GREEN ALGAL INFECTION OF AMERICAN HORSESHOE CRAB (*Limulus polyphemus*) EXOSKELETAL STRUCTURES

Running Head: Horseshoe Crab Green Algal Disease

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Abstract:

Degenerative lesions in the dorsum of the horseshoe crab (*Limulus polyphemus*) exoskeleton, eyes, arthrodial membrane, and base of the telson were documented in a population of wild caught laboratory animals. The disease can lead to loss of tissue structure and function, deformed shells, abnormal molting, loss of ocular structures, erosion of interskeletal membranes, and cardiac hemorrhage. Microscopy, histopathology, and in vitro culture confirmed the causative agent to be a green algae of the family Ulvaceae. Further research may explain how green algae overcome horseshoe crab innate immunity leading to external and internal damage.

Introduction:

The American horseshoe crab (*Limulus polyphemus)* is an aquatic arthropod (subclass: *Xiphosura*) whose evolutionary history has essentially remained unchanged for more than 200 million years. Hence, the horseshoe crab has been referred to as a 'living fossil' (Smith and Berkson, 2005). Despite being the closest living relative to the presumed extinct trilobite (an ancient aquatic arthropod), *L. polyphemus* is most closely related to terrestrial arthropods such as scorpions and spiders. Unlike true crabs, however, horseshoe crabs lack antennae, jaws and possess seven pairs of legs (instead of five as in decapod crustaceans), the first of which form chelicerae (used for grasping and crushing) (Walls et. al., 2002). *L. polyphemus*, which is one of four extant species of horseshoe crab, occupies the western Atlantic coast of North America from Maine south to the Yucatan peninsula and is the only species of Limulidae found in the United States.

Over the years, *L. polyphemus* has been intensively studied by researchers, and is important to many different industries. The bait fishery uses horseshoe crabs to catch eel and conch, principally in the Mid-Atlantic states, and have harvested more than 2.5 million horseshoe crabs annually (Smith et al., 2009). In agriculture, they have been used as a component of fertilizer and livestock feed (Shuster et al., 2004). Researchers have used *L. polyphemus* to study vision, the nervous system, invertebrate molting, cellular phagocytosis, and the embryological development of marine invertebrates (Shuster,et al., 2004). Their most recognizable use, however, is in the biomedical industry. A horseshoe crab's blood (known as hemolymph) contains circulating amebocytes that produce a substance called Limulus amebocyte lysate (LAL). This compound is used to detect extremely minute quantities of endotoxin, permitting its use to screen for endotoxin on medical devices, implants, and vaccines (Walls et. al. 2002).

Horseshoe crabs can be impacted by various pathogens including algae, fungi, cyanobacteria ,Gram-negative bacteria, and a variety of parasites (Nolan and Smith, 2009). One apparently common disease in both wild and captive horseshoe crabs is shell pathology caused by a green algal (*chlorophycophytal)* infection (Figure 1). Previous studies by Leibovitz and Lewbart (1987) localized the degenerative lesions caused by the green algae to the dorsum of the exoskeleton, the eyes (or ocelli), the arthrodial membrane (over the heart), and the base of the telson. The young algal zygotes use their rhizoidal processes to insert themselves between the chitinous lamina of the carapace (Figures 2 and 3), eventually penetrating the carapace of the animal, where it uses the same processes to destroy the crab's internal tissues and organs. This can cause shell deformities, abnormal molts, necrosis, degeneration of eye structures, perforations of the arthrodial membrane, and hemorrhaging from the heart.

This paper expands the earlier work of Leibovitz and Lewbart (1987) by providing images and further characterization of the algae and the disease that it produces. Further research can be applied to the development of methods to prevent and control the disease.

Materials & Methods:

Adult *Limulus polyphemus* specimens were obtained from the Marine Resources Center, Marine Biological Laboratory, Woods Hole, MA. The vast majority of these animals were recently dead or moribund and presented to the Laboratory for Marine Animal Health for necropsy. To examine living algal colonies, a sharp scalpel blade was used to remove algae from select areas of horseshoe crab exoskeleton. The samples were then placed on a glass microscopic slide with seawater, covered, and examined with a light microscope. For tissue histopathology, selected tissue specimens were fixed in 10 percent neutral buffered formalin for at least 24 hours.

Following fixation, specimens were dehydrated in graded ethanol and embedded in JB-4 (Electron Microscopy Sciences, Hatfield, PA, USA) plastic embedding medium. Finished blocks were sectioned with glass knives on a Sorvall JB-4 rotary microtome at 2-4 um and sections were stained with Polysciences (Warrington, PA, USA) JB-4 stain.

Tissue specimens were fixed in either straight glutaraldehyde formula or glutaraldehyde/barbiturate formula for transmission electron microscopy. After 1 to 3 hours in primary fixative at 4 degrees Celsius, specimens were rinsed in cold buffer at 15 minute intervals for 1 hour. Tissue specimens were then post-fixed in appropriately buffered 1% osmium tetroxide for 1 hour and dehydrated in a cold ethanol series. Specimens were embedded in Epon 812 (Luft, 1961).

Gold and silver sections were obtained with glass and diamond knives on a Sorvall MT2B ultramicrotome. Thin sections were placed on copper grids and stained 10-15 minutes each in 5-7% uranyl acetate and 0.2% lead citrate. Sections were viewed and photographed with a Zeiss-10 transmission electron microscope.

In vitro algal culture was accomplished by seeding sterile algal-grow culture medium with algal samples taken from infected horseshoe crabs. These cultures were incubated at room temperature under fluorescent light and examined several times per week to monitor algal growth.

Results:

Progressive and chronic degenerative lesions in the dorsum of the exoskeleton, the eyes (ocelli and large lateral eyes), arthrodial membrane, and the base of the telson were documented (Figures 1, 3-5).

Direct microscopic studies of the green algae from affected *Limulus* tissues, and of in vitro algal culture, revealed young germlings (zygotes) ability to extend their rhizoidal processes in and between the chitinous lamina that compose the horseshoe crab's exoskeletal surface structures and organs. Algal invasion of the exoskeleton could result in secondary bacterial and mycotic infections.

Morphological studies of the green algal organism, at both the light and electron microscopic levels, indicate that the pathogen belongs to the family Ulvaceae.

Discussion:

Histological sections were prepared from horseshoe crabs affected by green algal disease to further elucidate the pathogenesis of the disease. From these preparations, it was found that green algae (likely from the family Ulvaceae) were able to attach and insert themselves within the chitinous matrix of *L. polyphemus'* carapace. The algae was found to inhabit all chitinous surfaces of the animal, from the prosoma and opisthosoma, to the ocelli and telson. As the algal zygotes pushed rhizoidal processes into the carapace, deep areas of erosion (or algal pits) were formed that stretched from the epicuticle (the thin surface layer of the carapace) and into the exocuticle (the thick middle layer of the carapace). This created open wounds and made the crabs increasingly vulnerable to secondary infections from bacteria and fungi. The ill effects caused by the invading algae eventually overwhelmed the arthropods, causing them to succumb in many cases.

Green algal disease is documented as being one of the more common and thus important causes of morbidity in adult captive and wild caught *L. polyphemus* (Leibovitz and Lewbart 1987; 2003). The disease can lead to loss of tissue structure and function, including deformed shells, degeneration and loss of ocular structures, erosion of the arthrodial membrane, and cardiac hemorrhage. One of the reasons why algal disease may be so prevalent is that once horseshoe crabs reach maturity, they cease to molt (Harrington et. al., 2008). This is unlike the American lobster, *Homarus americanus*, which can effectively "molt out" of its epizootic shell disease (Smolowitz et al., 2005). Therefore, once epibionts like algae attach to the surface of *L. polyphemus*, they are unlikely to be dislodged unless taken off by an outside force (i.e. in a lab setting). This may not be an issue until there is a significant mat of algae covering the surface of the horseshoe's crab's carapace (see Figure 1). In a captive setting, water quality may play a significant role in controlling the propagation of algae in a closed system, thus contributing to the possibility of the horseshoe crab developing algal disease. In one study, horseshoe crabs were raised in live car containers in a saline pond containing decaying fish parts. The animals in the live car secreted a very thick dermal exudate in comparison to animals raised in a cleaner environment (Harrington et. al., 2008). The exudate has been shown to display immunological properties (Harrington et. al., 1999). This further highlights the importance of maintaining low levels of ammonia, nitrite and nitrate (and maintaining other water quality parameters in their respective ranges) in order to deter growth of epibionts like algae that contribute to significant pathology in the horseshoe crab.

The further characterization of green algal disease complements current research that has started to describe the innate immunity of the horseshoe crab epithelium. For instance, a recent paper nullifies the accepted thought that only Gram-negative bacteria possess LPS as they were

able to purify an LPS-like molecule (aLPS) from the green algae *Chlorella* (Armstrong et. al., 2006). An aLPS was shown to cause the exocytosis of amebocytes as well as initiate the coagulogen processing pathways, but with lower efficiency than bacterial LPS. However, the aLPS still produced a biologically relevant response, because the coagulin clot forms in the presence of algal cells, effectively retarding systemic dissemination of microbes that have penetrated the carapace (Conrad et al,, 2001; 2006). These findings could explain how green algae are able to cause such significant pathology compared to other epibionts found on *Limulus*.

As described above, there is evidence that horseshoe crabs produce an exudate from their hypodermal glands that display immunological properties. Its anti-biological activity, exemplified by its ability to lyse foreign cells such as mammalian erythrocytes through inserting itself into the foreign cell's plasma membrane, may contribute to this activity (Harrington et al., 2008). In addition to its anti-biological activity, the continuous production of the exudate can exert a mechanical action, entrapping and sweeping potential fouling organisms away from the solid surface of the cuticle (Harrington et al., 2008).

Further research is warranted to explain how green algae overcome innate immune defenses of to cause internal damage to the horseshoe crab and to further characterize the lesions produced by algal disease. There is a good possibility that the incidence and severity of green algal disease is related to the age of the horseshoe crabs, and, that older animals, with long molting intervals (or none at all), are most vulnerable (Duffy et al, 2006).

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Figures:

Figure 1. Gross image of the prosoma (cephalothorax) in the area of the large compound lateral eye (LE). Both the carapace and the eye are partially encased by green algae. Histology can reveal the severity of the algal hyphaes' tissue penetration.

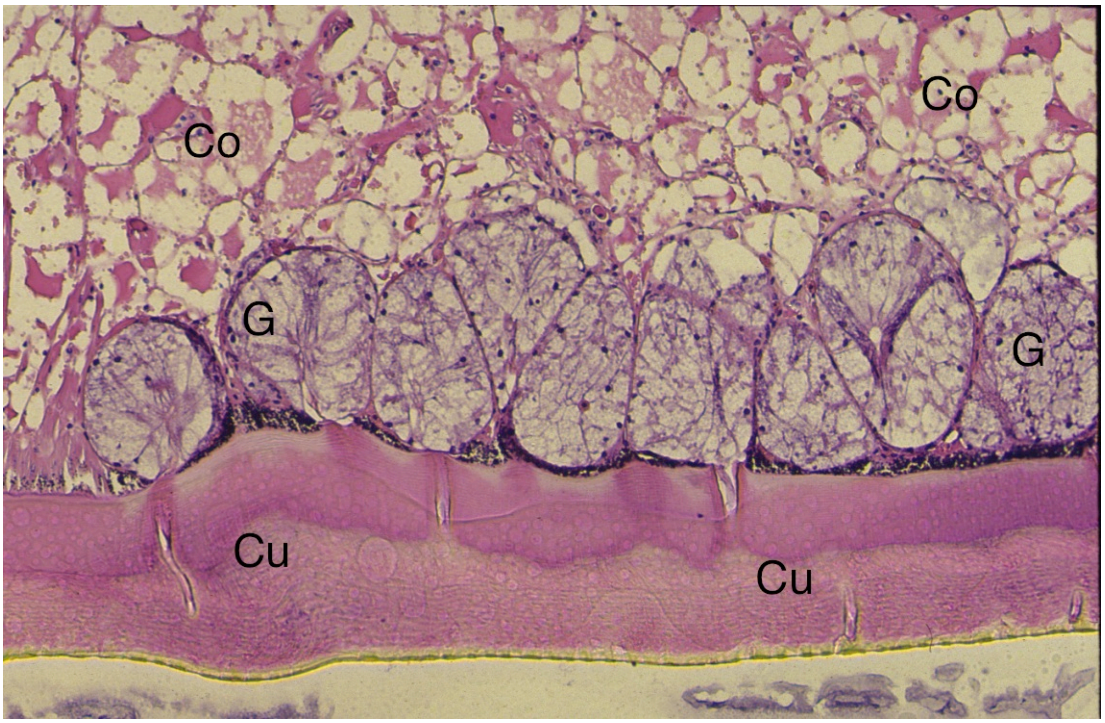

Figure 2. Histopathology of normal *Limulus* exoskeleton and underlying soft tissues. Note the smooth chitinous cuticle (Cu), glandular matrix (G), and thick layer of connective tissue (Co). These hypodermal glands possess tracts that allow for secretions to reach the surface of the carapace. Hematoxylin & eosin staining; 10X. Photomicrograph courtesy of S. Smith.

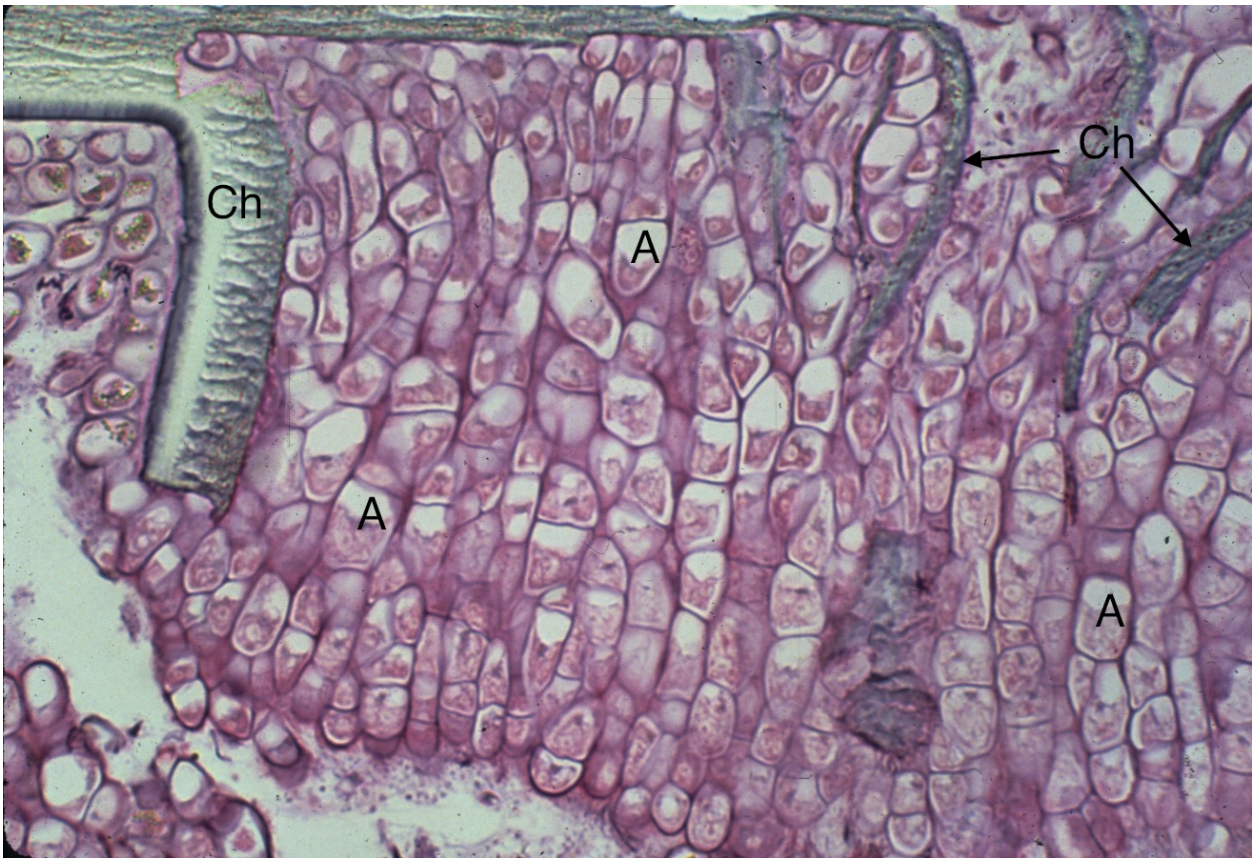

Figure 3. Histopathology of diseased chitin (Ch) showing invasive columns of green algal cells (A) elevating and displacing the acellular chitin. Polysciences JB-4 staining; 400X.

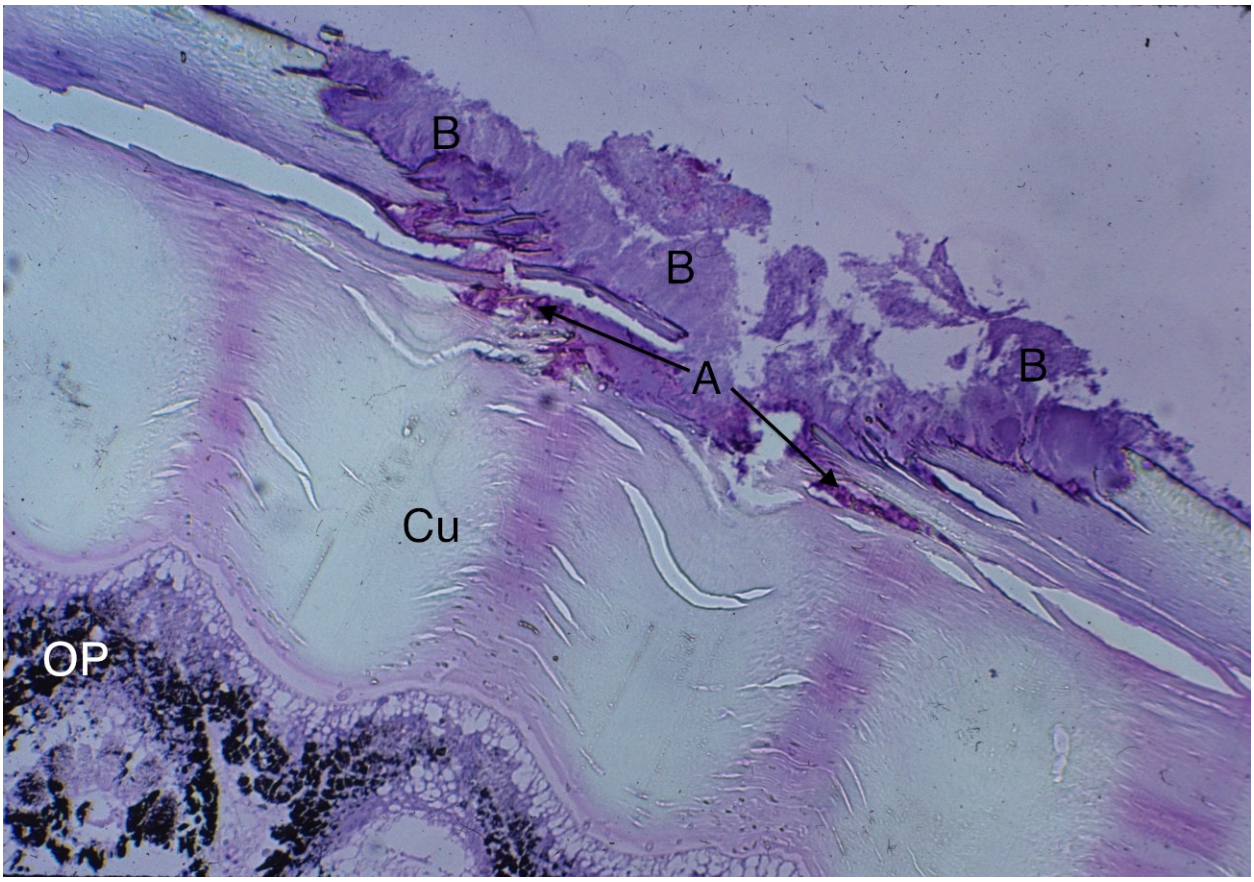

Figure 4. Histopathology micrograph of the ocellus affected by invasive green algae (A, arrows) and bacteria (B). The invasive organisms have eroded a pit-like lesion in the cuticle (Cu). Both the epicuticle and underlying exocuticle are affected. The ocular tissues, defined by the pigmented area (OP), remains directly unaffected. Polysciences JB-4 staining; 100X.

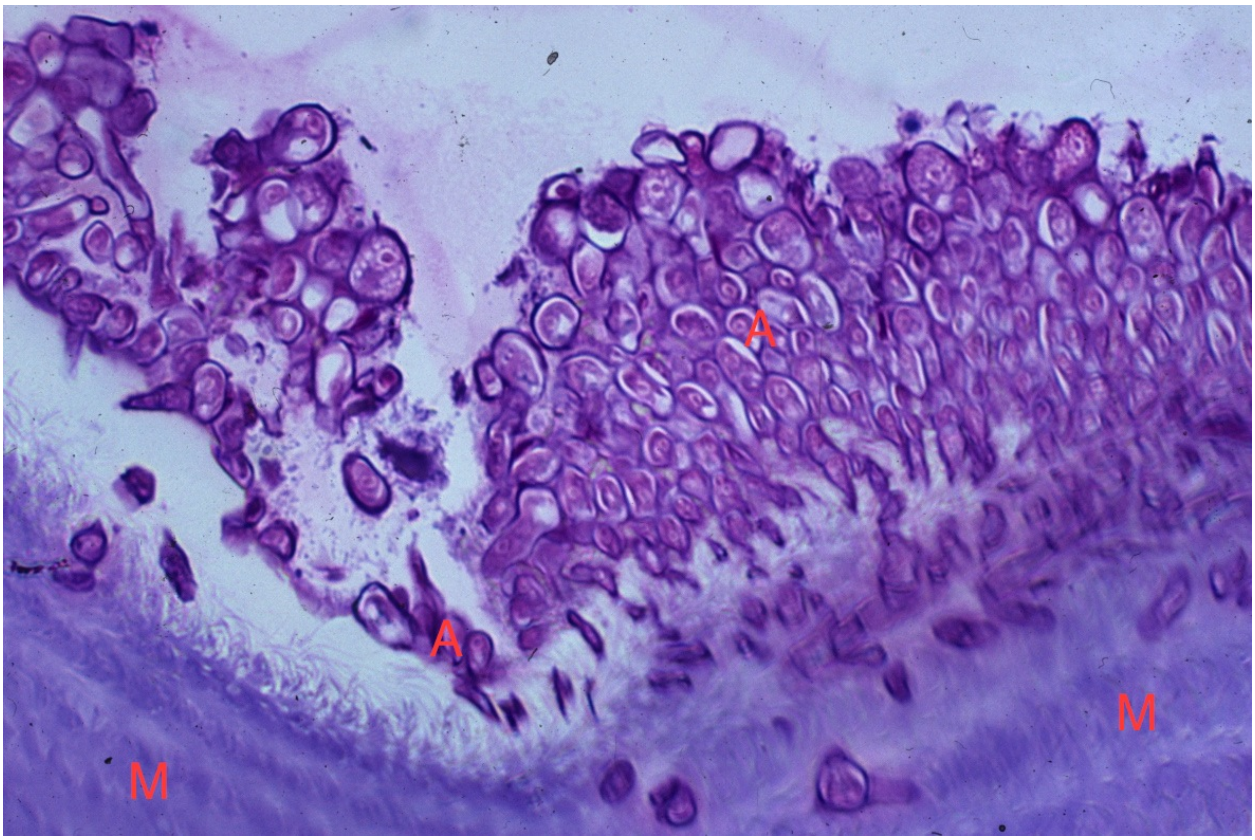

Figure 5. Histopathology of the telson ligament, or membrane (M), infected by green algae (A). Polysciences JB-4 staining; 400X.