Short Communication

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PII: S0022-2011(14)00180-3
DOI: http://dx.doi.org/10.1016/j.jip.2014.12.002
Reference: YJIPA 6617

To appear in: *Journal of Invertebrate Pathology*

Received Date: 9 July 2014
Accepted Date: 2 December 2014

Please cite this article as: Shinn, A.P., Mühlhölzl, A.P., Coates, C.J., Freeman, M.A., Metochis, C., Zoothamnium duplicatum infestation of cultured horseshoe crabs (Limulus polyphemus), *Journal of Invertebrate Pathology* (2014), doi: http://dx.doi.org/10.1016/j.jip.2014.12.002

Zoothamnium duplicatum infestation of cultured horseshoe crabs (*Limulus polyphemus)*

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Keywords: American horseshoe crab, epibiont, infestation, aquaculture, parasite

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ABSTRACT

An outbreak of the sessile peritrich *Zoothamnium duplicatum* in a pilot, commercial-scale *Limulus polyphemus* hatchery resulted in the loss of ~ 96% (40,000) second/third instar larvae over a 61 day period. Peritich growth was heavy, leading to mechanical obstruction of the gills and physical damage. The peritrichs were controlled without resultant loss of juvenile crabs by administering 10 ppm chlorine in freshwater for 1 h and the addition of aquarium grade sand; a medium into which the crabs could burrow and facilitate cleaning of the carapace. Peritrich identity was confirmed from a partial SSU rDNA contiguous sequence of 1343 bp (99.7% similarity to *Z. duplicatum*).

1. Introduction

The American horseshoe crab, *Limulus polyphemus*, is an ecologically and commercially valuable marine invertebrate that has been exploited by numerous industries for over a century. Horseshoe crabs have been used as an ingredient in fertiliser and livestock feeds, as bait for whelk (*Busycon spp*.) and eel (*Anguilla spp.*), and most notably, for the production of the highly sensitive endotoxin detection assay, Limulus Amebocyte Lysate (LAL) (reviewed by Walls et al., 2002). LAL is derived from a series of proteins stored within the cytoplasmic granules of *L. polyphemus* blood cells (amebocytes; Coates et al., 2012a). After blood (hemolymph) has been extracted from the animals they are returned to their capture point, and although the industry states that typical mortalities are between 5-15%, other studies suggest that these can be high as 30% (Hurton and Berkson, 2006; Leschen and Correia, 2010). Recent evidence indicates that extracting large volumes of blood may not result immediately in horseshoe crab death, but induces sub-lethal effects that diminish animal fitness (Anderson et al., 2013).

Given that horseshoe crabs appear relatively easy to house *ex situ* (Smith and Berkson, 2005; Coates et al., 2012b), there is commercial aquaculture interest in maintaining and rearing sustainable captive held populations of *L. polyphemus* (Carmichael and Brush, 2012; Kwan et al., 2014). There remains, however, a lack of knowledge regarding the aetiologies/pathologies of infectious/non-infectious diseases of captive and wild populations. Degenerative lesions on the shell (carapace) and soft tissues of horseshoe crabs (irrespective of age) appear to be the most frequently reported disorder, attributed to pathogenic algae, fungi and Gram-negative bacteria (reviewed by Nolan and Smith, 2009).

The objectives of this study were to characterise the aggressive epibiont infestation (*Zoothamnium duplicatum*) that covered the entire carapace of juvenile horseshoe crabs, and to develop a non-invasive treatment. The presented data offers a relatively rare insight into horseshoe crab-protozoan antibiosis. The abundance of *Z. duplicatum* most likely facilitated fouling within the hatchery, providing conditions favourable for the proliferation of other noxious microorganisms.

2 Materials and methods

2.1 Culture conditions

In February 2010, a spawning event in a commercial aquaculture facility was initiated in two 5,700 L (3.8 m diameter) polypropylene welded tanks each containing 82 adult *L. polyphemus* (ratio 1:1), representing wild stock originating from Delaware Bay, USA. Spawning continued until mid-April 2010 with the release of 750-1000 mL of eggs (~216 eggs mL^{-1}) every 2 days. The eggs were maintained in 6 L plastic tubs on a flow-through system (150 mL min^{-1} flow rate, 24°C, 30 ppt). After hatching (~42% post-hatch survival), the trilobites were transferred to 315 mm diameter × 150 mm high downwellers, the base of each lined with 50 µm mesh. The downwellers were arranged such that there were seven units per 200 L trough. Water was adjusted to 6.2 – 7.7 L per downweller, with a flow rate of 1 L min^{-1}. Approximately 2000 ± 10% trilobites were stocked into each downweller but as they moulted into first/second

instars, the numbers were decreased to 1000 ± 10% per unit. As the crabs grew, the size of the base mesh was increased accordingly up to 400 μm. Crabs were maintained on a blended diet of bloodworms, blue mussels (*Mytilus edulis*), mysid shrimp (*Americamysis bahia*) and *Artemia salina* at a rate of ~5 g of feed per downweller per day. Artificial seawater (Instant Ocean^{TM}) was used throughout the culture system (salinity; 32 ± 5 ppm). Up to 30% of water was exchanged weekly. Water quality properties were monitored using a Nutrafin® Master Test Kit: PO_4, Ca, NO_3, NH_3, pH, carbonate hardness, NO_2 and non-chelated iron Fe.

2.2 Farm led management of the epibiont outbreak

A series of chemical interventions were conducted on select numbers of crabs (n = 10 per replicate), to establish an effective regime for the management of the epibiont community. Treatments included 10, 20 and 50 ppm chlorine (Supermarket brand bleach) in freshwater (1 h); 25, 100, 300 and 1000 ppm formalin in freshwater (1 h); 0.5 ppm (1 h) and 50 ppm (dip) methylene blue; and, 13 mg L^{-1} PimaFix^{TM} (Mars Fishcare Inc.) (1 h) (see Table 1). Following treatment, crabs were transferred into clean, filtered 35 ppt seawater with or without sand substrate (Oolytic sugar sand, 0.2 - 1.2 mm diameter), and monitored over 48 h.

2.3 Specimen preparation

Five, moribund *L. polyphemus* second instars (selected randomly) were fixed in 10% neutral buffered formalin for 24 h before being wax embedded, sectioned longitudinally (5 μm sections) and stained with haematoxylin and eosin following standard procedures. Six, second instars with visible epibiont communities on their external surfaces were fixed in 95% ethanol for molecular analyses. The ectocommensal parasite community on a further four specimens was demonstrated macroscopically by immersing the animals in 5 ml 0.01% neutral red for 5 min before being fixed in 10% neutral buffered formalin and photographed. Specimens for scanning electron microscopy were fixed in 2.5% glutaraldehyde (0.1 M sodium cacodylate buffer) at 4ºC for 2 days and rinsed in 0.1 M sodium cacodylate for a further 24 h. Specimens were post-fixed in 1% osmium tetroxide (2 h) before being dehydrated through an alcohol series to 100% ethanol. Next, specimens were critical point dried using liquid carbon dioxide at 32°C, 75 bar pressure (Bal-Tec CPD 030

Critical Point Dryer). The dried specimens were mounted on 0.5" aluminium pin stubs (Agar Scientific Ltd., Essex, UK) using double-sided carbon tape (G3347N, Agar Scientific), sputter-coated with gold (Edwards Sputter Coater S150B) and viewed under a Jeol JSM 6460 LV SEM (accelerating voltage of 10kV).

2.4 Molecular methods

Three of the six infected *L. polyphemus* (carapace widths 5-7 mm) were immersed in DNA lysis buffer containing proteinase K (100 µg mL^{-1}) and left at room temperature until most of the stalked ciliates became detached (< 2 h). Post-treatment, DNA was extracted using a GeneMATRIX DNA extraction kit (EURx Poland) following the manufacturer's tissue protocol. Partial small subunit ribosomal DNA (SSU rDNA) sequences were amplified using the universal primers 390f, 870r (its complementary forward primer 870f) and 18gM, for which primer sequences and PCR conditions have been described (Freeman et al., 2004; Freeman and Ogawa, 2010). PCRs were performed in triplicate. Amplifications from three different extractions and all PCR products were sequenced in both forward and reverse directions using the original amplification primers. A contiguous sequence was constructed manually using ClustalX and BioEdit.

3. Results

3.1 Light and SEM examinations

Initially, mortality rates were low (<0.25% day^{-1}) until the crabs reached the second and third instar stages, when mortality rates rose sharply to >5% stock day^{-1}. Subsequent assessment of juveniles under an Olympus SZ40 dissecting microscope revealed a heavy epibiont burden on all crabs (Figure 1a). Rising nitrite concentrations (>3.3 mg L^{-1}) within the culture facility were addressed immediately by increasing flow rate to 1.5 L min^{-1} and lowering water temperature and salinity to 19°C and 25 ppt, respectively. Overall, water quality properties were satisfactory for the duration of the outbreak: 0.25 - 1 mg L^{-1} PO_4, 420 ± 60 mg L^{-1} Ca, 170 ± 20 mg L^{-}

^{1} carbonate hardness, < 0.1 mg L^{-1} non-chelated iron, 5-10 mg L^{-1} NO_3^-, 0 – 0.1 mg L^{-1} NH_3 and a pH range of 7.9 to 8.3.

Two weeks into the mortality event, a requested visit to the hatchery site was made to investigate the possible cause(s) for the juvenile horseshoe crab deaths. A random sample of second and third instars that were examined under a dissecting microscope (×4 magnification, Olympus SZ40) harboured heavy external growth of epibionts. Samples were stained on site with neutral red, a vital stain, to ascertain whether the attached epibionts were indeed viable (Figure 1a). Some of the epibionts on the carapace detached during the preparation of specimens for wax histology, but within the gill chamber, deleterious growth was evident. Gill histopathology revealed the over-accumulation of epibionts, causing a mechanical blockage to the extent that respiratory function was likely impaired (Figure 1b). Examination of the epibiont by light microscopy revealed a branched, sessile peritrich, colony-forming species consistent with the morphological features of the genus of *Zoothamnium* Bory de St. Vincent (Peritrichida: Vorticellidae) (Figure 1c). Typically, the zooids were ovoid in shape (Figures 1c, 2b), measuring 21.7 ± 1.3 (19.5 – 24.2) µm width × 31.8 ± 2.5 (28.4 – 35.8) µm length (mean ± 1 S.D followed by the range in parentheses; n = 20) not including the ciliary tuft were measured from SEM micrographs, connected to one another by a spasoneme, and attached to their host by irregularly "circular" anchoring discs measuring 16.9 ± 2.9 × 13.7 ± 5.0 µm (mean ± 1 S.D.; n = 5) in diameter. All juvenile crabs within the facility appeared to be colonised by *Z. duplicatum* and no specimens were sufficiently epibiont-free to serve as controls for comparison. SEM was used to confirm this observation and that growth was represented by a single species only (Figure 2a, b). Figures 2a, b show that epibiont growth was extensive such that the attachment discs anchoring colonies to the surface of the carapace occluded the existing pores (Figure 2c). It should be noted that filamentous *Leucothrix*-like bacteria could be seen alongside *Z. duplicatum* in the electron micrographs (Fig. 2), as well as other rod-shaped microbes.

3.2 Treatment

Of the treatment regimes that were tested (see Table 1), 10 ppm chlorine in freshwater for 1 h proved effective without the subsequent loss of crabs. Higher doses were equally effective, without loss, but not necessary to effect control. The other bath

treatments appeared to have no discernible impact on epibiont growth/viability. Following a pilot trial using sand in two test vessels, the extent of epibiont growth (scored on a scale of 1 to 10; Table 1b) was seen to decrease and so aquarium sand was added subsequently to each downweller so that crabs could burrow in it to dislodge epibionts or to discourage regrowth.

3.3 Molecular characterisation

A partial SSU rDNA contiguous sequence of 1343 bp was successfully obtained and is deposited within GenBank (HQ268528). Nucleotide Blast searches showed the sequence to be 99.7% similar to the sessile peritrich ciliate, *Z. duplicatum* (DQ662851).

4. Discussion

An epibiont outbreak within a commercial hatchery of juvenile horseshoe crabs resulted in gross mortality of stock (~96%) over a 61day period. *Z. duplicatum* was identified as the ectocommensal parasite and undoubtedly contributed to horseshoe crab deterioration. *Zoothamnium* is known to be a richly diverse genus with a greater amount of molecular diversity than is predicted by morphological data, a trait that exists within all major clades of sessiline peritrichs (Clamp and Williams, 2006). Therefore, the genetic distance between *Zoothamnium* spp., with respect to SSU rDNA sequences, can be large. The distance between *Zoothamnium niveum* (DQ868350) and *Zoothamnium arbuscula* (AF401523) over 1683 bp of SSU rDNA sequence data is 0.0689 (93.1% similarity). Thus, the 99.7% similarity in SSU rDNA seen here indicates with little doubt that the ciliate removed from the horseshoe crabs is *Z. duplicatum*.

Zoothamnium spp. infections have been associated previously with the mass mortality of larval crustaceans in hatchery settings, such as *Eriocheir sinensis* (see Wu and Feng, 2004), *Penaeus aztecus* and *Litopenaeus setiferus* (see Johnson et al., 1973), where heavy growth on the gills was thought to restrict oxygen exchange leading to anoxia. A mixed bacterial and protistan community on the gills of a South

African freshwater crab, *Potamonautes warreni,* composed of ~10% *Zoothamnium* sp. induced a series of deformities visible in gill histopathology (Schuwerack et al., 2001). Similarly, histology data gathered here depicts the overwhelming presence of *Z. duplicatum* within the gill filaments of *L. polyphemus* (Fig. 1b).

Roegge et al. (1977) was able to control an unidentified infection of *Zoothamnium* on *Macrobrachium acanthurus* larvae by bathing specimens in 15 ppt seawater containing 50 ppm formalin (24 h). Although short duration (1 h) baths of up to 300 ppm formalin were trialled, they were ineffective in controlling the ectoparasitic peritrichs on *L. polyphemus* (see Table 1). A concentration of 1000 ppm formalin was successful in killing *Z. duplicatum* but was also fatal to horseshoe crabs. Perhaps, the consequential removal of oxygen led to hypoxic conditions within the downweller. With respect to the animals studied here, the *Z. duplicatum* infestation was controlled eventually through the use of 10 ppm chlorine in freshwater for 1 h and maintained with the addition of aquarium sand into each culture vessel to allow crabs to burrow and facilitate self-cleaning. Provision of a substratum in aquaria, notably sand, can assist the survival of horseshoe crab juveniles (Hong et al., 2009). Horseshoe crabs, both juvenile and to a lesser extent, adults maintain their carapace through a number of mechanisms: frequent moulting, immersion in sediment while at rest and secretion of an anti-fouling agent. *L. polyphemus* are usually free from ectocommensals and macroscopic epibionts (extending for periods $\geq$10 years) by employing a series of hypodermal glands (Stagner and Redmond, 1975), which covers the entire surface of the organism's carapace in a surfactant-like, cytolytic glycoprotein exudate (Harrington et al., 2008). Admittedly, the *Z. duplicatum* growth was extensive, with many of these surface pores blocked by their anchoring discs (Figure 2c), it is likely, however, that the stocking density, organic loading and operational temperature of the culture system facilitated epibiont growth at a rate faster than it could be controlled ordinarily by carapace based secretions, further exacerbated by the absence of sand substrate and the presence of opportunistic fouling organisms.

Fouling agents, such as filamentous *Leucothrix* spp., are found commonly in culture systems of larval invertebrates (e.g. *Homarus americanus*). Outbreaks of *Leucothrix mucor* have been linked to crustacean mortalities on a number of occasions (reviewed by Shields, 2011). An array of microbes, including *Leucothrix*-like bacteria, were visible in the SEMs of moribund *L. polyphemus* (Fig. 2), therefore,

L. polyphemus mortalities were likely caused by a combination of fouling organisms, not just *Z. duplicatum* alone. Few studies exist regarding the pathogen profile of wild or captive horseshoe crabs. Discolouration (white to reddish-brown) of the soft tissues, such as the arthroidal membrane and gill lamellae, is related to tissue necrosis, lesion formation and subcuticular damages (Allender et al., 2008; Nolan and Smith, 2009). Invasive fungal hyphae were observed in lethargic crabs within an aquarium touch tank (Tuxbury et al., 2014). The fungus was identified as *Fusarium solani,* a plant pathogen that has been associated with mycosis in invertebrates and many domestic animals. Allender et al. (2008) also reported mycotic dermatitis in aquarium-based horseshoe crabs possessing irregular and ulcerated lesions on the carapace and joint spaces of appendages. While fungal infections appear to be restricted to captive animals, algal, cyanobacterial (*Oscllatoria* spp.), parasitic trematodes and Gram-negative bacterial (*Flavobacterium* and *Vibrio* sp.) infections have been linked to morbidity in wild populations (reviewed by Smith and Berkson, 2005). Chlorophycophytal (green algae) is the most characterised infection of *L. polyphemus* (Leibovitz and Lewbart, 2004; Braverman et al., 2012). Symptoms can range from superficial shell discolouration to cuticular erosion and tissue putrefaction.

The spawning event represented the first in the hatchery, and in the absence of reference data, the stocking density of juvenile crabs in each downweller was subsequently determined to be 5.5 – 7.0 g L^{-1}. The most likely source of *Z. duplicatum* was from the shucked *M. edulis* component in the juvenile diet. Although we did not screen mussels to verify that they were a potential source of infection, *Zoothamnium* spp. have been reported from the mantle cavity of molluscs (*e.g. Zoothamnium chlamydis* from *Chlamys farreri* (Hu and Song, 2001); *Zoothamnium vermicola* from *Meretrix* spp. (Ji et al., 2009)) and from inert substrates (the hull of wooden sailing vessels; Carlton and Hodder, 1995). As a future biosecurity precaution, it might be judicious to either ozonate or UV irradiate (*i.e.* 100 – 400 nm) live prey items to remove potential pathogens from their surface before they are fed to the crabs (Summerfelt, 2003).

While a number of recent advances have been made in maintaining horseshoe crabs *ex situ*, there remains a need to characterise further, the nutritional requirements, substrate compositions and stocking density of these animals, if success is be achieved in sustaining a commercially viable and healthy environment. The presented

data advertises the destructive impact of an epibiont outbreak (amongst other fouling organisms) on vulnerable horseshoe crab juveniles, and highlights a non-invasive treatment.

Acknowledgments

This study was supported financially by the University of Stirling and Marine Biotech Ltd. We are grateful to Mr. Linton Brown and Mr. Denny Conway from the Institute of Aquaculture, University of Stirling for their technical assistance in photography and the preparation of specimens for SEM. DNA analyses were performed in Malaysia (University of Malaya HIR grant No: UMC/625/1/HIR/027).

Conflict of interest statement

We declare that there are no conflicts of interest.

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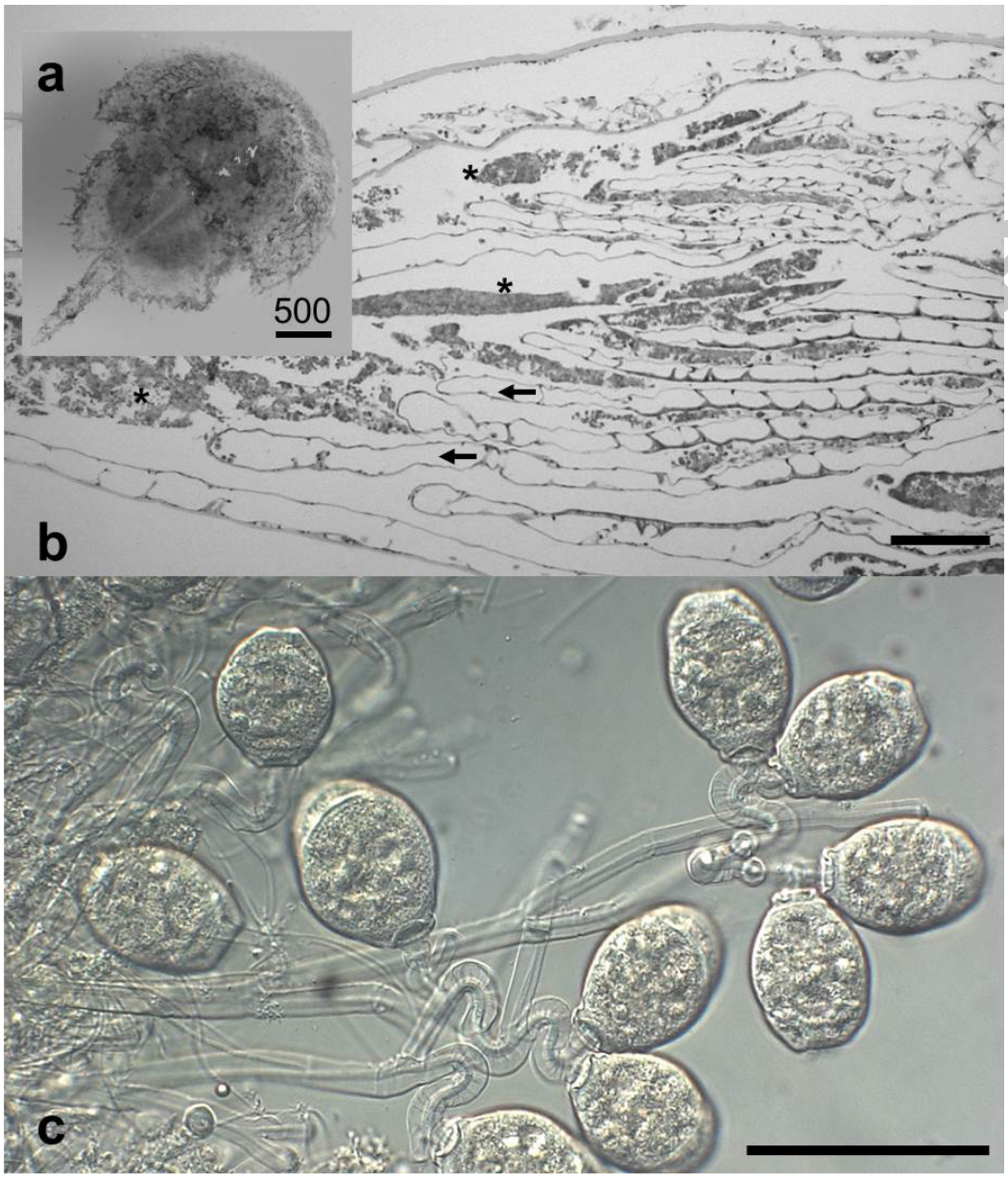
Figure legends

Figure 1 a-c. Light micrographs of juvenile *Limulus polyphemus* infected with the vorticellid *Zoothamnium duplicatum.* **a**, Neutral red stained second instar highlighting epibiont growth over the carapace; **b**, Histological section through the gill chamber of a second instar congested by a heavy growth of *Z. duplicatum* (*). The arrows point to separate gill lamellae; **c**, Branch forming colonies of *Z. duplicatum*. Scale bars: a = 500 μm, b = 50 μm, c = 50 μm.

Figure 2a-c. Scanning electron micrographs of *Zoothamnium duplicatum* on the carapace of the American horseshoe crab, *Limulus polyphemus.* **a**, Whole body view of a second instar of *L. polyphemus* with an evident heavy external growth of *Z. duplicatum*; b, an aggregation of contracted *Z. duplicatum* zooids; **c**, anchoring discs of the colony occlude pores (arrows) in the carapace, in areas of heavy growth, theoretically preventing the release of material from dermal gland cells that might otherwise assist in keeping the carapace free of fouling organisms. Scale bars: a = 500 μm, b = 50 μm, c = 5 μm.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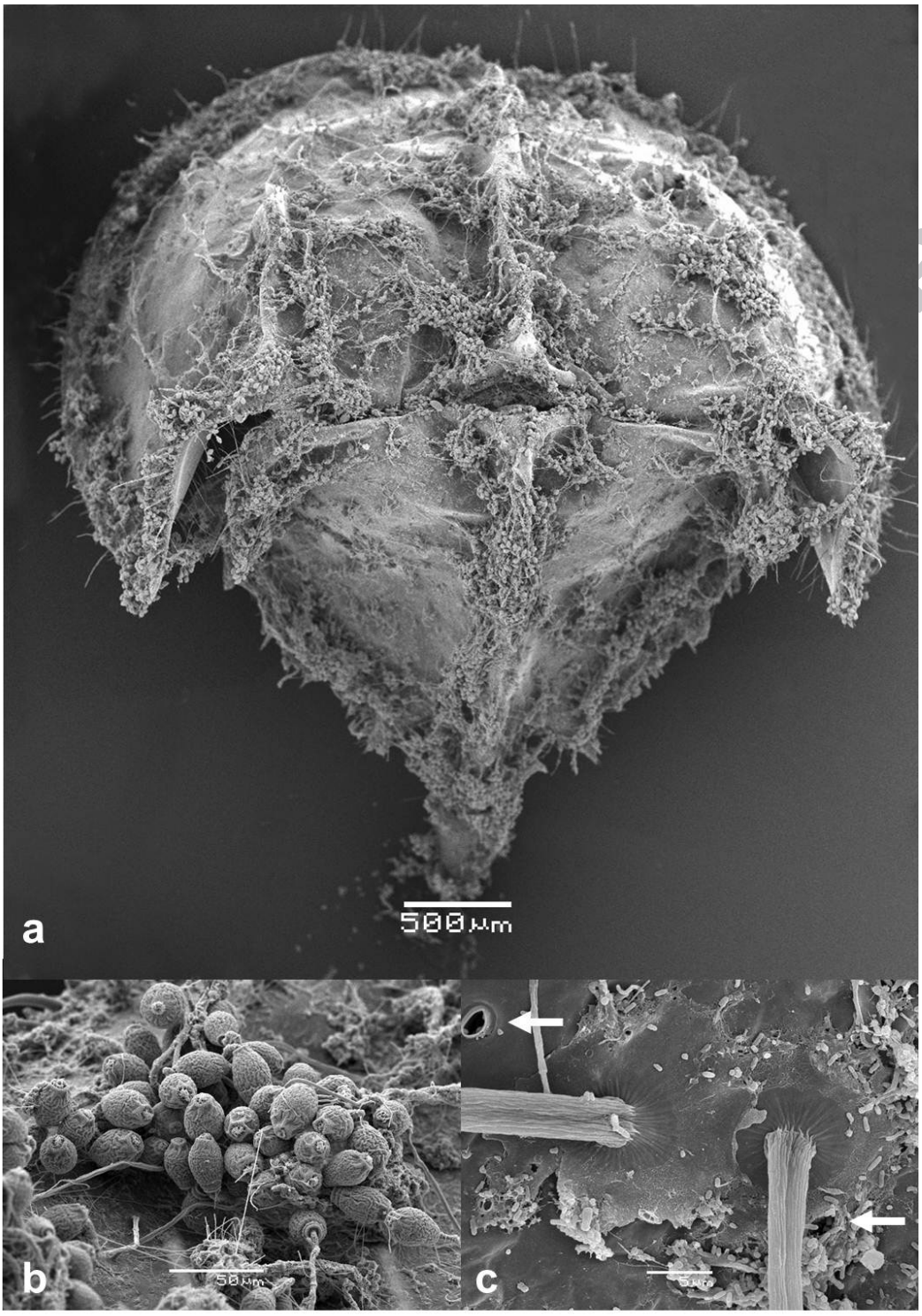
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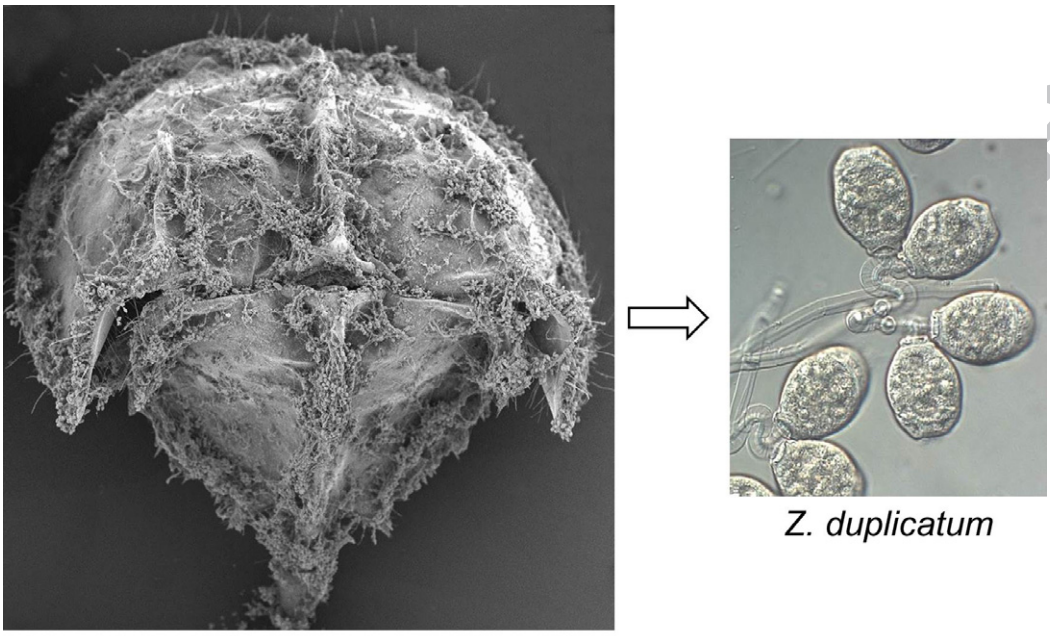
Graphical abstract



Research Highlights

- Mass mortality event in a pilot, commercial scale horseshoe crab hatchery
- A protozoan infestation of juvenile horseshoe crabs
- The protozoan was identified as the sessile peritrich, *Zoothamnium duplicatum*
- *Zoothamnium duplicatum* colonised extensively the carapace and gills
- Treatments with 10 ppm chlorine removed the epibiont burden

Table 1. Farm led treatment trials to reduce the visible *Zoothamnium duplicatum* growth on juvenile *Limulus polyphemus.* A) The efficacy of the treatment in removing the epibionts was assessed after 24 h (PEval) alongside host mortalities. A total of 10 animals per test run were used unless otherwise stated. B) The effect of including sharp sand in the culture vessels on epibiont growth. *Zoothamnium duplicatum* growth was scored (i.e. 1 = light; 10 = heavy) over 10 days after which each downweller was divided into two, one with sand (test) and the other without (control). Approximately 1000 juveniles were used in each test vessel at the start of the trial.

A) The efficacy of various bath treatments in controlling *Z. duplicatum* growth.

Treatment	**Mortalities (%)**							**PEval**[1]
(ppm; duration)	1 h	2 h	3 h	4 h	5 h	24 h	48 h	
Chlorine (10; 1 h) in FW	0	0	0	0	0	0	0	D[3]
Chlorine (10; 1 h) in FW	0	0	0	0	0	0	0	D[3]
Chlorine (20; 1 h) in FW	0	0	0	0	0	0	0	D[3]
Chlorine (50; 1 h) in FW	0	0	0	0	0	0	0	D[3]
Formalin (25; 1 h)[2]	0	0	0	0	0	0	0	A
Formalin (100; 1 h)[2]	0	0	0	0	0	0	16.7	A
Formalin (300; 1 h)[2]	0	16.7	16.7	16.7	16.7	16.7	32.5	A
Formalin (1000; 1 h)[2]	50	100	100	100	100	100	100	D
Freshwater (1 h)	0	0	0	0	0	0	0	A[4]
Methylene blue (0.5; 1 h)	0	0	0	10	10	20	20	A
Methylene (50; 10 s dip)	0	0	0	0	0	0	0	A
Oolytic sugar sand	0	0	0	0	0	0	0	A[5]
Pimafix™ (13; 1 h)	0	0	0	0	0	0	10	A
Control 1	0	0	0	0	0	0	0	A
Control 2	0	0	0	0	0	0	0	A
Control 3	0	0	0	0	0	0	0	A
Control 4	0	0	0	0	0	0	0	A

B) The effect of including sharp sand in the culture vessels on *Z. duplicatum* growth.

	Time (d)					
	D0	D4	D7	D10	D14	D17
Tank 1	8	6	8	8	5	5
Tank 2	1	8	4	6	5	7
Tank 1 + sand					2	1
Tank 2 + sand					2	1

[1] Evaluation of parasite viability at 24 h by exposing them to a drop of a hypersaline solution (80 ppt). Live parasites were seen to retract their cilia (A), whilst there was no response if these were dead (D).
[2] Only six animals per trial run used.
[3] Parasites had deciliated and appeared empty. A second evaluation at 48 h revealed that the number of attached epibionts had noticeably decreased as they decayed.
[4] A proportion of the parasite population had deciliated and were lethargic in their response to the hypersaline solution, however, they quickly recovered following their transfer back into clean 25 ppt seawater.
[5] Following the introduction of sand into the culture vessel, all the juveniles quickly buried themselves. This behaviour and the impact on epibiont burdens warranted further investigation.