle fibers (Wong, 2010).

Malozzi used pharmacology, electrophysiology, and antibody staining to investigate if ACh was the main excitatory neurotransmitter in *Limulus polyphemus.* She videotaped claw closure from nerve stimulation and added methyllycaconitine, an acetylcholine antagonist which resulted in muscle paralyzation (Malozzi, 2005).

Rory Fuller, Chris Vacher, and Yip Wong all conducted separate MQPs and injected juvenile horseshoe crabs with anti-AChR which resulted in paralysis. However, the paralysis of the horseshoe crab could be at the CNS level because ACh is known to be the primary neurotransmitter in the CNS of *Limulus polyphemus* (Fuller, 2006; Vacher, 2007; Wong, 2010)

Hypothesis for the Project

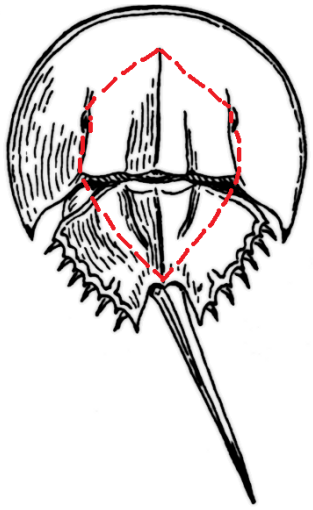
We hypothesized that Acetylcholine is the neuromuscular transmitter in *Limulus.* We set out to provide evidence for the presence of ACh and AChR in the NMJ of *Limulus polyphemus*, in support of our hypothesis. Immunohistochemistry was utilized, with antibodies against ACh and AChR . Appropriate secondary antibodies were used to detect the presence of ACh and AChR at the NMJ by transmission electron microscopy (TEM), confocal microscopy, and fluorescence microscopy

Materials and Methods

Dissection and Specimen Preparation for acrylic embedding

Whole, live 5mm horseshoe crabs were placed in a Petri dish with seawater and injected with 4% formaldehyde in 100 milliMolar phosphate buffer at pH 7.4, using a 0.5cc Insulin syringe (31 gauge needle) into the soft tissue found at the hinge of the crab. Care was taken not to push the needle all the way through the crab. The crab was held into place using tweezers in order to make the injection easier. Since the nervous system is ensheathed in an artery from the heart, the injection provides a direct and fast delivery of the formaldehyde. The injection was performed correctly if the crab swelled up due to the entry of the fluid. After fixation with 4% formaldehyde, the top of the shell was removed by cutting from the median eye, along side the eyes, to the back of the crab. Once the hinged piece of shell had been removed, the hinge muscle attached to the hinge was removed using tweezers.

Figure Four: Removal of Horseshoe Crab Hinge



Previous MQPs used leg muscle for investigation of NMJ in horseshoe crabs, but hinge muscle was used in this MQP because it is straighter, more parallel, and longer than any single leg muscle. The extirpated muscle was transferred to 4% buffered formaldehyde in glass vials and

refrigerated until the embedding step, when it was removed to a buffer rinse and dehydrated with ethanol.

Preparation of sample in Acrylic Resin

The samples put in acrylic resin were used for confocal microscopy and fluorescence microscopy. Acrylic resin was used instead of an epoxy because acrylic resin doesn't require the extra step of removing the resin for treatment of sample with fluorescence antibodies. Acrylic resins are more hydrophilic and therefore presumed to better preserve antigenicity.

The hinge muscle samples were removed from the 4% formaldehyde and soaked in 70% ethanol three times at 10 minutes each in order to dehydrate the muscle. The acrylic resin was made of 0.2g benzoyl peroxide paste (catalyst), 2 mL of methyl methacrylate monomer, and 8 mL of n-butyl methacrylate. The muscle tissues were soaked in 1:3, 1:1, and 3:1 resin to ethanol ratios for 1 hour in each different ratio at 4°C. The samples were then placed in an eppendorf tube filled with 100% resin and placed in an oven at 65°C-70°C overnight to cure. All resin transfers were performed in a fume hood due to the strong smell of uncured resin. Any used equipment or materials that came in contact with the resin was placed in the oven at 65°C-70°C overnight where it would cure and become inert. The following day the samples in the resin were removed from the eppendorf tube using a hammer blow to the tip of the tube to loosen the resin, a razor blade to cut off the tip of the tube, and a wooden applicator stick in the cut-off tip to push the cured resin out of the tube.

Preparation of sample in Spurrs Resin

Samples cured in spurrs resin were used for electron microscopy. Their first fixation was in either 4% buffered formaldehyde or 2% buffered glutaraldehyde, which is a better protein crosslinker than formaldehyde but may interfere with antigenicity . The aldehyde-fixed samples

were buffer rinsed and then fixed in 1% buffered osmium tetroxide for one hour. The osmium tetroxide oxidizes and fixes the double bonds in unsaturated fatty acids. As the osmium becomes reduced it changes from clear to brown/black and provides stability and electron density to lipids.

Spurrs resin is a low viscosity epoxy mixture of 10g VCD (vinylcyclohexene dioxide), 6g DER 736 plasticizer, 26g NSA (nonenoyl succinic anhydride), and 0.4g DMAE (dimethylaminoethanol). Unused mixture was kept in an airtight container in a freezer for future use.

The muscle tissues were soaked in 1:3, 1:1, and 3:1 resin to ethanol ratios for 1 hour in each different ratio at 4°C. The samples were then placed flexible molds of polyethylene or silicone rubber and cured at 65°C-70°C overnight. All resin transfers were performed while wearing gloves, in a fume hood, due to the strong smell of uncured resin and undetermined carcinogenicity of some of the uncured components.. Any used equipment or materials that came in contact with the resin, were cured 65°C-70°C overnight, which rendered them inert for easy and safe disposal. The following the molds were cut off or peeled away from the cured resin.

Figure Five: Hinge Muscle Samples in Spurrs Resin

Making sections on Microtome

Samples were trimmed using a jeweler's saw and industrial razor blades in order to remove excess resin around the tissue sample. The samples were trimmed so that the muscle would be cut either perpendicularly or parallel to the fibers. The best type of angle for find the NMJ on the muscle fibers was to cut parallel with the fiber. The sample was then mounted on a Sorvall Porter-Blum MT Ultra Microtome. Diamond and glass knives were used to cut the sample at different thicknesses. The diamond knife was used in order to cut thinner and more uniform sections. Resin slices float off the knife into a water reservoir called a boat. These sections can then be removed by surrounding them with a wire loop that picks them up in a film of water, or by dipping them out on a metal mesh grid, depending on the intended use.

The "semithin" sections about 0.5 μm thick were placed on Superfrost Plus Slides using a wire loop. The slides were placed on a hot plate for 1 minute at 80°C in order to dry the sample to the slide and evaporate any water. When the slide was dry, the sections were stained with 1% Toludine blue for 1 minute on the hot plate. The toludine blue serves as a contrast agent, and was added to the sections using a syringe filter. The slides were then rinsed with distilled water and placed on the hot plate to dry. Once dry, the sections were observed under the light microscope in order to see if the hinge muscle was presence in the cut section.

Sections used for the electron microscope were cut 60-90nm in thickness and placed on nickel grids. Sections used for fluorescence microscopy were cut 0.5μm in thickness and placed on a slide.

Figure Six: Cutting of Muscle Tissue Sample on Microtome. The block of resin (red arrow) in a metal chuck is carried by the microtome mechanism past the stationary knife (blue arrow), in this case a diamond knife.

Staining for fluorescent light microscopy

Rabbit anti-ACh

Five cut tissue sections (without osmium tetroxide in acrylic resin) were placed on two slides each. All the sections were blocked with normal goat serum (NGS) for thirty minutes and then rinsed with PBS for 10 minutes. "Blocking" is a pretreatment with non-immune serum from the animal that supplies the secondary antibody. It blocks later nonspecific binding of antibodies to naturally "sticky" sites. Both middle three sections on both slides were soaked with 1:10 dilution of Rabbit anti-ACh and the first and last sections were soaked with a 1:10 dilution of normal rabbit serum (NRS) as controls. The two slides were incubated at 4C overnight in a humidified chamber made from a sealed petri dish with moistened paper inside.

The next day, all the samples were soaked in PBS three times at 10 minutes each. All samples on one slide were soaked with a 1:10 dilution of goat anti-rabbit biotinylated secondary

antibody from the ABC peroxidase staining kit with PBS for thirty minutes. The samples were then rinsed twice at 10 minutes each in PBS and then soaked in ABC reagent for thirty minutes. The ABC reagent is composed of 45 µL of reagent (avidin), 45 µL of reagent B (biotinylated horseradish peroxidase), and 135µL of PBS. The slides were then rinsed in PBS twice at 10 minutes each and then were incubated in peroxide buffer and metal-enhanced Diamino-benzide (DAB) for seven minutes. If DAB turns black/brown, it means that horseradish peroxidase is releasing oxygen from the peroxide. The only way that HRP would be present is if the primary antibody is bound to the antigen and the secondary is bound to it and the HRP to it. Because the primary antibody was against ACh, brown product would indicate that ACh is present. The slide was then rinsed two more times in PBS at 10 minutes each and then rinsed in distilled water for 3 minutes. After rinsing, the slide was then observed under the light microscope.

All the samples on the second slide were treated with 1:10 dilution of Invitrogen Goat anti-Rabbit Alexa Fluor 488® in phosphate buffer saline (PBS) for thirty minutes. All the samples were then rinsed with PBS twice for 10 minutes each. The samples were all counterstained with a 1:10 dilution of DAPI with distilled water for 5 minutes and then covered with a 24 X 60mm cover slip in order to be viewed under the fluorescence microscope. DAPI is a nucleophilic dye that fluoresces in ultraviolet light, at wavelengths that do not interfere with the desired fluorescence but will allow observation of surrounding structures by switching to the ultraviolet filter set on the microscope.

Mouse anti-AChR

The anti acetylcholine receptor antibody that we used was a monoclonal antibody made in mice against the alpha subunit of human acetylcholine receptors. As overly specific as that might seem, several MQPs (Wong, Fuller, Vacher) had success paralyzing horseshoe crabs with

this antibody, even though it is made against a human antigen. The antibody was used to determine if sectioned muscle contained acetylcholine receptors.

Five cut tissue sections (without osmium tetroxide, in acrylic resin) were placed on two slides each. All the sections were blocked with normal goat serum (NGS) for thirty minutes and then rinsed with PBS for 10 minutes. Both middle three sections on both slides were soaked with 1:10 dilution of Mouse anti-AChR and the first and last sections were soaked with a 1:10 dilution of normal mouse serum (NRS). The two slides were placed in a Petri dish and placed in the refrigerator overnight with a wet paper towel covering them in order to prevent them from drying out. The next day, all the samples on both slides were soaked in PBS three times at 10 minutes each.

All the samples on the first slide were treated with 1:10 dilution of Invitrogen Goat anti-Mouse Alexa Fluor 555® in phosphate buffer saline (PBS) for thirty minutes. All the samples were then rinsed with PBS twice for 10 minutes each. The samples were all counterstained with a 1:10 dilution of DAPI with distilled water for 5 minutes and then covered with a 60mm cover slip in order to be viewed under the fluorescence microscope.

All samples on the second slide were soaked with a 1:10 dilution of poly-HRP with PBS for thirty minutes. The slides were then rinsed in PBS twice at 10 minutes each and then were incubated in peroxide buffer and metal-enhanced Diamino-benzide (DAB) for seven minutes. If the oxidized DAB turns black/brown, then the reagents are bound and AChR is present in the sample. The slide was then rinsed two more times in PBS at 10 minutes each and then rinsed in distilled water for 3 minutes. After rinsing, the slide was then observed under the light microscope.

Staining for Transmission Electron Microscopy (TEM)

Gold ultra thin sections that were placed on the nickel grids were etched with 10% hydrogen peroxide in distilled water to remove osmium tetroxide, which would interfere with antibody binding. The drops were placed on dental wax and the grids were placed onto the drops shiny side down for five minutes. The grids were then rinsed three times in distilled water for three minutes each and then five minutes for the third rinse. Then the grids were treated with block solution of 6% normal goat serum in 0.1M PBS-Tween-80 (0.05%) for thirty minutes. The grids were then rinsed three times in 0.1M PBS-Tween-80 (0.5%)-NGS (0.25%) at five minutes each. The sections were then incubated at 4°C for two hours in 1:10 dilutions of primary antibodies (Mouse anti-AChR and Rabbit anti-ACh) with 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%). Control grids however didn't get soaked in primary antibody, but were instead treated with a 1:10 dilution of NGS with 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%).

After two hours of incubation, the grids were rinsed three times with PBS solution at 5 minutes each. The grids were then soaked in a 1:100 secondary antibody conjugated to colloidal gold balls (GAM-10nm and GAR-25nm) overnight at 4°C. The grids were placed in Petri dishes and a wet paper towel covered them in order to prevent the grids from drying out. The next morning, the grids were rinsed two times at three minutes each in 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%). The sections were then rinsed five minutes in PBS and then rinsed two times in distilled water at five minutes each. The grids were then soaked in 2% glutaraldehyde for ten minutes. The purpose of the glutaraldehyde was to crosslink and stabilize the antibody complex that the colloidal gold spheres were attached to. Because the gold spheres were different sizes for the two different antibodies, it would be possible to tell which antibody was bound to which structure even though both antibodies were present. The sections were then rinsed twice with

distilled water at five minutes each and then allowed to dry on bibulous paper in order to be ready for heavy metal staining. Saturated ethanolic uranyl acetate (1:1 dH2O:EtOH) and 0.4% lead citrate in full 1.8mL microfuge tubes were spun free of sediment for 10 minutes at 10,000g in a microfuge. Drops of uranyl acetate were placed on dental wax and the grids were soaked in the uranyl acetate section - side down for five minutes. The sections were then rinsed three times in distilled water for five minutes each and then dried on bibulous paper. The grids were placed shiny side up on the dental wax and a drop of lead citrate was placed on top of the grids for 30 seconds. The grids were then rinsed three times in distilled water for five minutes each and then placed on bibulous paper to dry. The grids were finally placed in gelatin capsules individually in order to transport them to the electron microscope.

TEM Photography

Images were recorded on Kodak 4889 estar-base film with dimensions 3.5 by 4.25 inches. Film was developed in Kodak D19 developer for five minutes, rinsed with water for thirty seconds, and then fixed in Kodak rapid fix for four minutes. Once dry, the negatives were then scanned on a Micro-Tek Backlit Scanner and were converted to positives by the scanner.

Whole Mount Preparation for Confocal Microscopy

The hinge and legs were removed from 5mm horseshoe crab using small scissors. The legs were additionally cut by cutting off the toes at the second joint so that antibody solutions could enter from both ends. The hinge and legs were fixed in 4% paraformaldhyde overnight at 4°C in separate small glass vials.

The hinge and legs were then rinsed in 0.1M PB Tx (Triton X) six times in three hours. The first two rinses were five minutes apart. The 0.1M PB Tx was composed of 20mL 0.1M PB and 60µl Triton-X (0.3%) and was filtered using a syringe filter. Triton-X is a detergent that

helps solubilize tissues for antibody penetration. The hinge and legs were incubated over night in 1:40 primary antibody (Rabbit anti-ACh and Mouse anti-AChR) at 4°C. The control samples were incubated in a 1:40 dilution of NGS overnight at 4°C.

The next morning, all the samples were rinsed in 0.1M PB Tx six times in three hours with the first two rinses 5 minutes apart. The samples were then incubated overnight in the dark at 4°C in a 1:200 dilution of Alexafluor secondary antibodies (GAM and GAR). The next day, all the samples were rinsed with PB six times in three hours with the first two rinses five minutes apart. The hinge muscle was removed from the hinge and legs were then mounted on slides using GelMount and were allowed to rest for 30 minutes before being viewed on the confocal microscope. When not in use, the slides were kept refrigerated and covered with aluminum foil to prevent bleaching of the fluors.

Results

Transmission Electron Microscopy (TEM)

60-90µm thin sections were placed on nickel grids and double stained with Mouse anti-AChR and Rabbit anti-Ach. The secondary antibodies were tagged with nanogold particles with different sizes (GAM-Au 10nm and GAR-Au 25nm). Figure seven shows a transmission electron micrograph of horseshoe crab hinge muscle. The nerve is clearly seen next to the muscle fibers as proposed by Sherman and Fourtner. Unfortunately, no colloidal gold balls were observed anywhere in the sample but were expected to be present at the NMJ between the nerve and muscle fibers. If the colloidal gold balls were seen present at the NMJ, then it would provide evidence for the presence of ACh and AChR at the NMJ in the horseshoe crab's PNS.

Figure Seven. TEM 4,000X, crab hinge muscle with nerve next to muscle fibers

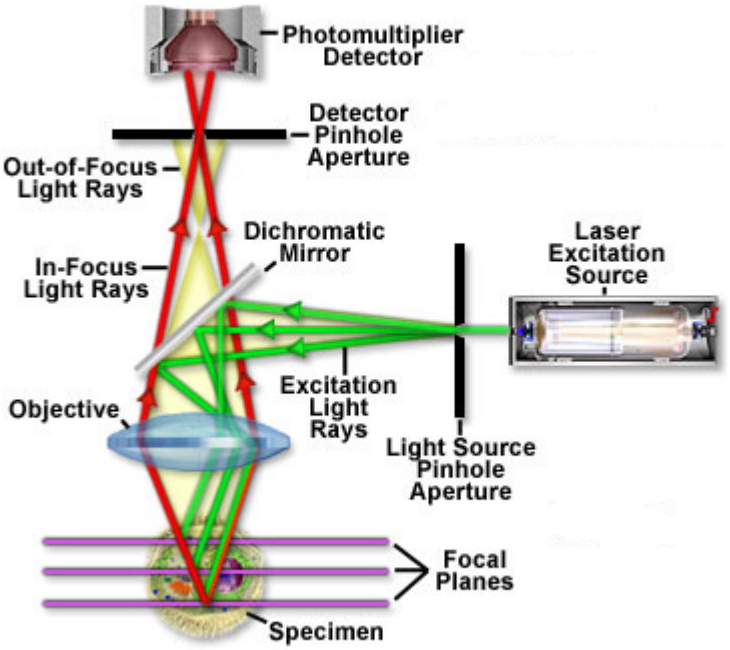
Confocal Microscopy

Fluorescence microscopy uses a high intensity light source with a specific wavelength that excites fluorescent markers in a sample. The fluorescent markers emit a longer wavelength which is a different color than the absorbed light. First a filter in the microscope only allows the

passage of radiation with the appropriate wavelength that matches the fluorescing markers in the sample. The atoms in the sample collide with the radiation and the electrons are excited to a high energy level. Light is emitted when the electrons relax to a lower level. In order for the fluorescing light to be visible, the light emitted from the fluorescing markers is separated from the brighter excitation light by a second filter. Now the fluorescing markers in the sample can be observed and fluoresce against the dark background (The Official Web Site of the Nobel Prize).

LSCM, or laser scanning confocal microscopy, excites the sample sequentially with a small, highly focused laser beam. Because detectors know the exact angle of the excitation beam as well as the fluorescence it causes, bits of data can be collected and transformed to sharpen the image and to take extremely thin optical sections that can be reconstructed (z stack) and rotated, as in figure 8.

Figure Eight. Principal Light Pathways in Confocal Microscopy



Horseshoe crab hinge muscle was treated with primary antibodies rabbit anti-ACh and mouse anti-AChR and secondary antibodies Goat anti-rabbit Alexa Fluor 488 ® and Goat anti-mouse Alexa Fluor 555 ®. Figure eight shows a whole mount of horseshoe crab hinge muscle viewed with the confocal microscope. Red fluorescence of Alexafluor 555 indicates presence of AChR and green fluorescence of Alexafluor 488 indicates presence of Ach. Figure nine shows where the ACh and AChR are being detected and the presence of the ACh and AChR are in the vicinity of the muscle fibers.

Figure Nine. Whole mounted horseshoe crab hinge muscle stained with Goat anti-rabbit Alexa fluor 488 ® and Goat anti-mouse Alexa Fluor 555 ® after treatment with Rabbit anti-ACh and Mouse anti-AChR viewed on confocal microscope.

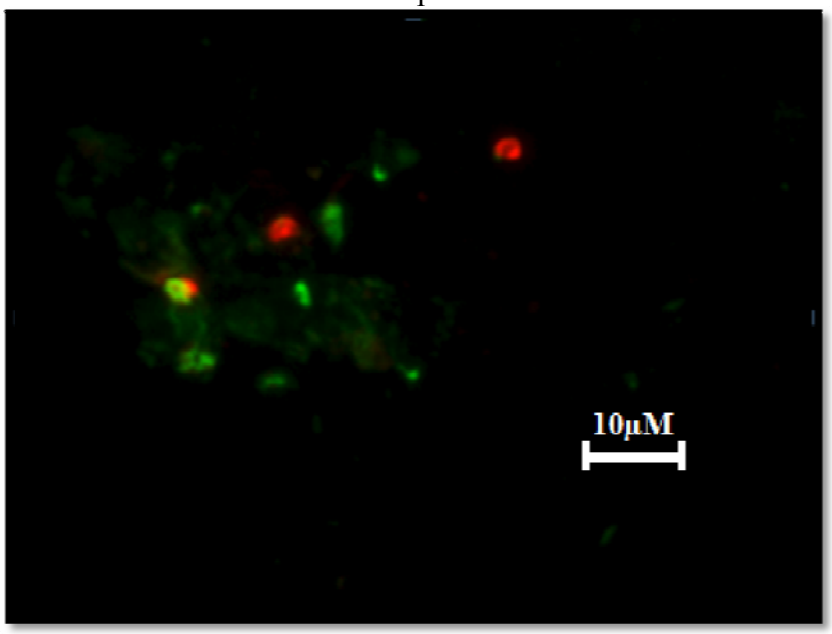
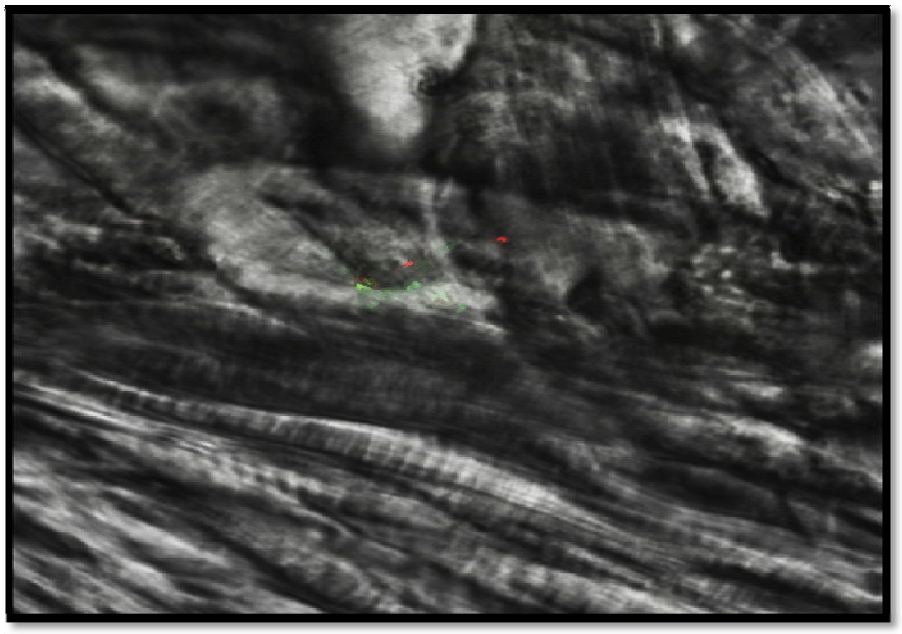


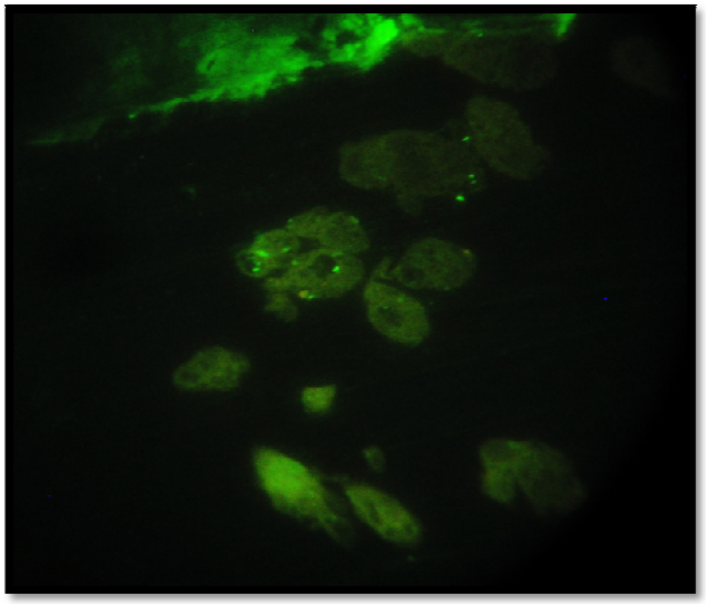
Figure Ten. Whole mounted horseshoe crab hinge muscle stained with Goat anti-rabbit Alexa fluor 488 ® and Goat anti-mouse Alexa Fluor 555 ® after treatment with Rabbit anti-ACh and Mouse anti-AChR viewed on confocal microscope. This is an overlay micrograph, showing the muscles as they appear in Differential Interference Microscopy (DIC) , with the red and green fluorescence also shown, but weakly. This is the same fluorescent complex as in Figure 8.



Fluorescent Light Microscopy

Tissue sections of horseshoe crab hinge muscle were first treated with the primary antibodies mouse anti-AChR and rabbit anti-ACh followed by the secondary antibodies Invitrogen Goat anti-mouse Alexa Fluor 555 ® and Invitrogen Goat anti-rabbit Alexa Fluor 488® in order to show the presence of ACh and AChR in horseshoe crab hinge muscle. The sections were also counterstained with DAPI to show the muscle fibers. Figure ten shows the section counterstained with DAPI and the location of the muscle fibers. Figure eleven shows a section stained with rabbit anti-ACh and GAR and the presence of ACh is shown as small green dots on the edge of the muscle fibers.

Figure Eleven. Section of nerve-muscle complex from horseshoe crab hinge muscle. Section stained with rabbit-ACh and GAR Alexafouor 488. The small bright spots are the sites of anti-ACh binding, where nerves would be located next to muscle. Each spot is 1-2 μm in diameter



Discussion

The purpose of this MQP was to provide evidence for the presence of ACh and AChR in the NMJ of *Limulus polyphemus*. Three methods were used to detect the presence of ACh and AChR at the NMJ; they were transmission electron microscopy (TEM), confocal microscopy, and fluorescent light microscopy. The confocal microscopy and fluorescent light microscopy samples were immunostained with two primary antibodies; Rabbit anti-ACh and Mouse anti-AChR. These samples were also treated with fluorescent secondary antibodies GAM and GAR. The electron microscopy samples however used the same primary antibody as the confocal and fluorescent light microscopy samples but the secondary antibody was linked to colloidal gold balls instead. The electron microscope was successful in showing the NMJ proposed by Sherman and Fourtner and the confocal microscopy and fluorescent light microscopy were successful in showing the presence of ACh and AChR in the vicinity of the muscle fibers.

Acetylcholine (ACh) and acetylcholine receptors (AChR) in vicinity of muscle fibers

Even though the confocal microscopy and fluorescent light microscopy wasn't able to show ACh and AChR at the NMJ, these methods were able to show that ACh and AChR were in the vicinity of the muscle fibers. Figure nine shows the green (ACh) and red (AChR) fluorescent spots of the Invitrogen Alexa Fluor 488 ® and Invitrogen Alexa Fluor 555® secondary antibodies fluorescing near the muscle fibers. These fluorescent spots indicate that AChR and ACh are present and since there are near the edge of the muscle, they could be where an axon is located. Figure Ten also shows ACh is in the vicinity of the muscle fibers since green spots can be seen fluorescing near the edge of the muscle fiber. Both these figures provide evidence that ACh and AChR are present near the muscle fibers which indicates that the NMJ should also

contain ACh and AChR. A closer image of the NMJ needs to be taken with the TEM in order to show the presence of ACh and AChR at the NMJ.

Observation of NM J with TEM

The goal of the TEM was to use colloidal gold balls of different sizes (GAM-10nm and GAR-25nm) in order to show the presence of ACh and AChR at the NMJ of horseshoe crabs. Figure seven shows a transmission electron micrograph at 20,000X of a NMJ. The muscle fibers are running parallel with the photograph and the nerves can be seen sitting on top of the muscle. Unfortunately no colloidal gold balls were seen at the NMJ or anywhere else in the grid. This may be because the colloidal gold balls weren't able to penetrate the section or the colloidal gold balls were not properly added to the section. Even though no colloidal gold balls were found in the section, the image in figure seven clearly shows the NMJ proposed by Sherman and Fourtner for *Limulus polyphemus*. They proposed that the NMJ for *Limulus polyphemus* consisted of sarcoplasmic evaginations that are penetrated by numerous axon branches. The NMJ in this image shows no synaptic cleft, space between the muscle fiber and nerve, as would be expected of *Limulus Polyphemus* NMJs since the muscle evagination penetrates the axon.

Future Experiments

Figure seven shows a beautiful image of the NMJ of *Limulus polyphemus* as proposed by Sherman and Fourtner, however the colloidal gold balls were nowhere in sight. A future project could again use horseshoe crab hinge muscle, testing more concentrations of primary and secondary antibodies. A positive control of vertebrate tissue (Frog or goldfish muscle) should precede any further TEM observations using the horseshoe crab. We know that the primary antibodies bind appropriately but not what the ideal concentrations of the secondaries should be. What works for confocal did not work for TEM, but it seems to just be a question of dosage.

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Empendymin in Horseshoe Crab Nerve Cords

A Major Qualifying Project Report

Submitted to the faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

_____

Sean O'Donnell

April 28, 2011

Approved by:

_____

Doctor Daniel Gibson, Advisor

Abstract

Ependymin is a vertebrate nerve growth factor that protects cultured neurons from Alzheimer's-like changes (Stovall, WPI Master's Thesis, 2006). A new antibody against an Epn octapeptide, KKETLQFR demonstrated Epn in the neuropil of juvenile horseshoe crabs (Dionne and Krzyzewski, MQP 2008). We tried to locate Epn ultrastructurally, using transmission electron microscopy with colloidal gold-tagged secondary antibodies, unsuccessfully. However, on the light microscope level, Epn was found for the first time among cell somata in the CNS, using horseradish peroxidase secondary antibodies. Fluorescence microscopy, both conventional and confocal, produced equivocal results.

Acknowledgements

I would like to thank Dr. Daniel Gibson for being my MQP advisor and providing me his knowledge, patience, and guidance throughout this project. I would additionally like to thank Dr. Michael Buckholt, and Abbie White for their assistance and for providing required materials and equipment. I would like to thank Vicki Huntress for her patience and help in running the confocal microscope. I would like to thank previous MQP students for the guidance that their projects provided.

Contents

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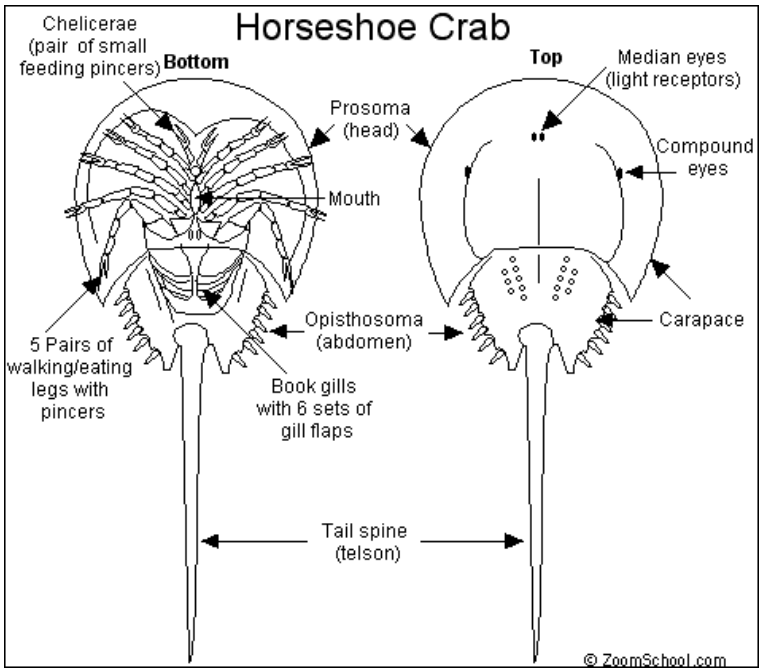
Introduction

Horseshoe Crabs

Horseshoe crabs, *Limulus polyphemus* are a member of the arthropod phylum meaning articulated body and the Merostomata class meaning thigh mouth, referring to the attachment of its legs around its mouth (Pisani et al., 2004). The horseshoe crabs although called crabs are not closely related to the crab and in fact are more closely related to scorpions and spiders with which they share these characteristics: chelicerae, 4 pairs of legs, book gills, and pedipalps. Horseshoe crabs have been living on earth for more than 360 million years and have changed very little over this time and are therefore often called living fossils. The species of horseshoe crab that was studied for this experiment was *Limulus polyphemus* the species native to the east coast of northern and central America. The other three species of horseshoe crab, *Tachypleus gigas, Tachypleus tridentatus,* and *Carcinoscorpius rotudicauda*, all live on the coast of Asia (Horseshoe Crabs, Ecological Research & Development Group).

Figure 1: Anatomy of *Limulus polyphemus*



http://www.enchantedlearning.com/subjects/invertebrates/arthropod/Horseshoecrab.shtml

Horseshoe crabs have four stages of their life cycle: egg, larval, juvenile, and adult. Adult females usually bury 4000 or more eggs in a nest in the moist sand in the intertidal zone. The length of the development period varies but is typically 14 days. Once hatched, they molt multiple times in the first year and around once per year after that. Usually they molt 16-17 times throughout the first 9-11 years of their life, and can live up to 20 years. (Horseshoe Crabs "A living fossil", Maryland Department of Natural Resources).

Horseshoe crabs have important uses in pharmacology and in physiology research. Their blood can be used in the production of the LAL (Limulus Amoebocyte Lysate) assay. This assay is used to check for the presence of endotoxins. Components of HSC blood cells are added to the substance and if bacterial endotoxins are present it will coagulate. It is a cheap and fast way of verifying the absence of bacteria from drugs, medical devices, and vaccines various compounds (McCartney et. Al., 1989). Also, HSCs are important models for

vision research, studies on the eyes of HSCs has led to the discovery of some functions of the human eye. (University of Delaware Graduate College of Marine Studies and Sea Grant, n.d.).

Ependymin

Ependymin is a neurotrophic factor that was first discovered as playing an important role in memory consolidation in goldfish. It was observed that certain proteins increased in relative percentage in the brain after learning events. It is a secreted protein that exists as a dimer and although it is known to function in memory consolidation its exact mechanism of action is unknown (Shashoua, 1976, 1991).

Project Goals

The goal of this project was to provide evidence for the presence of Epn in the central nervous system of *Limulus polyphemus,* and to provide transmission electron micrograph images indicating its presence. The method used to accomplish this goal was immunological: an antibody against Epn was then visualized by appropriate secondary antibodies for transmission electron microscopy, fluorescence microscopy, confocal microscopy, and light microscopy with horseradish peroxidase staining.

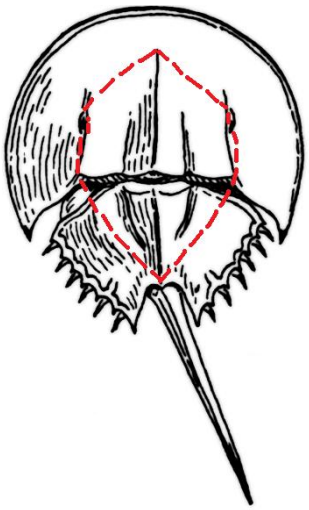
Materials and Methods

Dissection and Specimen Preparation

Primary fixative was 4% formaldehyde in 100 milliMolar phosphate buffer at a pH of 7.3 to 7.4. Juvenile horseshoe crabs in a Petri dish of filtered seawater were steadied with forceps and injected with 4% formaldehyde using a 0.5cc Insulin syringe into the hinge joint of the crab between the prosomal and opisthosomal segments. Care was taken not to penetrate through the other side. Since the nervous system is connected to the heart membrane, the injection provided a direct and fast delivery of the formaldehyde. The injection was performed correctly if the crab swelled up due to the entry of the fluid.

After fixation with 4% formaldehyde, the middorsal segment of shell was removed by cutting from their median eyes, alongside the lateral eyes, to the back of the crab. Once this hinge-containing piece of shell had been removed, the ventral nerve cord was pulled out using forceps and insect pins. The nerve cords were then placed in the refrigerator in a glass vial containing 4% formaldehyde.

Figure 2: Removal of Horseshoe Crab Hinge



Preparation of sample in Acrylic Resin

The samples put in acrylic resin were used for confocal microscopy and fluorescence microscopy. Acrylic resin (Methyl and Butyl Methacrylate, catalog #14520) was used instead of Spurr's epoxy because acrylic resin does not require the extra step of removing the resin for treatment of sample with antibodies for fluorescence or horseradish peroxidase color development. Acrylic resins are more hydrophilic and therefore preserve antigenicity better. Sellers of the acrylic resin (Electron Microscopy Sciences: emsdiasum.com) say that it cuts into sections with a rougher surface, thereby exposing more antigens.

The nerve cords were removed from the 4% formaldehyde and soaked in 70% ethanol three times at 10 minutes each in order to dehydrate the muscle. The acrylic resin was prepared by combining 0.2g benzoyl peroxide paste (catalyst), 2 mL of methyl methacrylate monomer, and 8 mL of n-butyl methacrylate. The muscle tissues were soaked in 1:3, 1:1, and 3:1 resin to ethanol ratios followed by 100% resin for 1 hour each at 4°C. The samples were then placed in an eppendorf tube in pure resin and cured overnight in a 65°C oven. All resin transfers were performed in the hood and any used equipment or materials that were exposed to the resin were placed in the oven to allow the resin to cure. The following day the samples in the resin were removed from the eppendorf tube using a hammer blow to the tip of the tube to loosen the resin, a razor blade to cut off the tip of the tube, and a wooden applicator stick in the cut-off tip to push the cured resin out of the tube.

Preparation of sample in Spurrs Resin

Samples cured in spurrs resin were used for electron microscopy. Their first fixation was in either 4% buffered formaldehyde or 2% buffered glutaraldehyde, which is a better protein crosslinker than formaldehyde but may interfere with antigenicity . The aldehyde-fixed samples were buffer rinsed and then fixed in 1% buffered osmium tetroxide for one hour. The osmium tetroxide oxidizes and fixes the double bonds in unsaturated fatty acids. As the osmium becomes reduced it changes from clear to brown/black and provides stability and electron density to lipids.

Spurrs resin is a low viscosity epoxy mixture of 10g VCD (vinylcyclohexene dioxide), 6g DER 736 plasticizer, 26g NSA (nonenoyl succinic anhydride), and 0.4g DMAE (dimethylaminoethanol). Unused mixture was kept in an airtight container in a freezer for future use.

The muscle tissues were soaked in 1:3, 1:1, and 3:1 resin to ethanol ratios for 1 hour in each different ratio at 4°C. The samples were then placed flexible molds of polyethylene or silicone rubber and cured at 65°C-70°C overnight. All resin transfers were performed while wearing gloves, in a fume hood, due to the strong smell of uncured resin and undetermined carcinogenicity of some of the uncured components.. Any used equipment or materials that came in contact with the resin, were cured 65°C-70°C overnight, which rendered them inert for easy and safe disposal. The following the molds were cut off or peeled away from the cured resin.

Figure 3: Nerve Cord Samples in Spurr's Resin

Making sections on an Ultramicrotome

Samples were trimmed using a jeweler's saw and industrial razor blades in order to remove excess resin around the tissue sample. The samples were trimmed so that the muscle would be cut either perpendicularly or parallel to the fibers. The best type of angle for find the NMJ on the muscle fibers was to cut parallel with the fiber. The sample was then mounted on a Sorvall Porter-Blum MT Ultra Microtome. Diamond and glass knives were used to cut the sample at different thicknesses. The diamond knife was used in order to cut thinner and more uniform sections. Resin slices float off the knife into a water reservoir called a boat. These sections can then be removed by surrounding them with a wire loop that picks them up in a film of water, or by dipping them out on a metal mesh grid, depending on the intended use.

The "semithin" sections about 0.5 µm thick were placed on Superfrost Plus Slides using a wire loop. The slides were placed on a hot plate for 1 minute at 80°C in order to dry the sample to the slide and evaporate any water. When the slide was dry, the sections were stained with 1% Toludine blue for 1 minute on the hot plate. The toludine blue serves as a contrast agent, and was added to the sections using a syringe filter. The slides were then rinsed with distilled water

and placed on the hot plate to dry. Once dry, the sections were observed under the light microscope in order to see if the hinge muscle was presence in the cut section.

Sections used for the electron microscope were cut 60-90nm in thickness and placed on nickel grids. Sections used for fluorescence microscopy were cut 0.5µm in thickness and placed on a slide.

Figure 4: Cutting of Muscle Tissue Sample on Microtome. The block of resin (red arrow) in a metal chuck is carried by the microtome mechanism past the stationary knife (blue arrow).

Staining for fluorescence microscopy

Five sections cut from a sample in acrylic resin and not treated with osmium tetroxide were placed on each two slides. All sections were blocked in NGS for 30 minutes. "Blocking" is a pretreatment with non-immune serum from the animal that supplies the secondary antibody. It blocks later nonspecific binding of antibodies to naturally "sticky" sites. The three middle sections on all slides were treated with at 1:10 dilution of anti-EPN antibody, the remaining two sections on each slide were treated with a 1:10 dilution of NRS. The two slides were placed in a covered petri dish with a wet paper towel and allowed to incubate in the refrigerator overnight. The following day the slides were rinsed in PBS three times for 10 minutes each.

All samples on the first slide were treated with a 1:10 dilution of GAR AlexaFluor 488 PBS for thirty minutes. All of the samples were rinsed again three times for 10 minutes each with PBS. All sections were then counterstained with a 1:10 dilution of DAPI in distilled water for 5 minutes then covered with a coverslip. DAPI is a nucleophilic dye that fluoresces in ultraviolet light, at wavelengths that do not interfere with the desired fluorescence but will allow observation of surrounding structures by switching to the ultraviolet filter set on the microscope.

All samples on the other slide were soaked with a 1:10 dilution of goat anti-rabbit biotinylated secondary antibody from the ABC peroxidase staining kit with PBS for thirty minutes. The samples were then rinsed twice at 10 minutes each in PBS and then soaked in ABC reagent for thirty minutes. The ABC reagent is composed of 45 µL of reagent (avidin), 45 µL of reagent B (biotinylated horseradish peroxidase), and 135µL of PBS. The slides were then rinsed in PBS twice at 10 minutes each and then were incubated in peroxide buffer and metal-enhanced Diamino-benzide (DAB) for seven minutes. If DAB turns black/brown, it means that horseradish peroxidase is releasing oxygen from the peroxide. The only way that HRP would be present is if the primary antibody is bound to the antigen and the secondary is bound to it and the HRP to it. Because the primary antibody was against Epn, brown product would indicate that Epn is present. The slide was then rinsed two more times in PBS at 10 minutes each and then rinsed in distilled water for 3 minutes. After rinsing, the slide was then observed under the light microscope.

Staining for Transmission Electron Microscopy (TEM)

Gold ultra-thin sections that were placed on the nickel grids were etched with 10% hydrogen peroxide in distilled water by placing drops of 10% hydrogen peroxide on dental wax and placing the grids onto the drops shiny side down for five minutes. The grids were then

rinsed three times in distilled water, twice for three minutes and once for five minutes. Then the grids were treated with block solution of 6% normal goat serum in 0.1M PBS-Tween-80 (0.05%) for thirty minutes. The grids were rinsed three times in 0.1M PBS-Tween-80 (0.5%)-NGS (0.25%) for five minutes each. The sections were incubated at 4°C for two hours in 1:10 dilutions of primary antibody (rabbit anti-ependymin) with 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%). Control grids however did not get soaked in primary antibody, but were instead treated with a 1:10 dilution of NGS with 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%).

After two hours of incubation, the grids were rinsed three times with PBS solution for 5 minutes each. The grids were then soaked in a 1:100 dilution of secondary antibody conjugated to colloidal gold balls (GAR-25nm) overnight at 4°C. The grids were placed in Petri dishes with a wet paper towel in order to prevent the grids from drying out. The next morning, the grids were rinsed two times at three minutes each in 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%). The sections were then rinsed five minutes in PBS and two times in distilled water at five minutes each. The grids were then soaked in 2% glutaraldehyde for ten minutes. The grids were then soaked in 2% glutaraldehyde for ten minutes. The purpose of the glutaraldehyde was to crosslink and stabilize the antibody complex that the colloidal gold spheres were attached to. Next, the sections were rinsed twice with distilled water at five minutes each and allowed to dry on bibulous paper in order to be ready for heavy metal staining. Saturated ethanolic uranyl acetate (1:1 dH2O:EtOH) and 0.4% lead citrate in full 1.8mL microfuge tubes were spun free of sediment for 10 minutes at 10,000g in a microfuge. Drops of uranyl acetate were placed on dental wax and the grids were soaked in the uranyl acetate section - side down for five minutes. The sections were then rinsed three times in distilled water for five minutes each and then dried on bibulous paper. The grids were placed shiny side up on the dental wax and a drop of lead

citrate was placed on top of the grids for 30 seconds. The grids were then rinsed three times in distilled water for five minutes each and then placed on bibulous paper to dry. The grids were finally placed in gelatin capsules individually in order to transport them to the electron microscope.

TEM Photography

Images were recorded on Kodak 4889 estar-base film with dimensions 3.5 by 4.25 inches. Film was developed in Kodak D19 developer for five minutes, rinsed with water for thirty seconds, and then fixed in Kodak rapid fix for four minutes. Once dry, the negatives were then scanned on a Micro-Tek Backlit Scanner and were converted to positives by the scanner.

Whole Mount Preparation for Confocal Microscopy

Nerve cords were removed from 5mm horseshoe crabs using small scissors and insect pins. The nerve cords were fixed in 4% paraformaldhyde overnight at 4°C in separate small glass vials. Next, the nerve cords were rinsed in 0.1M PB Tx (Triton X) six times in three hours. The first two rinses were five minutes apart. The 0.1M PB Tx was made by combining of 20mL 0.1M PB and 60µl Triton-X (0.3%), and was filtered using a syringe filter. The nerve cords were incubated over night in 1:40 primary antibody (Rabbit anti-EPN) at 4°C. The control samples were incubated in a 1:40 dilution of NGS overnight at 4°C. Incubations were done on a rotating platform (nutator) in the refrigerator.

The next morning, all the samples were rinsed in 0.1M PB Tx six times in three hours with the first two rinses 5 minutes apart. The samples were then incubated overnight in the dark at 4°C in a 1:200 dilution of Alexafluor secondary antibody (anti-EPN). The next day, all the samples were rinsed with PB six times in three hours with the first two rinses five minutes apart. The nerve cords were then mounted on slides using GelMount and were allowed to rest for 30

minutes before being viewed on the confocal microscope. When not in use, the slides were kept refrigerated and covered with foil to prevent degradation of the light-sensitive fluorescent dyes.

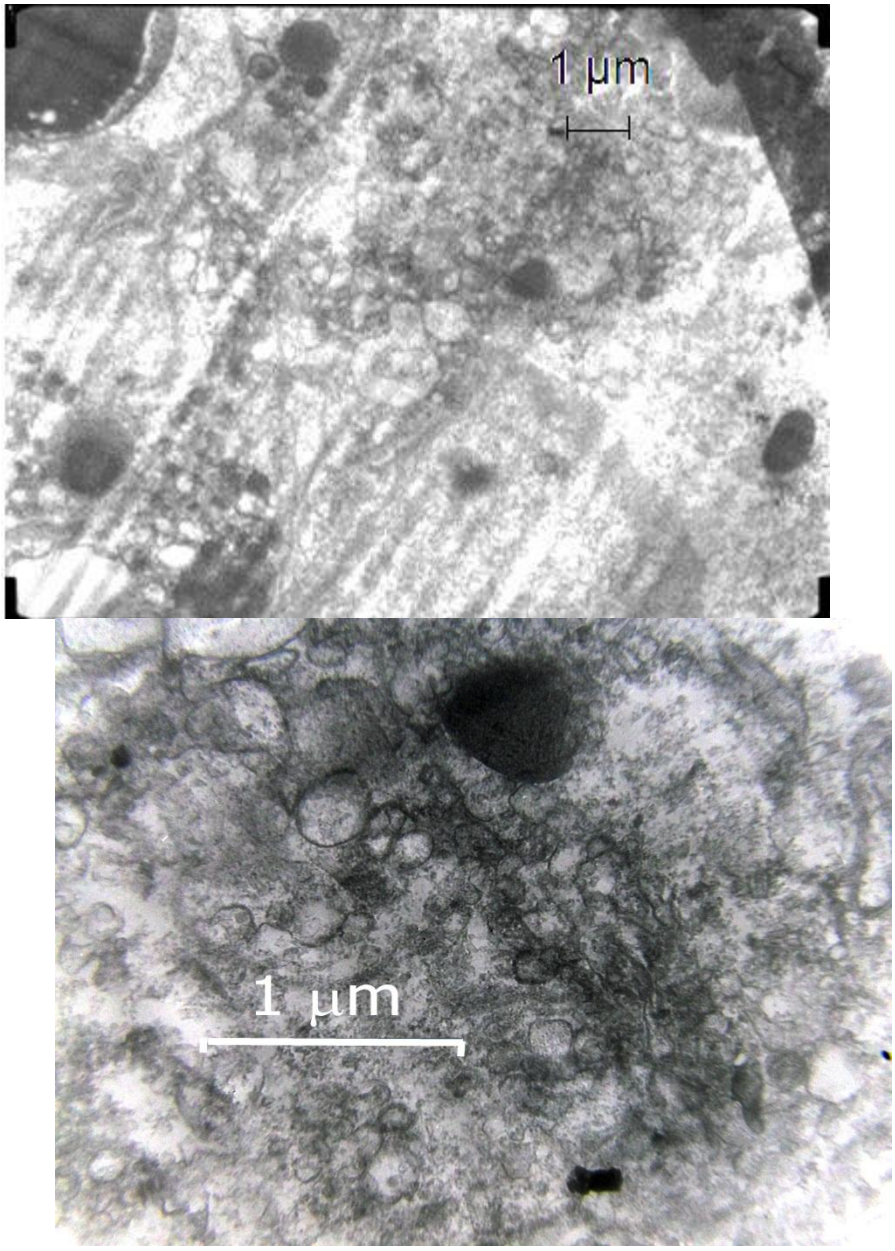
Confocal microscopy was used with blue light excitation of the Alexafluor 488 antibody, producing emission of a yellow-green light when present.

Results

Transmission Electron Microscopy (TEM)

Ultra-thin sections were placed on nickel grids and treated with anti-EPN primary antibody and Immunogold secondary antibody with 25nm gold particles attached. Figure 5 shows a transmission electron micrograph of a horseshoe crab nerve cord. The ganglion nuclei can be seen towards the center however, no colloidal gold particles were present. If the colloidal gold particles were present, this would have confirmed anti-EPN immunoreactivity. Colloidal gold's absence could be explained by inadequate concentration of one or another of the antibodies and does not rule out the presence of Epn.

Figure 4: TEM of nerve; light circular areas are axon profiles.



Confocal Microscopy

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markers in the sample. The atoms in the sample collide with the radiation and the electrons are excited to a high energy level. Light is emitted when the electrons relax to a lower level. In order for the fluorescing light to be visible, the light emitted from the fluorescing markers is separated from the brighter excitation light by a second filter. Now the fluorescing markers in the sample can be observed and fluoresce against the dark background.

LSCM, or laser scanning confocal microscopy, excites the sample sequentially with a small, highly focused laser beam. Because detectors know the exact angle of the excitation beam as well as the fluorescence it causes, bits of data can be collected and transformed to sharpen the image and to take extremely thin optical sections that can be reconstructed (z stack) and rotated (Nobel Prize).

Horseshoe crab nerve cords were treated with an Anti-EPN primary antibody and an AlexaFluor 488 secondary antibody. Figure 6 shows a whole mount of horseshoe crab nerve cord viewed with the confocal microscope. The green streaks indicated by the arrows are fluorescence from the AlexaFluor 488 antibodies and are indicative of anti-EPN immunoreactivity.

Figure 5: Whole mount of horseshoe crab nerve cord viewed on the confocal microscope. Photograph at right is for orientation. Red arrows point to streaks of anti-Epn Immunoreactivity. Autofluorescence was a problem, particularly from the nerve cord connective tissue sheath.

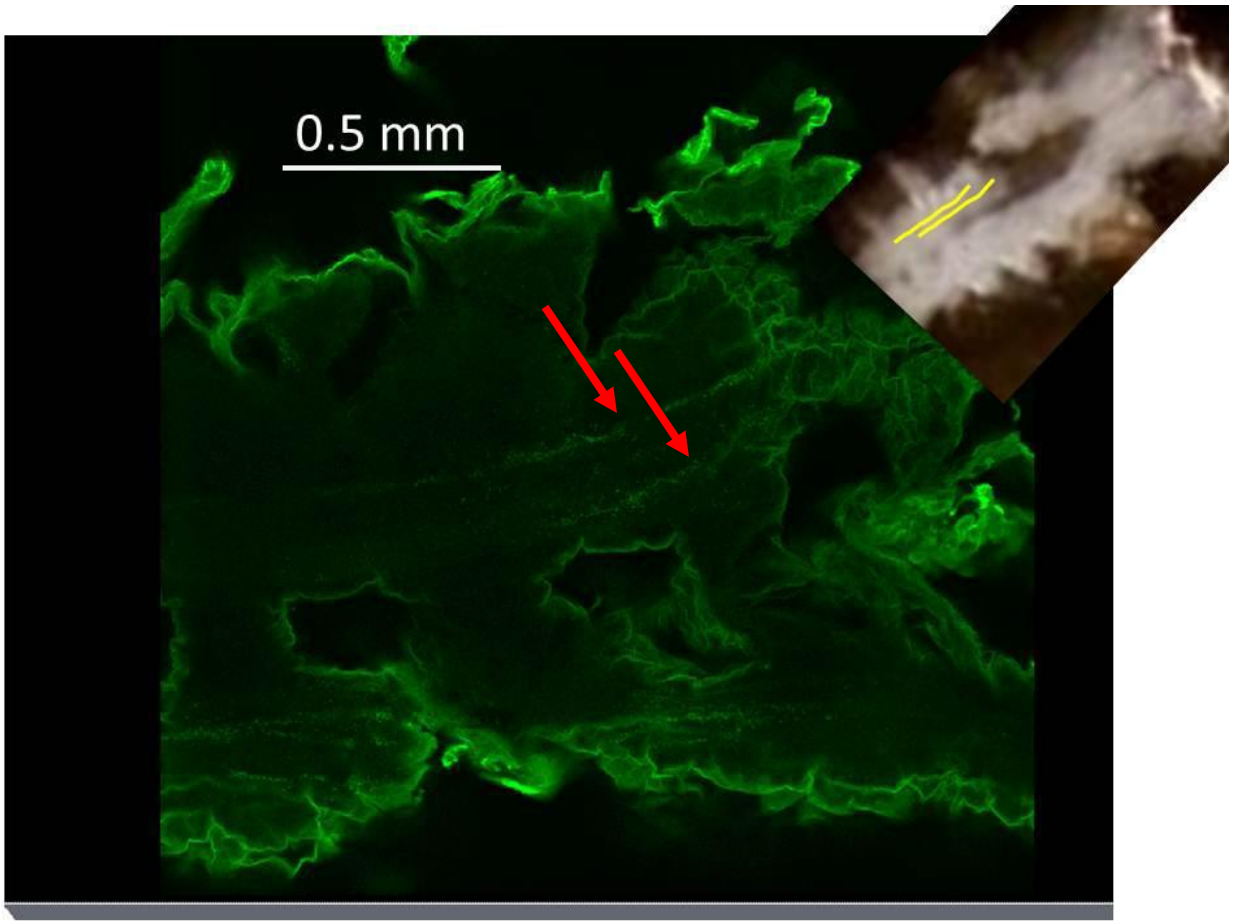
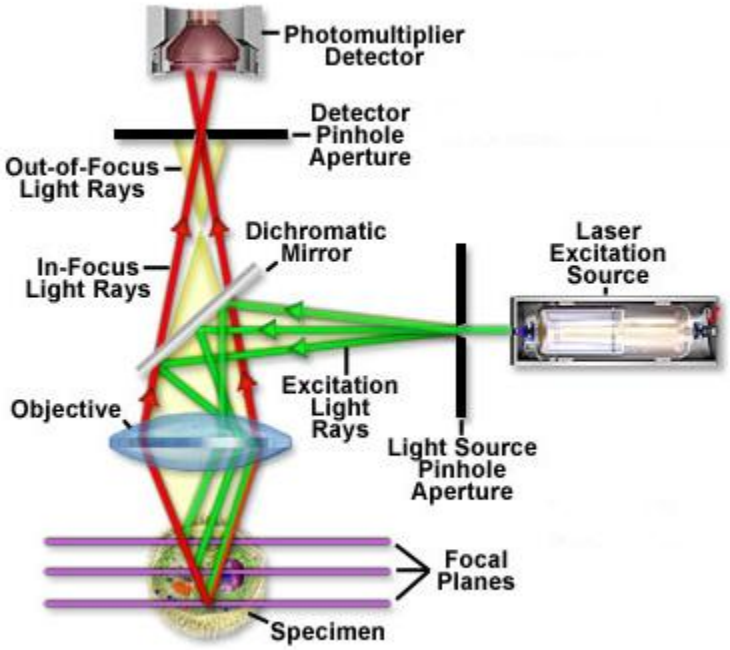

0.5 mm

Figure 6: Diagram of confocal microscope



Horseradish Peroxidase (HRP)

Semi-thin sections on glass slides were stained with the Pierce ABC Peroxidase Kit for HRP. Anti-EPN immunoreactivity appears as a brown precipitate. Figures 8, 9, and 10 show sections of nerve tissue stained for HRP. All three show brown precipitates indicative of anti-EPN immunoreactivity. Figure 8 shows brown precipitates in both the neuropil and the ganglion. Figure 9 is a low magnification toluidine-blue stained picture for orientation, showing nuclei and axons of the nerve cord. Figure 10: HRP stained nerve tissue counterstained with toluidine blue shows tight concentrations of precipitate almost as if in vesicles.

Figure 7: HRP stained nerve tissue counterstained with basic fuchsin. Circles identify nuclei for scale. Brown precipitate indicating Epn is apparently extracellular.

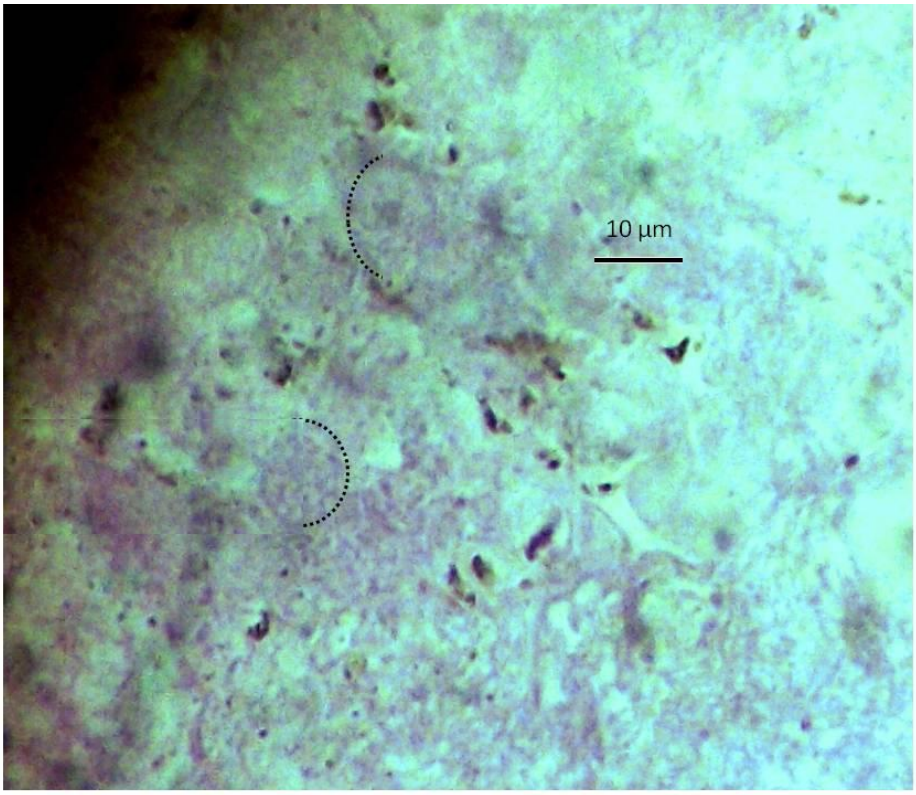


Figure 8: Low magnification picture of tissue stained for HRP activity. . Toluidine blue stain. Brown precipitate not visible at this magnification.

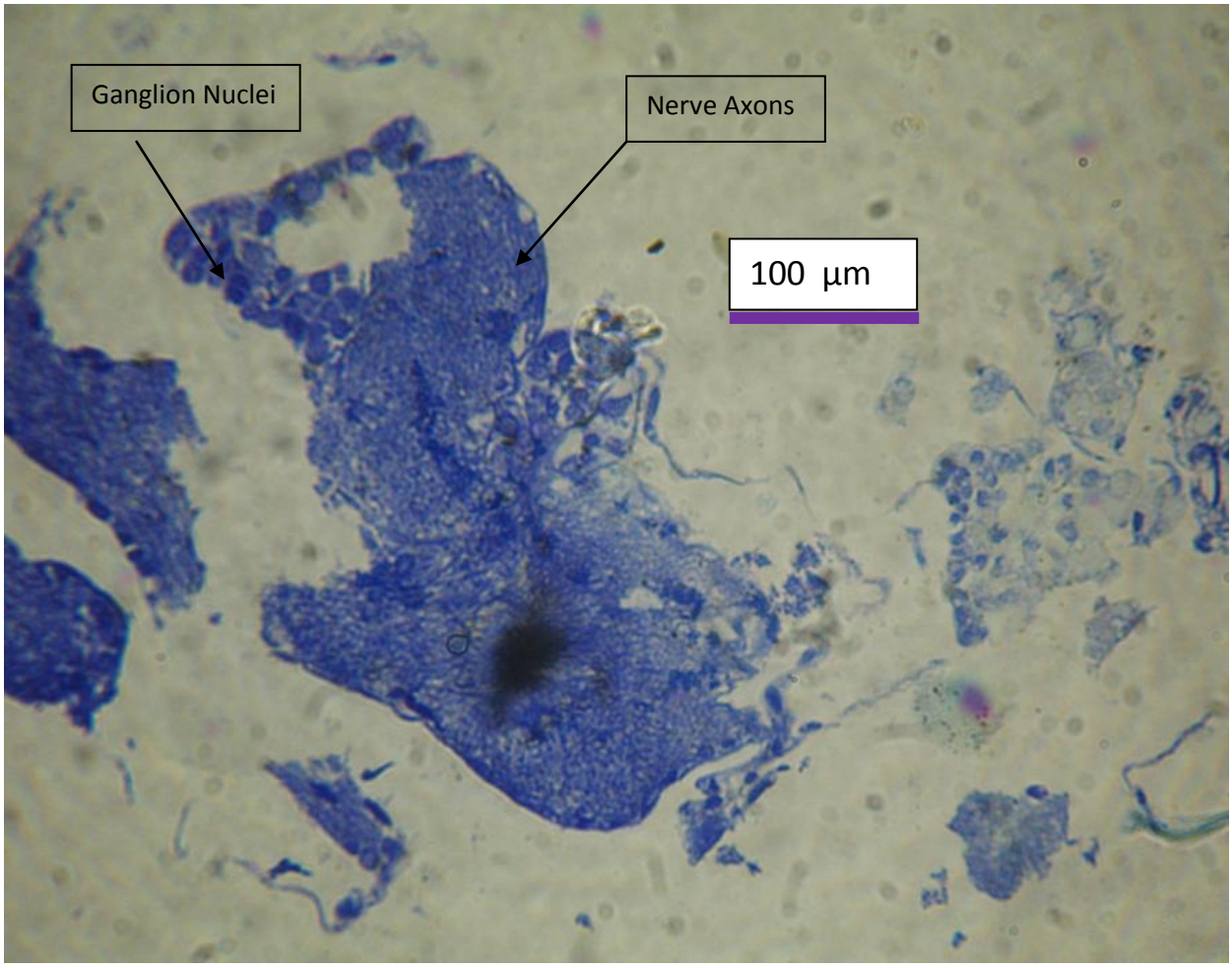
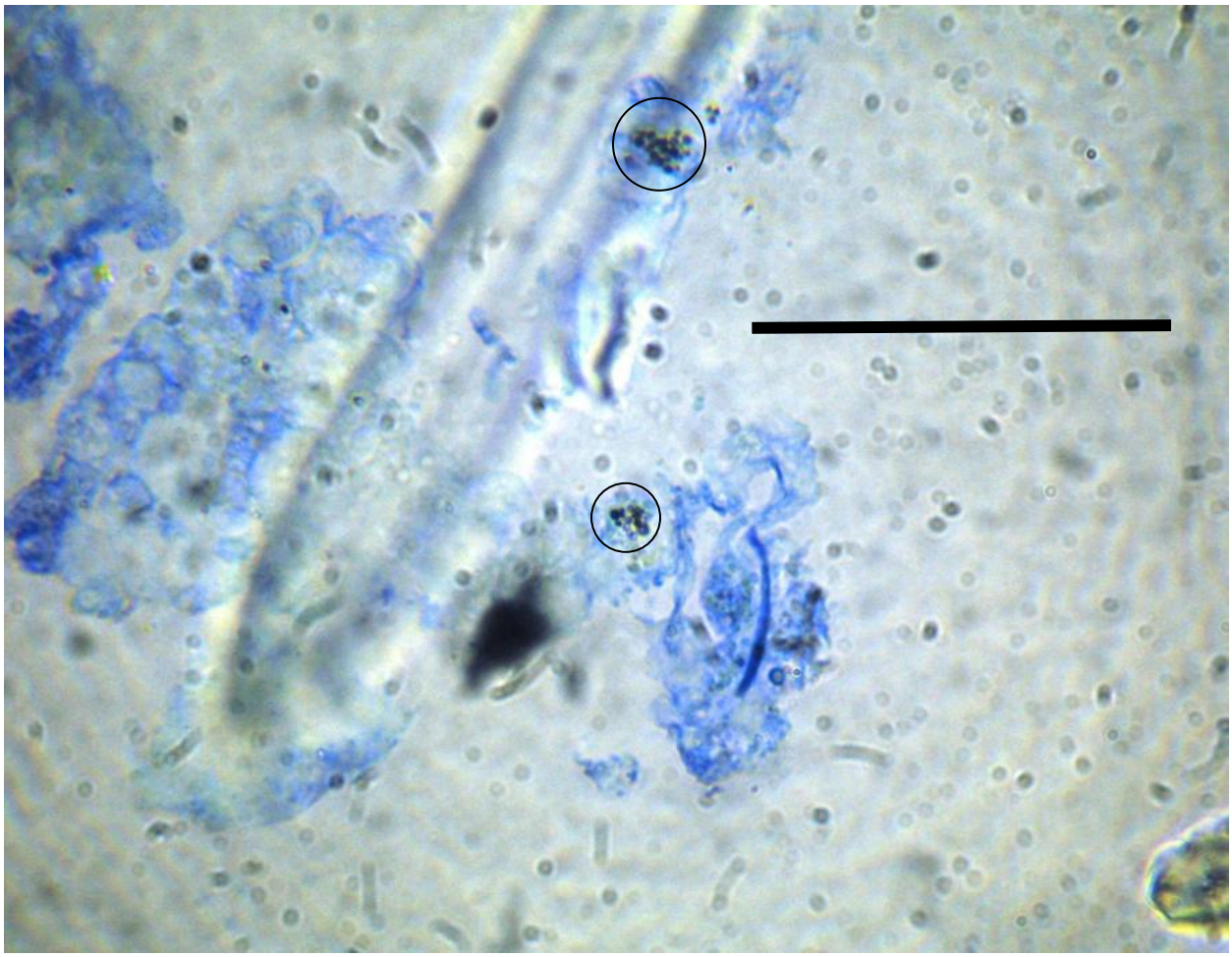


Figure 9: Circles emphasize concentrations of HRP precipitate in the somatic (cell-body containing area) of the CNS. Nuclei show as light blue ovals. Bar is 100 µm.



Fluorescence Microscopy

HRP development of brown precipitate proved to work better as an indicator of ependymin than did fluorescence microscopy. Slides that were prepared for fluorescence did not yield interpretable results, most often because of interference by autofluorescence.

Discussion:

The goal of this project was to provide evidence for the presence of EPN in the central nervous system of *Limulus polyphemus*, and to provide transmission electron micrograph images indicating its presence. The methods used to accomplish this goal were transmission electron

microscopy, fluorescent microscopy, confocal microscopy, and light microscopy with HRP staining. For transmission electron microscopy, ultrathin sections were treated with a GAR anti-EPN primary antibody and the secondary antibody ImmunoGold, which has colloidal gold balls attached to it. For fluorescent and confocal microscopy, semi-thin sections were treated with GAR anti-EPN primary antibody, and an AlexaFluor 488 fluorescent secondary antibody. For light microscopy semi-thin sections were treated with the Pierce ABC Peroxidase kit, they were first treated with anti-EPN primary antibody, and then were treated with a biotinylated anti-rabbit secondary antibody.

The confocal microscopy was successful in showing anti-EPN immunoreactivity in the whole mounts of the nerve cord, shown as streaks in figure 6. Light microscopy with HRP was successful in showing Ependymin in both the ganglion and the neuropil and also showed clumps of Ependymin grouped together as if in a vesicle for secretion. Fluorescent and electron microscopy did not show any anti-EPN immunoreactivity. Electron microscopy did not show any colloidal gold, and even if the primary antibody did not bind some nonspecific binding of the ImmunoGold secondary antibody would be expected.

Ependymin is present in both the neuropil and the ganglion of HSCs

Light microscopy with HRP staining was successful in providing evidence for the presence of EPN in both the neuropil and the ganglion of HSC. Figures 8, 9, and 10 show brown precipitates in both the neuropil and the ganglion. These sections were treated with the Pierce ABC Peroxidase kit and the brown precipitate is indicative of anti-EPN immunoreactivity in both the neuropil and the ganglion of the HSC. Previous MQPs have provided evidence for its presence in the neuropil but none has shown its presence in the ganglion before.

Ependymin is present in vesicles in the central nervous system

Light microscopy with HRP staining was successful in providing evidence for the presence of EPN in vesicles in the central nervous system. Figure 9 shows a section of nervous tissue with HRP staining. The brown precipitates are indicative of anti-EPN immunoreactivity; the groups of brown precipitates indicate that the EPN is in a vesicle. This evidence suggest that the EPN is a secreted molecule in HSCs as Shashoua has shown in goldfish and other vertebrates (Shashoua, 1991).

Future Experiments

The transmission electron micrographs show nerve tissue but do not show any colloidal gold balls indicative of anti-EPN immunoreactivity. Future experiments would use the nerve cord of *Limulus polyphemus* and stain with anti-EPN primary antibody and immunogold secondary antibody but do an increased concentration of secondary antibody such as 1:40. Hopefully, this would increase the chance of the secondary antibody body and show anti-EPN immunoreactivity on the electron microscope level.

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