

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

3 **Running Head: Horseshoe Crab Green Algal Disease**

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19 **Abstract:**

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

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38 **Introduction:**

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

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

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

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

75 **Materials & Methods:**

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

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

87 Tissue specimens were fixed in either straight glutaraldehyde formula or
88 glutaraldehyde/barbiturate formula for transmission electron microscopy. After 1 to 3 hours in
89 primary fixative at 4 degrees Celsius, specimens were rinsed in cold buffer at 15 minute intervals
90 for 1 hour. Tissue specimens were then post-fixed in appropriately buffered 1% osmium
91 tetroxide for 1 hour and dehydrated in a cold ethanol series. Specimens were embedded in Epon
92 812 (Luft, 1961).

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

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

102 **Results:**

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

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

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

113 **Discussion:**

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

125 Green algal disease is documented as being one of the more common and thus important
126 causes of morbidity in adult captive and wild caught *L. polyphemus* (Leibovitz and Lewbart
127 1987; 2003). The disease can lead to loss of tissue structure and function, including deformed
128 shells, degeneration and loss of ocular structures, erosion of the arthroal membrane, and
129 cardiac hemorrhage. One of the reasons why algal disease may be so prevalent is that once
130 horseshoe crabs reach maturity, they cease to molt (Harrington et. al., 2008). This is unlike the
131 American lobster, *Homarus americanus*, which can effectively “molt out” of its epizootic shell
132 disease (Smolowitz et al., 2005). Therefore, once epibionts like algae attach to the surface of *L.*
133 *polyphemus*, they are unlikely to be dislodged unless taken off by an outside force (i.e. in a lab
134 setting). This may not be an issue until there is a significant mat of algae covering the surface of
135 the horseshoe’s crab’s carapace (see Figure 1). In a captive setting, water quality may play a
136 significant role in controlling the propagation of algae in a closed system, thus contributing to the
137 possibility of the horseshoe crab developing algal disease. In one study, horseshoe crabs were
138 raised in live car containers in a saline pond containing decaying fish parts. The animals in the
139 live car secreted a very thick dermal exudate in comparison to animals raised in a cleaner
140 environment (Harrington et. al., 2008). The exudate has been shown to display immunological
141 properties (Harrington et. al., 1999). This further highlights the importance of maintaining low
142 levels of ammonia, nitrite and nitrate (and maintaining other water quality parameters in their
143 respective ranges) in order to deter growth of epibionts like algae that contribute to significant
144 pathology in the horseshoe crab.

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

148 able to purify an LPS-like molecule (aLPS) from the green algae *Chlorella* (Armstrong et. al.,
149 2006). An aLPS was shown to cause the exocytosis of amebocytes as well as initiate the
150 coagulogen processing pathways, but with lower efficiency than bacterial LPS. However, the
151 aLPS still produced a biologically relevant response, because the coagulin clot forms in the
152 presence of algal cells, effectively retarding systemic dissemination of microbes that have
153 penetrated the carapace (Conrad et al., 2001; 2006). These findings could explain how green
154 algae are able to cause such significant pathology compared to other epibionts found on *Limulus*.

155 As described above, there is evidence that horseshoe crabs produce an exudate from their
156 hypodermal glands that display immunological properties. Its anti-biological activity,
157 exemplified by its ability to lyse foreign cells such as mammalian erythrocytes through inserting
158 itself into the foreign cell's plasma membrane, may contribute to this activity (Harrington et al.,
159 2008). In addition to its anti-biological activity, the continuous production of the exudate can
160 exert a mechanical action, entrapping and sweeping potential fouling organisms away from the
161 solid surface of the cuticle (Harrington et al., 2008).

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

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239 **Figures:**

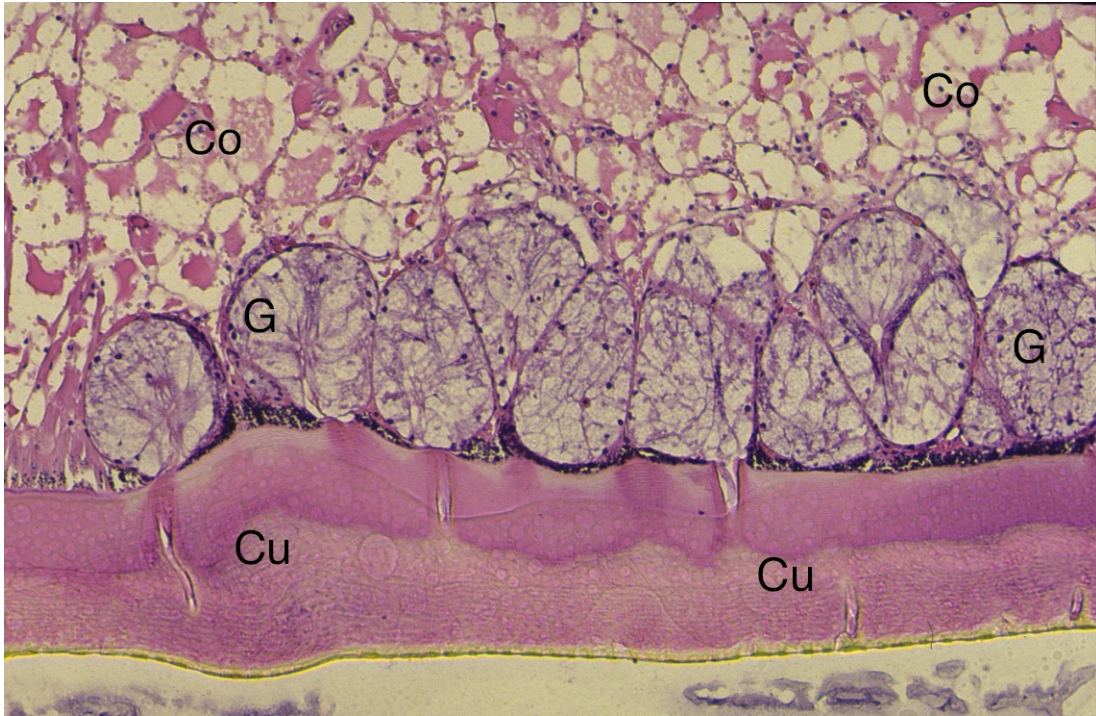


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241 Figure 1. Gross image of the prosoma (cephalothorax) in the area of the large compound lateral

242 eye (LE). Both the carapace and the eye are partially encased by green algae. Histology can

243 reveal the severity of the algal hyphaes' tissue penetration.



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246 Figure 2. Histopathology of normal *Limulus* exoskeleton and underlying soft tissues. Note the

247 smooth chitinous cuticle (Cu), glandular matrix (G), and thick layer of connective tissue (Co).

248 These hypodermal glands possess tracts that allow for secretions to reach the surface of the

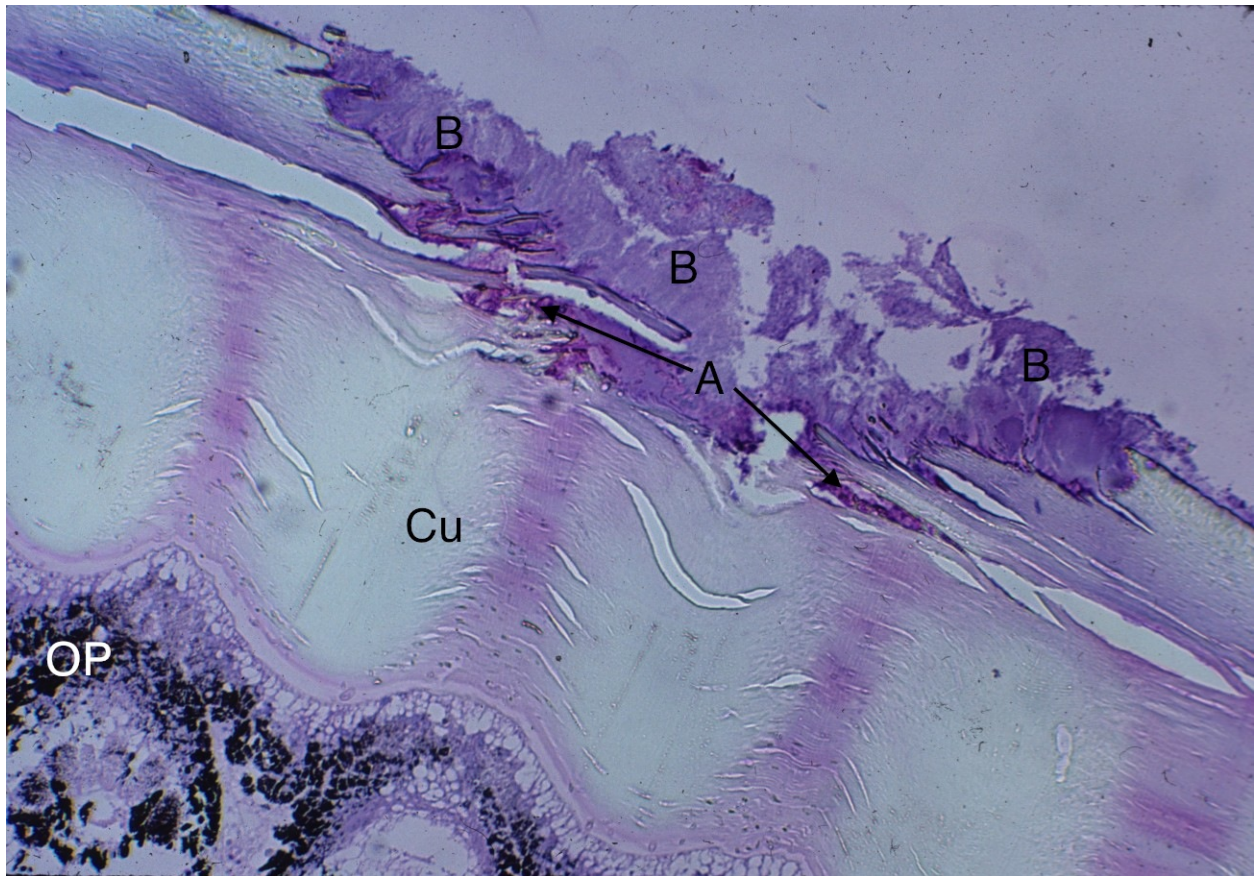
249 carapace. Hematoxylin & eosin staining; 10X. Photomicrograph courtesy of S. Smith.



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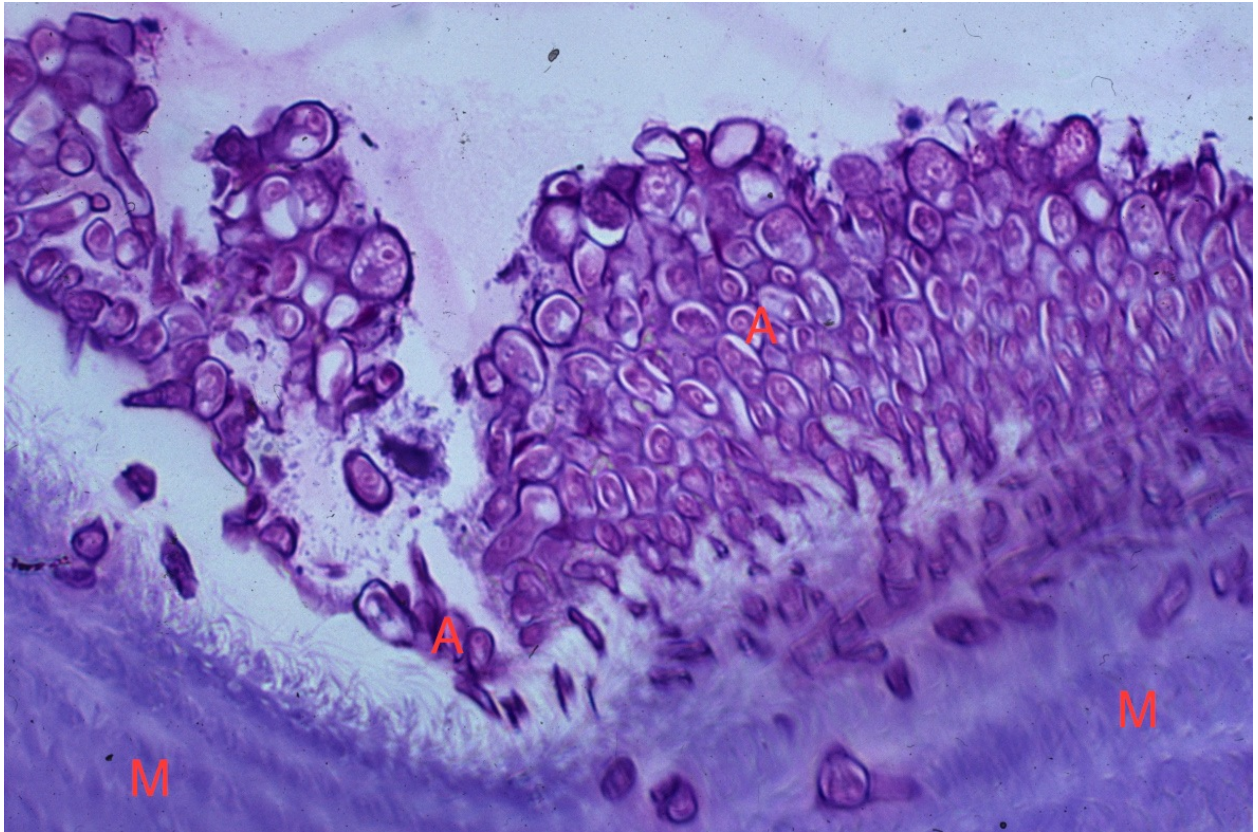
251 Figure 3. Histopathology of diseased chitin (Ch) showing invasive columns of green algal cells

252 (A) elevating and displacing the acellular chitin. Polysciences JB-4 staining; 400X.



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



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259 Figure 5. Histopathology of the telson ligament, or membrane (M), infected by green algae (A).

260 Polysciences JB-4 staining; 400X.

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