Limb Regeneration in Horseshoe Crabs

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Abstract

It is well known that horseshoe crabs, like many invertebrates, are able to regenerate ablated appendages. The regeneration process, however, is not well documented. Regeneration was observed morphologically and immunohistologically. It was hypothesized that ependymin (EPN; neurotrophic factor) and/or juvenile hormone (JH) would contribute to regeneration at ablation sites and could be demonstrated by immunohistochemistry. Ablated appendages and their respective regenerated stumps were examined by both light and electron microscopy. It was found that the post-ablation tissue retracts into the remaining limb where regeneration begins. New shell is formed in between two layers of living tissue. JH immunoreactivity was found in blood cells in the regenerating stumps. Anti-EPN immunoreactivity was not detected in regenerating stumps.

Acknowledgements

We would like to thank the Biology/Biotechnology department at Worcester Polytechnic Institute for the opportunity and resources to successfully complete this project. Our gratitude is extended to the University of Massachusetts Medical School for the use of their Electron Microscopy Facility; especially Dr. Greg Hendricks. Finally, we would like to thank our advisor, Dr. Daniel Gibson, for his help, guidance, and support throughout this project. It was a pleasure.

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Chapter 1: Introduction

1.1. Horseshoe Crab

The horseshoe crab, although it has a hard shell and claws like other crabs, is classified as a chelicerate arthropod. Often referred to as "living fossils" because their body form has not changed in over 300 million years, horseshoe crabs belong to the lineage of giant sea scorpions that lived at the same time as the extinct primitive arthropod, the trilobite. Horseshoe crabs are particularly useful in this study because they, like many invertebrates, are able to regenerate appendages that have been wounded or removed from their bodies (by natural/environmental causes or otherwise) (Figure 1).

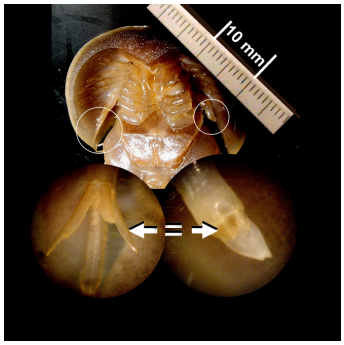

Figure 1 - Two year old crab regenerating its 4th leg (original leg left; regenerating stump right) Courtesy of Dr. Dan Gibson, WPI

The species of horseshoe crabs that is found along the western Atlantic and Gulf coasts is *Limulus polyphemus*. The habitat of *Limulus polyphemus* ranges from Nova Scotia to the Yucatan, but the center of its population is Delaware Bay (University of Delaware College of Marine Studies and Delaware Sea Grant, no date (n.d.).; Riska, 1981).

1.1.1. Anatomy

The body of the horseshoe crab is divided into three parts: the prosoma, the opisthosoma, and the telson (tail). Under a hard, protective exoskeleton, the prosoma contains an intestinal tract, a nervous system, a tubular heart, excretory glands,

connective tissue, and cartilaginous plates. Two large compound eyes, along with other sensory receptors, are located on top of the prosoma. On the underside of the prosoma there are six paired appendages, originating around the mouth. The first pair, the chelicera, is used for placing food in its mouth. The second pair, the pedipalps, is the first pair of ambulatory legs in all juvenile crabs and adult females. In adult males they are modified to form pincers that are used to grip the female's opisthosoma. Following these are four pairs of legs used for locomotion (The Ecological Research and Development Group, 2006).

As shown in Figure 2, the opisthosoma is connected to the prosoma on a hinge. The opisthosoma contains a large number of muscle groups, including those involved in the operation of book gills and the telson. The first pair of the six book gills is called the operculum, and it houses the opening of the genital pores through which eggs or sperm are released. The operculum also serves to protect the other five pairs of book gills which are used not only for "breathing" but as well as for propulsion when swimming. Lastly, the telson, or tail, is used to help itself turn over if capsized and also for steering (The Ecological Research and Development Group, 2006; Abrahamsen and Leo, 2005; University of Delaware College of Marine Studies and Delaware Sea Grant, n.d.).

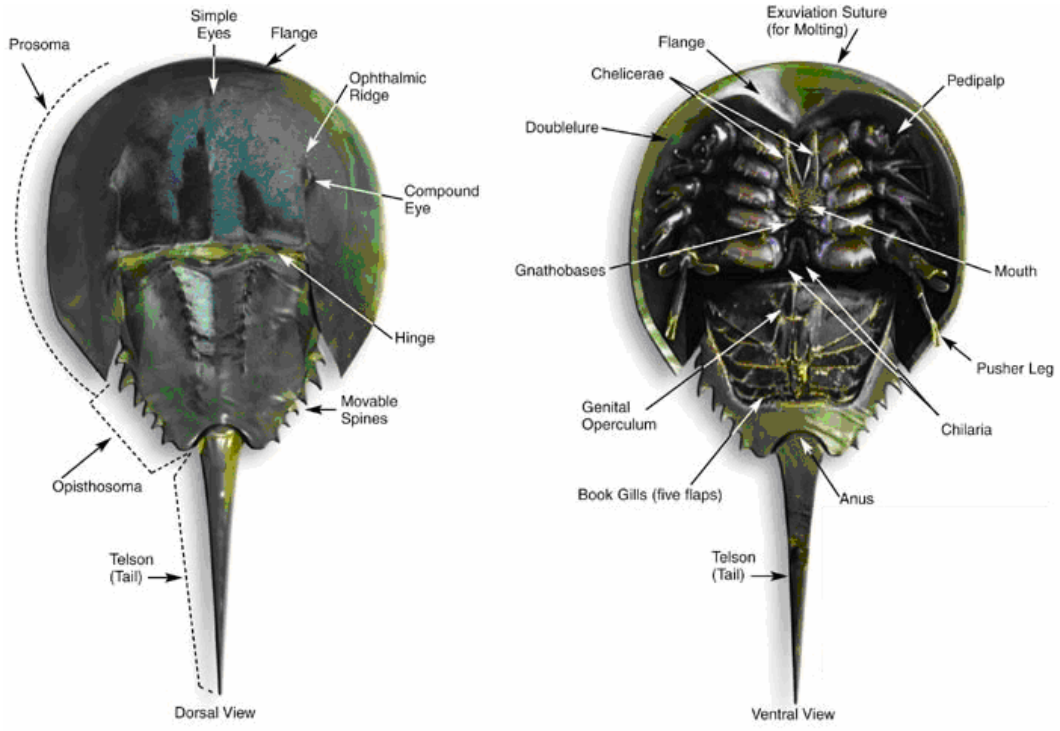

Figure 2 - Anatomy of a male horseshoe crab (University of Delaware College of Marine Studies and Delaware Sea Grant, n.d.).

1.1.2. Life Cycle

Newly laid horseshoe crab eggs are usually 1.5 mm in diameter, opaque, pastel-green, blue, gray, brown, or pink in color, and can be found buried in the damp sand on the shoreline. After fertilization, the eggs develop into trilobite larvae. With moisture supplied by the tides and the warmth of the sun, after approximately two weeks the eggs will have molted four times before hatching. Upon hatching, 3 mm larvae emerge and swim around until after about 20 days, when they settle to the intertidal flats to begin their first molt. Within their first two years, horseshoe crab juveniles appear to molt an average of seven to nine times, then annually in the years to follow in deeper waters. During their final molt, which for males is their sixteenth molt in their ninth year and for females their eighteenth molt in their tenth year, they will have reached sexual maturity

(Flynn, 1997; University of Delaware College of Marine Studies and Delaware Sea Grant, n.d.).

Mature, adult male horseshoe crabs will range from 7-9 inches across and 13-16 inches long. The larger, mature, adult females will range from 9-12 inches across and 16-20 inches long. To avoid predators, horseshoe crabs will bury themselves underneath the sand and emerge at night for feeding and spawning. As long as the crab is upright, their helmet-like shell also protects them from shorebirds. Aside from predation and disease, the largest cause of death for horseshoe crabs is from the bait fishing industry. Horseshoe crabs are harvested as bait for eel and conch fishing. Egg bearing females are particularly prized as bait; a piece of chopped up crab is put into each "pot", or enclosure, to attract eels or conch. Some horseshoe crabs also die during capture and bleeding for medical uses. If a horseshoe crab is fortunate to avoid these tragedies it may live to 18 years of age (University of Delaware College of Marine Studies and Delaware Sea Grant, n.d.).

1.1.3. The Horseshoe Crab's Current Importance in Medicine

In the world of medicine horseshoe crabs are currently most famous for their blood. The blood of a horseshoe crab contains an amebocyte lysate, known as *Limulus* amebocyte lystate (LAL). This lysate is known to have a coagulation mechanism that is stimulated by trace amounts of bacterial endotoxin (lipopolysaccharide, LPS) (Zhang *et al.*, 1994). In medicine today, LAL is isolated from horseshoe crab blood cells and used to detect gram-negative bacteria in injectable medications (www.horseshoecrab.org, 2006). Research suggests that the LAL test will one day be used in a clinical setting to detect infections in patients (Zhang *et al.*, 1994).

1.2. Ependymin (EPN)

Ependymin is a secreted glycoprotein composed of 216 amino acids. It exists as a disulfide-linked dimer, first discovered in the extracellular fluid in the brains of goldfish. Goldfish ependymin has a β form which is glycosylated, and a non-glycosylated γ form (Shashoua, 1991). The discovery of ependymin was the result of observed changes in the patterns of protein synthesis detected after learning activities (Shashoua, 1976). It is principally found in extracellular fluid in the brain and in cerebrospinal fluid (Shashoua, 1985). While ependymin functions in ways that are similar to known neurotrophic factors (NTFs), the exact means by which the nervous system utilizes ependymin is unknown. However, the increased turn over rate of ependymin in goldfish, after learning has taken place, suggests that one of the roles of ependymin is in the synaptic changes that occur during long term memory formation (Shashoua, 1976).

1.2.1. The Role of Ependymin in Tissue Regeneration in Invertebrates

Invertebrates are known for their ability to regenerate body parts. One such invertebrate, whose regeneration has been studied, is the sea cucumber, *H. glaberrima.* While regenerating intestinal tissue, the sea cucumber was found to over-express an ependymin-like protein (Suarez-Castillo *et al.*, 2004). This observation implies that the ependymin-like protein is likely to play a vital role in tissue and organ regeneration in the sea cucumber.

The horseshoe crab is another example of an invertebrate that is able to regenerate body parts, particularly its appendages (Clare *et al*., 1989). Additionally, ependymin has been found in horseshoe crabs by means of partial DNA sequencing (Cruikshank *et al*.,

1993). Using the anti-ependymin antibody SHEILA, (developed in rabbit against an 18-mer peptide from the carboxy terminus of the protein) ependymin has been observed in ganglion, neuropils, extracellular spaces around axons, intracellular membrane complexes, and connective tissue in juvenile horseshoe crabs (Barroso, 1999). In contrast, ependymin was not found in neuronal tissue in horseshoe crabs during the embryonic stage. This suggests that ependymin may be expressed intermittently during horseshoe crab growth and development. Further research has yielded results that show ependymin to be present (via staining with SHEILA antibody) in the blood cells of a wounded adult male horseshoe crab (Costigan and Gallant, 2004). The presence of ependymin could indicate its necessity of it in tissue regeneration in the horseshoe crab. If ependymin does in fact play a role in tissue regeneration, then blocking EPN activity post-injury should prevent regeneration.

1.3. Juvenile Hormone (JH)

All arthropods molt to grow. Some undergo a drastic metamorphosis at the terminal molt (e.g. caterpillar → pupa → butterfly) while others maintain a similar appearance throughout their lives (Figure 3). Molting is preceded by a spike of steroid hormone called ecdysone, but growth between molts is linked to the lipid-based juvenile hormone (JH), in those arthropods where it has been investigated. Such arthropods include those in the taxa Insecta and Crustacea, while the role of JH in Chelicerata has not yet been

Figure 3 - Freshly molted horse shoe crab. A clear increase in size, but lack of maturation into adult form is apparent. This phenomenon is suspected to be caused by JH as in insects and crustaceans.

clearly defined. In insects JH has been found to permit growth, while prevent maturation such that high levels of the hormone at the time of the molt results in an increase in the size of the animal, while its juvenile state is maintained. Conversely, low levels of JH results in the maturation of the insect into an adult form post-molt (Wigglesworth, 1940). This hormone has also been observed to stimulate the reproductive process and the subsequent sexual behavior in of adult insects of many species (Rose *et al.*, 2001). In crickets, JH has even been found to be involved in the stimulation of the formation of nervous tissue (Cayre *et al.*, 1994).

Crustaceans possess a form JH that is unepoxidated called methyl farnesoate (MF) (Laufer *et al.*, 1987). This hormone has been found to have similar effects in crustaceans as JH has in insects. The maintenance of juvenile state during the molting cycle has been observed in juvenile Spider crabs (*Libinia emarginata*). Like insects, high levels of MF prior to the molt, results in a larger juvenile crab, while lower levels results in the maturation of the crab into its adult form (Rotllant *et al*., 2000). MF has apparent roles in reproduction in several species of crustaceans. In particular, female crustaceans boast higher levels of MF during vitellogenesis, the formation of the yolk in the ovum (Wainwright *et al*., 1996). In male spider crabs mating behaviors have been linked to increased levels of circulating MF (Sagi *et al.*, 1994). Finally, in green crabs (*Carcinus maenus*), MF levels were observed to increase in crabs that were coping with exposure to disease or a difficult living environment (Lovett *et al.*, 1997).

The exact role of JH, or it analogs, in Chelicerata has not been identified at this time. However, the presence of an epoxidated JH-like hormone has been found in subadult horseshoe crabs (but, as is to be expected, not in adult horseshoe crabs)

particularly in the larval digestive tract and blood cells by HPLC, capillary electrophoresis, and immunohistochemistry (Levin, 2003).

1.3.1. The Role of JH in Tissue Regeneration in Invertebrates

At this time the role of JH in tissue regeneration in invertebrates, if any, is unknown. There is reason to believe that there may be a correlation between the presence of JH and regeneration, however. This is based on data obtained in a telson regeneration experiment, which resulted in considerable expansion and normalization of shape (a pointed end) of the telson immediately following a molt. Significantly less regeneration was observed to occur during periods prior to molting (Clare *et al*., 1989). Increased levels of circulating JH or JH at the wound site would be consistent with involvement in the regeneration process.

Chapter 2: Materials and Methods

Two sets of horseshoe crabs were studied during the course of this experiment. The first set of crabs collected from a salt marsh in Falmouth, Massachusetts. Fourteen crabs were collected in total, five of which had a body length (excluding the telson) of 5mm, while the remaining nine crabs were 8mm in length. The crabs were placed in separate wells of a six well Petri dish filled with filtered sea water (each well was approximately 3cm in diameter). In order to maintain conditions that were conducive to the growth and activity of the crabs, they were kept in an incubator. The incubator kept the water temperature of the horseshoe crabs' environment at approximately 70°C throughout the telson regeneration period. Heat was provided by a 60 watt light bulb

suspended above the incubator. The first set of horseshoe crabs underwent surgery to remove a small section from their telsons.

A second set of four horseshoe crabs was collected in early January. At the latitude of Cape Cod, horseshoe crabs remain buried and inactive during the winter months except during warm spells when daytime temperatures exceed 20°C for several days. This set included crabs with body lengths (excluding the telson) of approximately 2.5cm, 3cm, 6cm, and 8.5cm. The horseshoe crabs were kept in aerated 0.45µm filtered sea water in a small aquarium with each other (along with several other crabs not used in the experiment). The crabs from the second set underwent surgery to remove two legs each. All crabs from both sets were fed dehydrated tubifex worms and dehydrated brine shrimp every other day throughout the course of the experiments. The diet of the 5mm and 8mm animals was supplemented weekly with TetraMin® fish flakes to ensure that they were receiving food particles that were small enough for them to swallow.

2.1. Ablation Procedures

2.1.1. Telson Ablation

Each of the fourteen horseshoe crabs from the first set underwent surgery to amputate approximately 1mm from the tip of the telson. The horseshoe crabs were removed from their wells and placed on a piece of dental wax. They were then positioned with their telsons flat against the wax and the tips were ablated using a razor blade. After the surgery each crab was returned to its well immediately.

2.1.2. Telson Stump Ablation

The horseshoe crabs were allowed a 45 day telson regeneration period. At the end of this period the regenerated tissues (stumps) were then ablated. As was done with the initial ablation, the horseshoe crabs were removed from their wells and placed on a piece of dental wax. They were then positioned with their telsons flat against the wax and the regenerated telson stumps were amputated using a razor blade. After the surgery each crab was returned to its well immediately.

2.1.3. Leg Ablation

The four horseshoe crabs from the second experimental set each had two legs ablated. The horseshoe crabs were removed from their aquarium and placed on their backs in a shallow container filled with 1cm of filtered sea water. From the left side of their bodies (when the crab is in it natural position, face down) the second leg was removed at the first joint, which is just below the intersection of the pincers (Figure 4), using small Vannas-type surgical scissors. From the right side of their bodies the third leg was ablated at the second joint (Figure 4), in the same manner previously described.

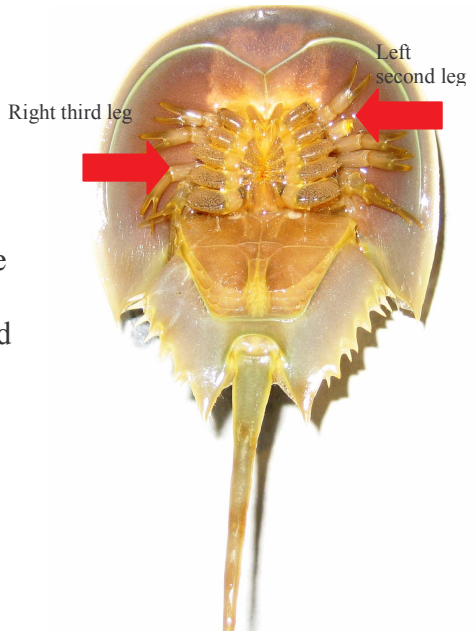

Figure 4 - Horseshoe crab (6cm body length) with its second left leg ablated at the first joint and its third right leg amputated at the second joint.

Immediately following their operations, the horseshoe crabs were returned to the aquarium. The surgery seemed to be traumatic for the specimens; two days post ablation the largest crab (8.5cm) died, and at 8 days post ablation the smallest crab (2.5cm) died.

2.1.4. Leg Stump Ablation

The horseshoe crabs in the second specimen set were allowed two regeneration periods. This was done to ensure the collection of at least one regenerated stump before the either of the crabs died. The third leg on the right side of the crabs was allowed a 40 day regeneration period. At the end of this regeneration period the two remaining horseshoe crabs underwent a stump ablation surgery. This surgery was performed in the same manner as the initial ablations; however the target tissue was the regenerated stumps from the previously removed third legs. The regenerated leg stumps from the second leg on the left side of the crabs were allowed a 60 day regeneration period before the second ablation surgery. This surgery was performed in the same manner previously described. The tissue was immediately fixed for immunohistochemistry (see 2.2. Fixation and Embedding).

2.1.5. Incubation and Algae Removal

Due to the incubation heat source's emission of white light, the horseshoe crabs used in the telson ablation experiments began to grow algae on their shells. Presumably, the algae growth was stressful; this and the trauma of the surgery most likely contributed to the death of seven of the fourteen crabs during the telson regeneration period. In order to help rid the horseshoe crabs of the algae, the remaining seven live crabs were washed in a 10% solution of Clorox® chlorine bleach and diluted with filtered sea water. Each crab was carefully placed into the wash solution for 30 seconds and then thoroughly rinsed in sea water. Algae were then manually removed from the shell, gills, legs, and telsons of the horseshoe crabs using tweezers. They were then placed back into the 10% Clorox ® solution for an additional 10 seconds to kill any residual algae that was not able

to be removed from the crabs. They were again thoroughly rinsed in filtered sea water and placed back into their respective wells. In effort to prevent any further growth of the algae on the crabs, the six well plates used to house the crabs were covered with a thin piece of black plastic. This allowed for the transfer of heat, while blocking the light source. There were no subsequent crab deaths after the Clorox® treatment, prior to the amputation of the regenerating stumps at the end of the regeneration period.

2.2. Fixation and Embedding

2.2.1. Epon-Araldite

Ablated original appendages and stumps were immediately fixed in a solution of 1% paraformaldehyde in a 0.1 M phosphate buffer at pH 7.4 and then refrigerated overnight. This served to cross-link proteins and preserve the integrity of the structure of the tissue. Next, the specimens were rinsed for five minutes in a 1:1 solution of 200mM Phosphate Buffer: Reagent Grade Distilled Water. To stabilize and enhance the lipid membrane by cross-linking unsaturated fatty acids, the specimens were post-fixed in 1% osmium tetroxide at room temperature for one hour. The specimens were next rinsed twice with reagent grade distilled water (rdH2O) for five minutes each. The specimens were dehydrated at room temperature in an ethanol (EtOH) series (70%, 90%, 100%, 100%, 100%) for 10 minutes at each concentration. This served to remove all traces of water from the tissues, as embedding media is not soluble in water, and soft or fluid-filled tissue would be easily deformed. The specimens were then rinsed twice for 10 minutes each with propylene oxide, at room temperature. The low molecular weight epoxy,

propylene oxide, served to remove the alcohol from the dehydrated specimens and aid in the infiltration of the viscous epoxy-resin, Epon-Araldite (EA) (Abrahamsen and Leo, 2005). The specimens were then infiltrated with a solution of 1:2 EA: propylene oxide for one hour, followed by infiltration with a solution of 2:1 EA: propylene oxide. Next, the specimens went through two changes of 100% Epon-Araldite, for one hour each, before curing in polypropylene or polyethylene containers overnight at 60°C.

2.2.2. LR White

LR White resin is an acrylic, hydrophilic embedding agent which is soluble in alcohol. Unlike epon-araldite, LR White polymerizes around the tissue instead of binding to it and it also tolerates rapid, partial dehydration. Therefore, antigenicity of the specimens is better preserved. Ablated stumps for LR White embedding were immediately fixed in a solution of 4% paraformaldehyde in a 0.1 M phosphate buffer, and then refrigerated for two hours. Next the specimens were rinsed in a 1:1 solution of 200mM Phosphate Buffer: Reagent Grade Distilled Water for 5-10 minutes, followed by a rinse in Reagent Grade Distilled Water (rdH_2O) for 5-10 minutes, both at room temperature. The specimens were then dehydrated in 70% EtOH and 100% EtOH for 15 minutes each, before infiltrating with a solution of 1:1 EtOH: LR White and refrigerating overnight. Next, the specimens were placed in 100% LR White overnight, followed by a final change into 100% LR White using gelatin capsules before curing overnight at 60°C.

2.3. Microtomy/Sectioning

Once the samples have been embedded in either Epon-Araldite or LR White, they are ready to be trimmed. A jeweler's saw and a razor blade were used to trim away the excess medium surrounding the tissue sample. Samples were sectioned using a Sorvall MT-2 Ultramicrotome with a diamond knife. The embedded tissue was positioned in the microtome, and then sectioned at a thickness of 0.5 μm (semi-thin) for light microscopy and 50-90 nm (ultra-thin) for electron microscopy. For light and fluorescence microscopy, semi-thin sections were collected with a wire loop and dried onto Fisher Frost Plus glass slides on a hot plate (80°C). For electron microscopy, ultra-thin sections were collected on nickel grids of varying mesh configurations and densities. After blotting dry on bibulous paper, the grids were stored on a magnetic strip.

2.3.1. Light Microscopy

Once sections have been properly mounted on slides, staining or antibody detection steps can be carried out to find out what is inside the tissue. To better examine morphological features of the tissue, the sections were stained with Toluidine blue, which stains membranes and nuclei dark blue, for one minute on the hot plate. The dye was then rinsed off with distilled water (dH_2O) and the slide was allowed to air dry before viewing with a light microscope. For each embedded sample, at least one stained slide was made for viewing under a light microscope to ensure that the desired part of the tissue was being cut.

2.3.2. Fluorescence Microscopy

Fluorescence microscopy was used to view the auto-fluorescing parts of the tissue (such as the shell). For fluorescence microscopy, control slides made for the immunohistochemistry experiments were used. (See 2.4. Immunohistochemistry for slide preparation details.).

2.3.3. Transmission Electron Microscopy (TEM)

For electron microscopy, grids were stained with electron dense solutions to enhance contrast. The grids were floated section side down on drops of 50% ethanolic Uranyl Acetate (UAc) for five minutes. Next, the grids were washed three times in drops of dH_2O. The grids were then post-stained by floating them section side down in drops of 0.2% lead citrate for less than a minute. This was done in a closed container as to prevent CO_2 from precipitating with the lead. Finally, the grids underwent two washes in drops of dH_2O and were blotted dry, before being viewed under a transmission electron microscope.

2.4. Immunohistochemistry

The general procedure of immunohistochemistry includes applying a primary antibody to bind to the antigen of interest. Then, a secondary antibody, which is complexed with biotin or a fluorophore, is applied. Finally, a developing substrate is applied before viewing by microscope.

Undyed slides that came from the Epon-Araldite embedded specimens needed a few additional steps than did the slides from the LR White embedded specimens.

Because the epoxy resin, unlike LR White, binds to the specimen itself, an "etching" procedure was carried out in an attempt to expose the potential antigenic sites. The epoxy resin was removed from the section by incubation for three minutes in a Dissolving Solution (50 mL EtOH, 50 mL propylene oxide, 5.0 g NaOH pellets, stirred vigorously for five minutes) (Barroso, 1999). The sample was then rinsed three times in 95% EtOH for two minutes each, followed by a three minute rinse in 50% EtOH, and a five minute rinse in rdH_2O. Then, the sample was treated for 5-7 minutes with 1% $NaIO_4$, made fresh in rdH_2O, and rinsed three times for three minutes each with rdH_2O.

After "etching" the epoxy resin embedded samples, these slides and the LR white slides were equilibrated with a solution of 0.1 M phosphate buffered saline (PBS) for 20 minutes. An ABC Peroxidase Staining kit from Pierce was used. To block non-specific binding sites, the samples were treated for 20 minutes with blocking buffer (normal goat serum in 0.1M PBS) from the ABC kit (Pierce, Rockford, IL).

2.4.1. Primary Antibody

A primary antibody binds to the target protein. Two different antibodies were used in this study. The rabbit anti-ependymin polyclonal antibody (SHIELA) was diluted 1:50 in blocking buffer. The rabbit anti-juvenile hormone antibody was diluted 1:10 in blocking buffer. Slides that were used as a control received blocking buffer in place of the primary antibody. Slides were incubated in primary antibody or blocking buffer, overnight in a humidity chamber at room temperature. After incubation, the slides were rinsed in PBS three times for 10 minutes each. The subsequent steps depended on the type of microscopy that was going to be used.

4.2.2. Secondary Antibody

A secondary antibody against the species in which the primary antibody is made will bind to the primary antibody. The secondary antibody is complexed with a reporter (enzyme, fluorescence marker, gold particles, etc.) to allow for easy detection. The type of secondary antibody and the subsequent steps depended on the type of viewing.

Light Microscopy Immunohistochemistry

For light microscopy, the tissue was incubated at room temperature for 30 minutes with the ABC kit's Biotinylated, Affinity Purified Goat-Anti Rabbit Secondary Antibody conjugated with horseradish peroxidase (HRP). The slides were then rinsed in PBS three times for 10 minutes each. Next, the tissue was incubated for 100 minutes at room temperature with freshly-made ABC Reagent, which uses an avidin-biotin complex to amplify the signal sensitivity. After three rinses in PBS for 10 minutes each, the samples were ready for the substrate. An ImmunoPure® Metal Enhanced DAB Substrate Kit from Pierce was used to detect the HRP, using 1X nickel enhanced diamino-benzidine (DAB) in Stable Peroxide Buffer. The tissue was incubated in this freshly-made DAB working solution for 15 minutes at room temperature. The development of a brown precipitate would indicate the presence of oxidized DAB. Therefore, HRP bound to the secondary antibody which would be bound to the primary antibody, would in turn be bound to the antigen of interest. Some slides were counterstained with toluidine blue and basic fuchsin for 30 seconds each and then viewed using a conventional light microscope.

Fluorescence Immunohistochemistry

For fluorescence microscopy, an Alexa Fluor® 488 Goat Anti-Rabbit SFX kit (Invitrogen) was used. The tissue sections were incubated with the Image-iT™ FX signal enhancer for 30 minutes at room temperature. The slides were then rinsed in PBS three times for 10 minutes each. Next, the tissue sections were incubated at room temperature for 100 minutes with the Alexa Fluor® Goat Anti-Rabbit secondary antibody. After the slides were rinsed in PBS three times for 10 minutes each, they were incubated for 15 minutes with the nuclear counterstain 4', 6-diamidino-2 phenylindole dihydrochloride (DAPI). The slides were then rinsed in PBS three times for 10 minutes each, coated with glycerol, and a cover slip was placed over the tissue sections. The slides were then viewed under a Zeiss research fluorescence microscope.

Chapter 3: Results

3.1. Gross Observations

During the course of this study, regeneration was examined beginning immediately after appendages were ablated, and ending when regenerating stumps were removed for examination at least 45 days after the initial ablation. When appendages were first removed a thick fluid, that was yellowish in color, oozed from the wound site. Within one minute of the ablation procedure, a clot had formed over the wound and no additional oozing liquid originating from the wound site was

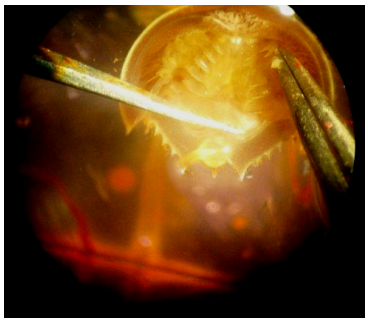

Figure 5 - Clot formation one mintute post-ablation in an 8mm body length crab.

observed (Figure 5). One day post-ablation the flesh had retracted into the remaining shell. Approximately two weeks post-ablation the regenerating flesh was observed to be even with the shell. Although some swelling of the wounded appendage was apparent, the cause of the extended flesh was presumed to be tissue re-growth. After the appendages had been allowed to regenerate for one month and beyond they began to normalize in shape. Regenerating telsons showed signs of reforming their pointed tips and regenerating claws showed early separation of pincers (Figure 6).

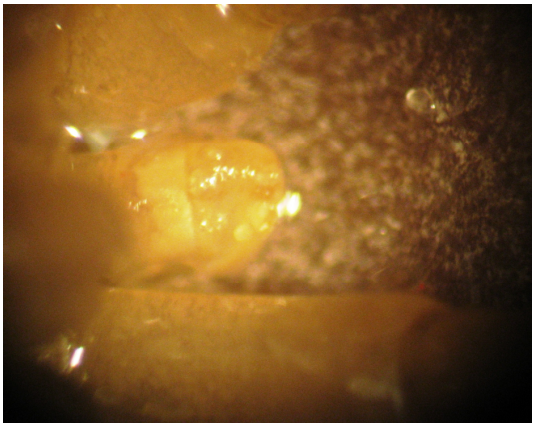

Figure 6 - Leg 2 regenerating stump from 3cm body length crab 30 days post claw ablation.

Three of the 8mm crabs that had undergone telson ablation procedures molted during their regeneration period. The molts occurred 15 days, 23 days, and 31 days after the initial ablation surgery. In these crabs a significant increase in telson length, ranging from 0.4-0.8cm, was observed immediately after the molt. Additionally, the physical shape of the telson normalized (not shown); it returned to a pointed end instead of a blunt end. Unfortunately, all three crabs died before their regenerated stumps were able to be collected.

3.2. Microscopy

Light and fluorescence microscopy were initially utilized to examine semi-thin cross sections of the regenerated stumps. It was observed that the regenerating shell was forming between two apparently living layers of tissue. This was first observed by light microscopy when semi-thin sections were stained with toluidine blue (Figure 7). This observation was confirmed with the use of fluorescence microscopy. Because horseshoe crab shell auto-fluoresces, excitation 490nm, it was clear to see that new shell had in fact formed at the wound site (Figure 8). Tissue was visible (fluorescing a yellowish hue) on both the proximal and distal sides of the new shell relative to the stump in anatomical orientation.

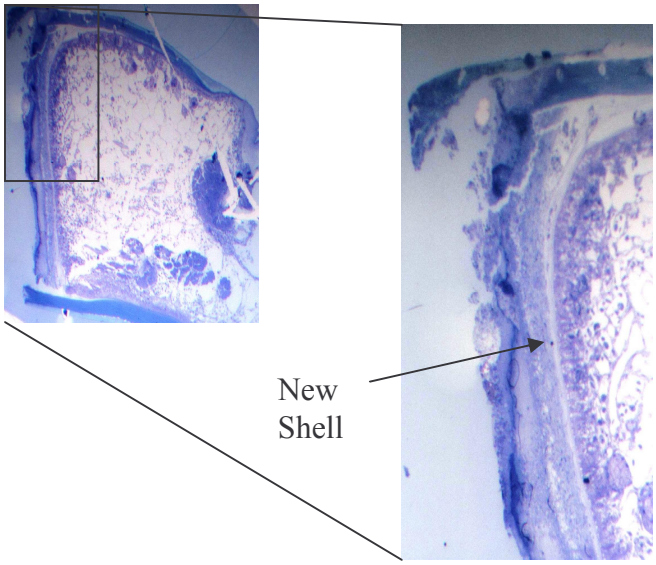

Figure 7 - Light micrograph at 100X (left) and 1000X (right), showing the cross-section of ablated stump with generation of new shell in between layers of living tissue. (Inset 50X200 µm)

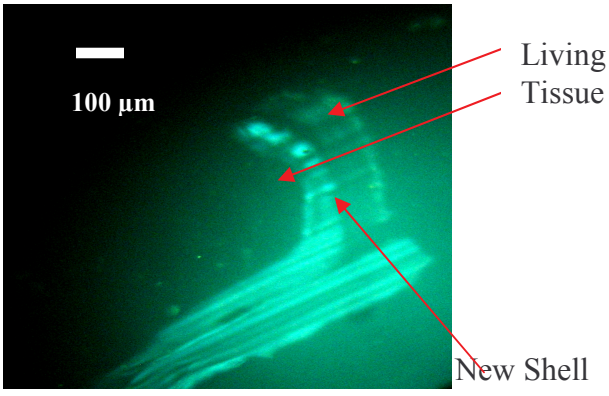

Figure 8 - Fluorescence Micrograph at 400X. Auto-fluorescing shell, excitation 490nm, showing the cross-section of ablated stump with generation of new shell in between layers of living tissue.

Further exploration of the regenerating shell and the surrounding tissue was done using TEM. The newly forming shell itself was more cellular than initially anticipated. In addition to stratified tissue there were also several nucleated and granulated cells present (Figure 9). The cellular composition of the tissues on the proximal and distal sides of the regenerating shell, in respect to the stump in anatomical orientation, differed in general appearance. The tissue on the immediate proximal side of the new shell was composed of tightly packed cells with clear secretory granules (Figure 10). Comparatively, the tissue found on the immediate distal side of the regenerating shell was composed of cells that were much less tightly packed, but more granular (Figure 11).

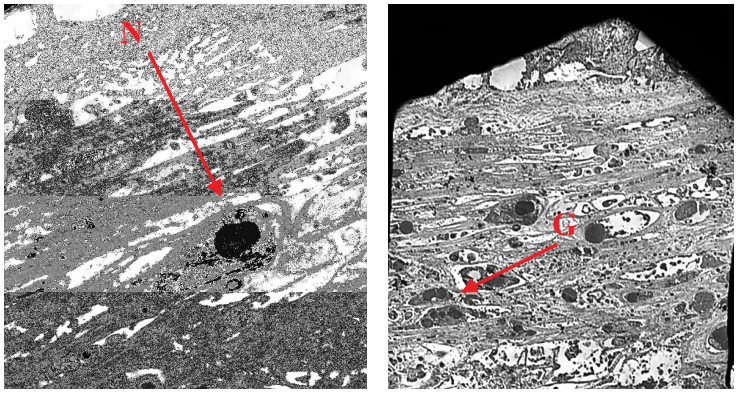

Figure 9 - Newly forming shell with nucleated (N) and granulated cells (G). Left micrograph at 4,400X and right micrograph at 1,000X.

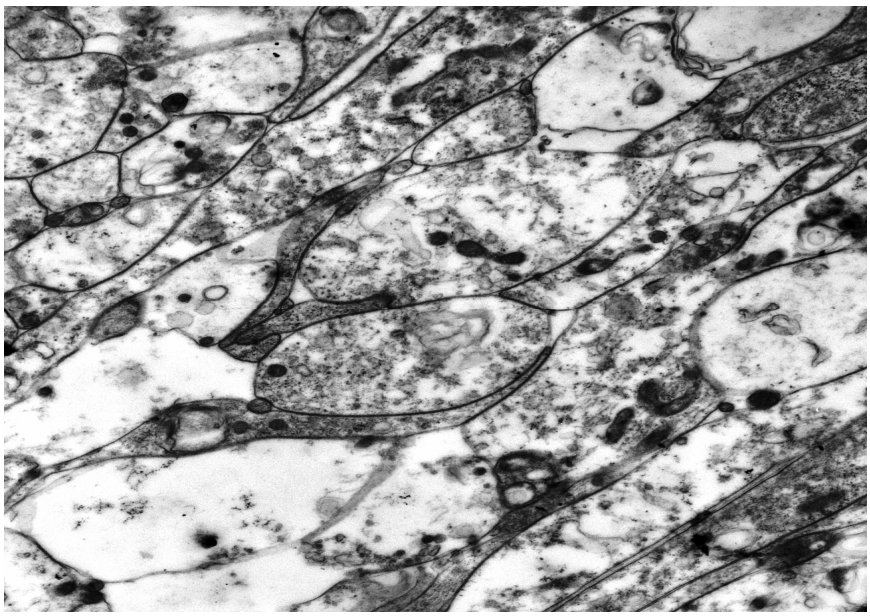

Figure 10 - TEM micrograph at 4,400X of packed cells with clear secretory granules. (proximal to new shell).

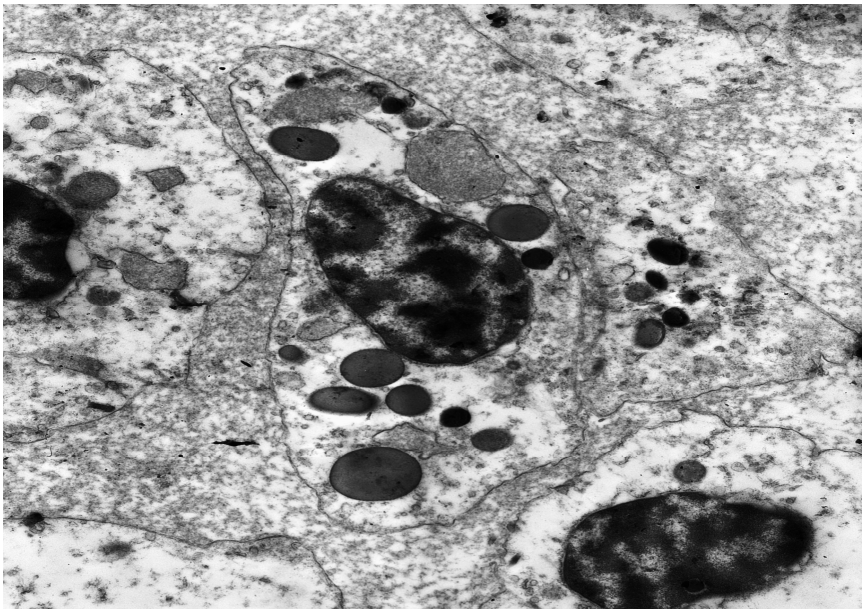

Figure 11 - TEM micrograph at 4,400X of less packed granular hemocytes. Note dark granules. (distal to new shell).

3.3. Immunohistochemistry

3.3.1. Ependymin

The anti-Ependymin to the carboxy-terminus that was used in the immunohistochemistry experiments did not display immunoreactivity in the tissues in the semi-thin sections.

3.3.2 Juvenile Hormone

The anti-JH was immunoreactive with a JH-like molecule in amebocytes in the regenerating stump. There were three amebocytes in one of the stumps that displayed a fluorescent signal in response to JH. The amebocytes were not located directly at the regeneration site. They were found in the periphery in the tissue that is proximal to the new shell.

Chapter 4: Discussion

4.1. Gross Observations

It was observed that after the horseshoe crabs underwent ablation surgeries that their flesh retracted into the remaining shell within the first 24 hours after the injury, rather like clot retraction in vertebrates. It is likely that expression of fluid from the clot results in the original shrinkage, and the retracted tissue is then protected from further damage while regenerating. Retraction may also make it easier for peripheral hemocytes to access the wound site and help with healing.

Approximately one month into the regeneration period, crabs who had their appendages ablated just below the claw were beginning to show signs of regenerating pincers (refer to Figure 6). Based on this observation, differentiation of the growing limb appears to occur before enlargement; pincers form first and then the limb extends to its original length. This mechanism may prove to be advantageous to the crab since the re-growing pincers, though shorter than the others, would be functional while the limb is in the process of regenerating. Additionally, if the crab molts for the final time, maturing into its adult form (thus halting significant regeneration), while still regenerating the limb, by forming pincers first it would be left with a functional, but abbreviated, limb.

4.2. Microscopy

Several granular hemocyte cells that could be pluripotential were found at the regeneration sites of the ablated appendages. Hemocytes, such as the ones observed in the regenerating stumps, could be cells which will differentiate into nerve, muscle, and shell tissue at the regeneration site.

The newly regenerating shell was re-growing between two apparently living layers of tissue, both of which contained granulated cells, at least some of which were hemocytes. Regeneration in this manner would allow the horseshoe crabs to regenerate their wounded appendage as quickly as possible (both from the outside and the inside of the newly forming shell). If this is in fact the mechanism by which regeneration occurs, it is likely that the tissue on the outside of the shell would be creating the newly forming shell and the tissue on the inside would be involved in the formation of muscle, nerve,

and other related tissues. Re-grown shell will subsequently be replaced from within, so the cells that are found on the outside of the shell during regeneration will be sloughed off.

4.3. Immunohistochemistry

Our attempts to detect ependymin in regenerating limbs with immunohistochemistry experiments were unsuccessful. However, as with many scientific experiments, many factors can contribute to negative findings. One reason for not finding any immunoreactivity for ependymin may have been due to a faulty primary antibody. The only primary anti-ependymin antibody available at the time was one that had been left over from a previous study and had been kept frozen, over which time the antibody may have lost its integrity. Neurotrophic factors can act at extremely low concentrations, so ependymin may have actually been present but below the detection threshold for our antibody.

To better understand if ependymin plays any role in the regeneration of horseshoe crab limbs, a new antibody, which was designed based on the active region of the molecule (KKETLQFR : Stovall, 2006, M.S. Thesis, WPI), was ordered (New England Peptide, Gardner MA: newenglandpeptide.com). We hope that this new antibody is a better reporter of active ependymin, and can be used to experimentally inhibit regeneration.

Future experiments with Juvenile Hormone (JH) could also provide more insight into the horseshoe crab's regeneration process. As previously discussed, JH has been found to have a significant effect in the maturation of other arthropods. The presence of

JH in a horseshoe crab regenerating stump suggests that it may have a role in the regeneration process. Treatment of live horseshoe crabs with anti-JH could provide useful supporting data for our conjecture, as well as a better general understanding of its role in *Limulus*. JH is secreted at extremely low levels in other arthropods (e.g., *Manduca sexta*) and therefore may be difficult to detect under any circumstances (Dr. Walter Goodman, University of Wisconsin, personal communication).

As noted in our gross observations, it also seems likely that the act of molting and/or the preceding growth period may play an essential role in regeneration. This is thought to be true based on observations made on three crabs, with ablated telsons, which had molted during the regeneration period. These crabs displayed significant regeneration of the wounded tissue immediately after they had molted. Additional evidence for this was reported by Clare *et al.*(1989) in similar experiments. Experiments extending through one or more molts are required to examine this contention. Also, use of the smallest/youngest crabs would be advantageous being that they have shorter molt cycles.

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