

Review

I recommend revision of the manuscript and publication in this journal. There is a relative paucity of literature on treatment options for HSC (and invertebrates generally). Well-designed studies such as this are desperately needed in this field. Most of the revisions I recommend are for the review and description of histologic findings and immune response. Also, I think there should be additional explanation for the methodology (specifically why the telson membrane was selected, why the length of time was selected, and why evaluation of suture reaction is important in this species). I have included additional general and specific comments below. I would be happy to review this manuscript again, and help the authors in further histologic interpretation, if necessary/desired.

General comments:

Please further research and describe the immune response in HSC. The immune effector cell of these animals is not called an amebocyte in references that I have reviewed (or the cited reference: Bursey 1977. Histological response to injury in the horseshoe crab). References use the terms “granulocyte” or “hemocyte” in HSC. I would prefer these terms (particularly hemocyte), but if the authors have good references that use “amebocyte,” that is fine. Although LAL stands for “limulus amebocyte lysate,” I don’t believe “amebocyte” is commonly used as a term for their inflammatory cells. Also, “granuloma” is not part of the immune system of HSC as they do not have macrophages or fibroplasia as part of their immune response. The inflammatory response the authors are likely observing is a “coagulum,” which is a specific formation incited by the activation of coagulen, a protein in their hemolymph that results in formation of an insoluble coagulum that entraps antimicrobial enzymes released from hemocyte granules.

Likewise, please elaborate on the histopathology findings. Some of the findings described in the figure legends are not evident in the images. Some of the lesions in the images are not described in the findings. Also, please include labels on the figures to denote epidermis, artifact spaces, spaces previously occupied by suture, coagulum formation, inflammatory cells, etc. The figures need to be oriented appropriately (i.e. epidermis should be across the top), and generally should be sharpened and white balanced.

Please include a statement on the necessity of determining the most appropriate suture material in this species. In my experience, captive HSC rarely die of primary trauma/physical injury that would have been cured with suturing traumatic wounds. Usually underlying infection is the primary cause of disease. Perhaps the findings here can be extrapolated to other chelicerate arthropods to increase the significance of the results?

How did you choose the suture site (in the telson membrane)? Can you comment on why you chose soft cuticle as a site for the experiment rather than hard cuticle? Do you see more injury in regions of soft cuticle? I would have thought that traumatic lesions would be more common in the hard cuticle.

How did you choose the length of time to evaluate this animal for dehiscence? Would the cuticle typically degenerate in 6-12 days? Or does it take longer?

Line by line comments are below:

- Line 34: Abstract: It is unclear what is meant by “opisthosoma to telson membrane” in the abstract. Are the authors indicating a portion of the exoskeleton or full thickness through the animal? Is the margin of the opisthosoma at the articulation with the telson reviewed or the whole opisthosoma? Please clarify in the abstract.
- Line 54: It is surprising that HSC have physiological and anatomical similarities to humans. Please provide a reference for this statement.
- Amebocyte spelling – is “amebocyte” preferred over “amoebocyte?”
- Line 66: Is this statement true? The Smith 2017 reference indicates that only some populations are declining, while others are stable. Consider rewording this statement for accuracy (e.g. “Some populations of HSC are declining...”). If some populations are stable, perhaps this species is not under the threat of extinction? Also, the population declines have not been persistent according to the Smith 2017 paper cited – some populations were declining (e.g. Delaware Bay), but are now stable due to conservation efforts.
- Line 90-92: I think this is a great idea.
- Line 97-98: How common is trauma as a primary problem in captive HSC? In my experience, this is an uncommon primary disease in HSC. Although they experience trauma, they frequently have other, underlying issues (e.g. infection) that predispose them to trauma/death.
- Throughout the manuscript, there is variation in *L. polyphemus* vs *Limulus polyphemus*. I believe *L. polyphemus* is appropriate after the genus is spelled out once.
- Fig. 1 is excellent
- Is there a reference for clove oil analgesia/anesthesia in HSC? If not, maybe include ref of pers comm?
- Line 153: how much clove oil was used?
- Line 153: Does “overdose” mean euthanasia? Please clarify
- Line 153-157: I’m a little confused – were all the animals euthanized as suggested on line 153, or were the controls allowed to survive as suggested on line 158?
- Line 164: Histologic description of the membrane can be found here: Fahrenbach WH. Merostomata. In: Harrison FW and Locke M, eds. *Microscopic Anatomy of Invertebrates*. New York: Wiley-Leiss; 1999;8A:21-115. If the authors do not have access to this book, I can provide a PDF copy for their use. Even though normal histology references are available, I believe that it is still important for the authors to include their normal histologic description of the telson membrane as the aforementioned text is out of print and their control data is relevant.
- Line 169: These animals don’t make granulomas as there are no histiocytes nor fibroplasia in the HSC inflammatory response. If “granuloma” is a term the authors would like to use, they should specifically define what is meant by “granuloma.” I prefer the term “hemocyte coagulum” or “granulocyte coagulum” as these terms reflect the immune response.
- Table 1: further descriptions of “activated amebocytes (or granulocytes/hemocytes)” is necessary. Exactly what morphological changes are observed? In HSC, hemocytes discharge their cytoplasmic granules upon activation, resulting in agranular hemocytes histologically (i.e. hemocytes that have clear cytoplasm lacking eosinophilic granules). Activated hemocytes also can become elongate (as opposed to their normal spherical appearance). Please elaborate on changes to inflammatory cells and cite references to indicate these changes reflect “activation.”
- Line 180: Please cite references for heart rate, if available. According to Fahrenbach WH. Merostomata. In: Harrison FW and Locke M, eds. *Microscopic Anatomy of Invertebrates*. New York: Wiley-Leiss; 1999;8A:21-115, *L. polyphemus* has a mean heart rate of 34 bpm with a maximum of

51 bpm. This is not in agreement with the statement “30bpm was very active.” It seems to me that the 30 bpm is normal activity and the 8 bpm is quiet?

- Line 191: Should a “membrane” include the epidermis? I would think the “membrane” would be the cuticle (i.e. bilayered and including epi and endocuticle).
- Line 194: The hemolymph sinuses are subjacent to the epidermis. Please clarify.
- Line 196: Please include what signs of inflammation HSC would have grossly as the typical lesions of inflammation (redness, etc.) would not be expected.
- Figure 2: There is some artifact (likely dust) on the slide in the image, and the image is not in focus. Additionally, it needs white balancing.
- Figure 3: Please crop the image so that the artifact in the top right of the epicuticle
- Line 423: Figure Legend 3: If only two layers are shown, please either indicate which layer is not shown, or reword the legend, something like: “Normal histology of the telson membrane showing the outer epicuticle and inner endocuticle that are separated by a distinct feathered margin (arrow)”
- Figure legends: please be specific – please use “epidermis” instead of “epithelium” where appropriate. This is applicable to figure legend 4, 6, and 7. I don’t believe the epithelium in figure 5 is epidermis. Revisit than image.
- Figures: For all figures, please rotate the images so that the epidermis is across the top, parallel to the top of the image.
- Figure 4: please rotate image so that epidermis is across the top. Also please adjust figure legent (line 425) to reflect this change.
- Figure 5: the legend indicates that an “A” should be labeled on the figure, but I don’t see this on the version I have. Also, what are the luminal structures that fill the majority of the field? Please label those, as well as the inflammation. What is the “hypertrophied epithelium” in this image? I do not think this is epidermis, and I don’t think you showed any other control epithelium? This figure legend needs substantial revision to explain the image. I’m not sure there is artefactual separation. Are the holes in the image where previous suture was?
- Figure 6: Please use epidermis. There is too much necrosis/artifact to appreciate hypertrophy based on this image. Also, please indicate what the large clear spaces are (previous location of suture [did you polarize this region?], artifact, etc.)
- Figure 7: please put the epidermis paraallel to the top of the image. Also, there is some coagulum formation on this slide (pink homogeneous extracellular fluid-like material). If that is the epidermis, there is also total loss of the cuticle, which should be described here and in the results if present.
- Table 1 and Table 2 Legend/title (line 417 and line 418): Typically titles of tables should be stand-alone (e.g. include animal species studied, etc.).
- Lines 202-208: Please elaborate on the histology findings. Please describe necrosis (there is karyorrhectic debris in slide 7), coagulum formation, loss of the cuticle, etc. Also please describe if hemocytes discharge granules in any of the reactions. “Granuloma” is described in the methods, but not in the results (I would prefer this term were not used generally, however).
- Line 210-211: How long were the previous sutures held in place?
- Line 212-213: The cuticle is absent and not attached to the epidermis in Fig. 7. I assume the cuticle was eroded in this case, no? I am unsure of the orientation or source of epithelium in Fig. 5, but cuticle may be absent in this case too.

- Lines 215-216: Please describe changes to the epidermis in results if you believe they are there – this will have to be compared to controls and highlighted with higher mag images (possible insets?).
- Line 225: Where is this terminology from? The cited Bursey 1977 uses “hemocytes” instead of amoebocytes, and “coagulum” instead of hyalinization. If using different terminology, please include a reference. Do you believe you had hyalinization? Please expand on the presence or absence of “hyalinization,” and define this term. I think this terminology is analogous to coagulum formation?
- Line 234: Here inflammatory cells are called hemocytes – I prefer this terminology, but will leave it up to the authors if they can find substantial relevant literature supporting use of the word “amoebocyte” over “hemocyte.”
- Line 251: Can the author comment on how clove oil might affect histology? Pentobarbital tends to cause mild artifact in the heart, and preserves the remaining tissues well. I have not reviewed any animals euthanized with clove oil, to my knowledge, and would be interested to know if there are artifacts (I would assume so if it is a hepatotoxin)?