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FISH PARASITOLOGY - SHORT COMMUNICATION

Molecular confirmation of *Henneguya adiposa* (Cnidaria: Myxozoa) and associated histologic changes in adipose fins of channel catfish, *Ictalurus punctatus* (Teleost)

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Abstract

Henneguya adiposa is one of ten known, closely related myxozoan species that parasitize a variety of tissue sites in the channel catfish, *Ictalurus punctatus*. Reported to specifically target the adipose fin, *H. adiposa* is not associated with morbidity or mortality, although detailed descriptions of its associated histologic pathology are lacking. The objective of this work was to confirm the presence of *H. adiposa* within fin lesions of affected channel catfish using DNA sequenced from histologic sections obtained by laser capture microdissection, as well as to describe pathologic changes induced by infection. The parasite formed large, white, elongate, nodular plasmodia that caused localized tissue damage and incited a granulomatous inflammatory response within a deep connective tissue layer at the base of the adipose fin. Myxospores released from ruptured plasmodia into adjacent tissue were observed to migrate superficially in tracts through the skin, indicating a portal of exit for environmental dispersal. Defects in the connective tissue layer created by ruptured plasmodia were infiltrated by granulomatous inflammation and fibroplasia, suggesting lesion resolution by scar formation over time. Sequencing of the 18S rRNA gene amplified from excised myxospores confirmed the myxozoan's identity as *H. adiposa*, with 100% similarity to the reference sequence from previous published work.

Keywords Henneguya adiposa · Histopathology · Laser capture microdissection

Introduction

Myxozoans comprise a group of over 2000 endoparasitic metazoans belonging to the phylum Cnidaria. Degraded by the transition to a parasitic life cycle, these morphologically and functionally simplified organisms share two-host life cycles that most commonly utilize fish as intermediate and oligochaete worms as definitive hosts. Representatives infect virtually all classes of fish, in all aquatic environments, with remarkably variable tissue tropism. While most infections are innocuous, some cause severe disease and significant economic losses in cultured and wild fish. Following infection, pre-sporogonic stages may proliferate as single cells before reaching final sites of sporogonic development, which occurs in either multinucleated plasmodia or unicellular pseudoplasmodia. Sporogony results in the production of a morphologically diverse array of myxospores unified by the presence of one or more polar capsules containing extrudable filaments. However, due to convergence in myxospore morphologies, features such as host preference, tissue specificity, developmental features, and increasingly, molecular sequence data are required for proper identification (Okamura et al. 2015).

Over 200 *Henneguya* species have been characterized to varying extents in freshwater and marine fishes (Eiras 2002; Eiras and Adriano 2012). Ten of these have been described in the channel catfish, *Ictalurus punctatus*, from commercial aquaculture operations in the southeastern USA, where static earthen ponds and the mixing of fish age classes provides an

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ideal environment for the perpetuation of myxozoan life cycles. The different *Henneguya* spp. infect a variety of tissues including the gills, skin, gall bladder, and adipose fin (Rosser et al. 2016). Elongate myxospores with two shell valves, each possessing a caudal projection, and two apical polar capsules develop within histozoic polysporic plasmodia that are usually large and cyst-like (Eiras 2002; Lom and Dyková 2006; Fiala et al. 2015). Most significant among these is *Henneguya ictaluri*, the cause of proliferative gill disease (PGD) in catfish, which results in severe respiratory distress and significant economic losses in the form of lost feed days and direct mortality (Pote et al. 2000). Other species, including *Henneguya adiposa*, produce disfiguring lesions, but are thought to have little to no adverse effects on fish health (Griffin et al. 2009; Rosser et al. 2016). While initial descriptions were based entirely on myxospore morphology, partial 18S small subunit rRNA gene sequence data is now available for most documented species affecting channel catfish (Lin et al. 1999; Pote et al. 2000; Griffin et al. 2008, 2009; Rosser et al. 2014, 2015). However, in situ studies linking sequence data from myxospores to specific tissue sites is not well documented and remains a matter of conjecture.

First described by Minchew (1977), *H. adiposa* infects the adipose fin of the channel catfish. The ultrastructure of its large plasmodia and asynchronous sporogenesis was reported shortly thereafter by Current (1979). Griffin et al. (2009) further refined descriptions of myxospore morphology and reported its 18S small subunit rRNA gene sequence, which revealed a monophyletic clade for the *Henneguya* spp. infecting North American ictalurids. Despite investigations into actinospore diversity in catfish ponds, the definitive host and actinospore stages of *H. adiposa* have yet to be identified (Hanson et al. 2001; Rosser et al. 2014). To supplement the existing literature concerning the morphology and molecular characteristics of *H. adiposa*, this report details histologic features of myxospores in situ and descriptions of pathologic changes induced in channel catfish. In addition, presence of the parasite within lesions was confirmed using laser capture microdissection, coupled with myxozoan-specific PCR and sequencing.

Materials and methods

Channel catfish fingerlings reared in 264-L flow-through tanks supplied by pond water spontaneously developed lesions consistent with *H. adiposa* infection (Minchew 1977; Current 1979; Griffin et al. 2009). Twenty-five adipose fins with gross lesions suggestive of *H. adiposa* infection were sampled from fingerlings and fixed in either 10% neutral buffered formalin ($n = 10$), 70% ethanol ($n = 10$), or 95% ethanol ($n = 5$). Prior to histologic processing, fins were sectioned transversely and placed into tissue cassettes. Formalin-fixed samples were processed routinely in a Sakura Tissue-Tek VIP 5 tissue processor (Sakura Finetek USA Inc., Torrance, CA). Ethanol-fixed samples were prepared by programming the processor to avoid any contact with formalin, which might interfere with molecular analysis as a result of DNA crosslinking. Processed tissues were then embedded in paraffin wax, sectioned at 4 μm thick, and stained with hematoxylin and eosin (H&E), Giemsa, and a modified Brown-Hopps Gram stain for light microscopic observation.

Individual plasmodia were excised from ethanol-fixed, H&E stained sections using laser capture microdissection (LCM). Histologic sections were prepared on MMI nuclease-free membrane slides and the plasmodia collected using an MMI CellCut Plus® microdissection system (Molecular Machines & Industries, Eching, Munich, Germany). DNA was extracted from excised plasmodia using a Qiagen QIAamp DNA Mini kit according to the manufacturer's protocols (Qiagen, Hilden, Germany). Conventional PCR targeting the 18S small subunit rRNA gene was performed using previously published primers and methods to confirm the presence of *H. adiposa* in adipose fin sections (Griffin et al. 2008, 2009). The PCR products were purified by gel extraction (QIAquick Gel Purification Kit, Qiagen), sequenced commercially (Genewiz, South Plainfield, NJ), assembled and edited using Geneious® 10.1.3 (Kearse et al. 2012), and used in a Blastn search for somewhat similar sequences in the National Center for Biotechnology Information non-redundant nucleotide (nr/nt) database for myxozoan identification. Sequence data was deposited in GenBank (MK253077).

Results

Consistent with histologic descriptions by Grizzle and Rogers (1976), the base of the adipose fin is composed of a typical stratified epidermis and circumferential superficial dermis of irregular collagen bundles. Oriented perpendicular to the base is a deeper connective tissue layer formed by distinct parallel bundles of dense regular collagen separated by areolar fibers and fibroblasts. The core of the fin is comprised of a mixture of adipose and elastic connective tissue.

Grossly, multifocal, spindyloid to nodular, white, plasmodia were most numerous at the fin base (Fig. 1). Microscopically, expansile plasmodia up to approximately 500 × 1000 μm were localized to the deep connective tissue layer where they displaced and effaced collagen bundles (Fig. 2a). Plasmodia were limited by 4–6 μm thick, pale eosinophilic walls with striations compatible with pinocytotic channels (Lom and Dyková 1992), and often further enveloped by thin, 2–3 cell thick, fibrous capsules (Fig. 2b, c). Myxospores demonstrated asynchronous development with immature, pansporoblastic stages located peripherally

Fig. 1 Gross image of nodular, white *Henneguya adiposa* plasmodia up to approximately 1 mm in length at the base of the adipose fin of a fingerling channel catfish

within plasmodia and mature myxospores located centrally (Fig. 2b, c). Intact plasmodia elicited minimal to mild granulomatous inflammation within the adjacent collagenous tissue that occasionally extended into the central adipose core of the fin (not shown). Ruptured plasmodia released myxospores into adjacent tissue where myxospore filled tracts sometimes extended through the overlying dermis and epithelium (Fig. 2d). More intense granulomatous infiltrates, dominated by macrophages and lymphocytes, with infrequent multinucleated giant cells (not shown), surrounded ruptured plasmodia and free myxospores (Fig. 2e). In more advanced lesions, ruptured plasmodia and lytic collagen fibers were replaced by extensive granulomatous infiltrates and fibroplasia that effaced the normal tissue architecture (Fig. 2f). Myxospores were characteristic of the genus *Henneguya*, with two shell valves, two polar capsules containing coiled polar filaments, two long superimposed caudal processes, a central binucleated sporoplasm, and a clear posterior vacuole (Fig. 3a, b). Polar capsules stained magenta and deep purple with Giemsa and Gram stains, respectively (not shown). A 2058-bp segment of the 18S small subunit rRNA gene was 100% similar to the 2022 bp reference sequence for *H. adiposa* (EU492929) at the nucleotide level, confirming the presence of *H. adiposa* within lesions.

Discussion

Relatively few myxozoan parasites, including the many *Henneguya* species, cause significant disease in fish. While previous work suggested *H. adiposa* did not have adverse health effects on catfish, detailed histopathologic evaluations were not performed (Griffin et al. 2009). Among the fish examined in this study, lesions were often severe, but localized to the base of the adipose fin where plasmodia disrupted the normal tissue architecture, destroyed dermal and deep collagenous layers, and incited a granulomatous inflammatory response. While the granulomatous response induced by *H. adiposa* is similar to that described with other myxozoan diseases in fish (Sitjà-Bobadilla et al. 2015), this appeared to occur late in the course of infection when numerous large plasmodia were present and particularly when plasmodia were ruptured. In catfish suffering from *H. ictaluri* induced PGD, cartilage damage has been observed before a host inflammatory response develops. It has been hypothesized that collagen damage results initially from proteases released by trophozoite stages of the parasite (Lovy et al. 2011). In turn, collagen fragments incite a pronounced inflammatory response that further contributes to collagenolysis during later stages of disease as potentially seen here (Castillo-Briceno et al. 2009; Griffin et al. 2010; Lovy et al. 2011). The extensive presence of fibroplasia in some lesions suggests resolution may ultimately occur via fibrous scarring.

Mature myxospores of histozoic myxozoans may be either released into host tissue, discharged outside the host, encapsulated, or destroyed by the host's inflammatory response (Lom and Dyková 1992). Host death and decomposition are commonly required for the release of myxospores produced internally by many histozoic myxozoans, such as *Myxobolus cerebralis* (Eszterbauer et al. 2015). Antemortem release of myxospores from ruptured plasmodia located immediately adjacent to epithelial surfaces of the gills and skin, including *Henneguya* and *Myxobolus* spp., is frequently assumed, although histopathologic documentation is limited (Walsh et al. 2012; Rosser et al. 2019). An unusual observation in these catfish involved the release of myxospores into tissue following plasmodial rupture, accompanied by the formation of subcutaneous tracts and migration of myxospores through multiple layers of collagen and epithelium to reach the skin surface. Findings suggest death of the host is not required for environmental dissemination of myxospores and perpetuation of the parasite's life cycle. Mechanisms involved in the release of *H. adiposa* are unknown but could include direct tissue compression and atrophy induced by enlarging plasmodia, damage induced by products of the cellular inflammatory response, or the secretion of an unknown protease by myxospores (Lom and Dyková 1992; Lovy et al. 2011; Sitjà-Bobadilla et al. 2015). Although the inflammatory response was intense, plasmodia were confined to deep connective tissue layers and did not result in atrophy of the overlying dermis or epithelium in the 25 fins examined. Although not observed microscopically, skin perforation associated with myxospore release could provide a portal of entry for opportunistic bacterial infection.

Most myxozoans infecting fish exhibit a high degree of host, tissue, and organ specificity. Tissue selection is a useful taxonomic indicator for histozoic myxozoans and occurs independently of its migration route to final sites of myxospore production (Molnár and Eszterbauer 2015). This is reflected in the remarkably specific localization of *H. adiposa* plasmodia to a distinct deep layer of connective tissue unique to the base

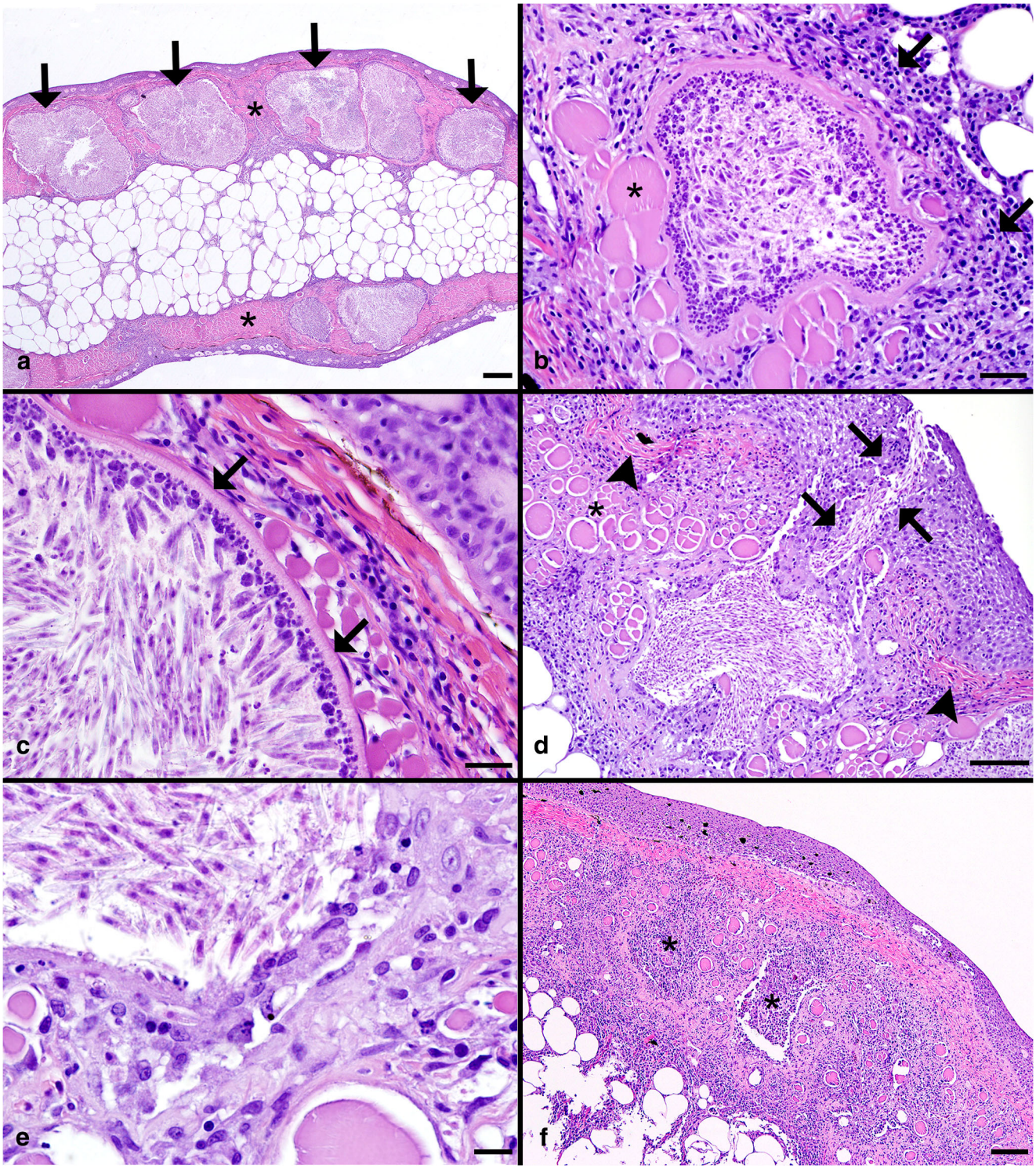

Fig. 2 Histological lesions in the adipose fin of a fingerling channel catfish infected with *Henneguya adiposa*. **a** Plasmodia (arrows) multifocally expand and efface the deep dermal collagenous layer (asterisks) (H&E, bar = 200 μm). **b** Mild inflammation (arrows) disrupts the deep collagenous layer (asterisk) and surrounds an intact plasmodium (H&E, bar = 20 μm). **c** Plasmodia consist of an eosinophilic wall (arrows) with peripheral immature and centrally located mature myxospores (H&E, bar = 20 μm). **d** A ruptured, collapsed plasmodium incites a granulomatous inflammatory response, with free myxospores forming a tract (arrows) through the dermis and epidermis. Note the severe disruption of the dermis (arrowhead) and deep collagenous layer (asterisk) by the intense inflammation (H&E, bar = 50 μm). **e** Free myxospores are surrounded by chronic inflammation (H&E, bar = 10 μm). **f** Ruptured plasmodia in the deep collagenous layer are replaced by granulomatous inflammation (asterisks) and fibroplasia in more advanced lesions that isolate the few remaining collagen fibers (H&E, bar = 100 μm)

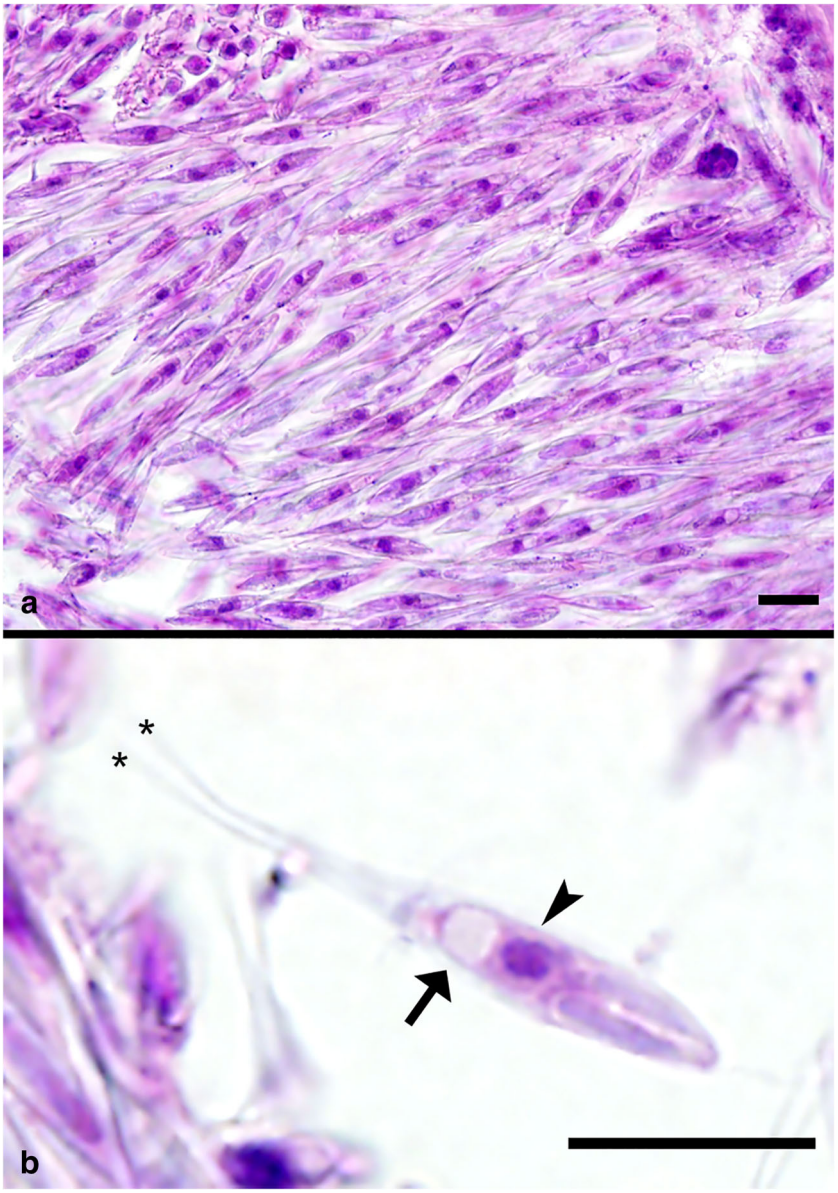

Fig. 3 High-magnification images of *Henneguya adiposa* myxospores and their morphologic features. **a** Densely packed mature myxospores fill a plasmodium (H&E, bar = 10 μm). **b** Myxospores possess typical features of a *Henneguya* sp., including paired elongate polar capsules, a basophilic binucleated sporoplasm (arrowhead), clear posterior vacuole (arrow), and two shell valves with two long, tapering caudal processes (asterisks) (H&E, bar = 10 μm)

of the adipose fin. Although unknown at present, it is presumed that *H. adiposa* utilizes similar portals of entry and migrates in a manner similar to that of the closely related *H. ictaluri*. Previous work demonstrated pre-sporogonic stages of *H. ictaluri* in the skin, buccal cavity, gill, and gastric wall within 24 h of experimental challenge, with stages also detected later in the heart and vasculature of the liver, suggesting a hematogenous component to its dissemination (Belem and Pote 2001). With the exception of the gill, the site of *H. ictaluri* maturation, the organism could not be detected after 96 h (Belem and Pote 2001). Most myxozoans start development in blood vessels before reaching their final site of sporulation, although the mechanisms behind tissue homing and site specificity have not been determined for myxozoans (Molnár and Eszterbauer 2015; Okamura et al. 2015; Rosser et al. 2016). Similar work has shown that pre-sporogonic stages of *Ceratonova shasta* and *Sphaerospora truttae* enter via the gills and migrate hematogenously before reaching the intestines and kidney, respectively (Holzer et al. 2003; Bjork and Bartholomew 2010).

Light microscopic features of *H. adiposa* myxospores were characterized originally by Minchew (1977) and supplemented with ultrastructural descriptions by Current (1979). More recent work refined their morphologic characterization, determined the 18S rRNA gene sequence, and investigated the phylogeny of *H. adiposa*, determining it to be most closely related to *H. ictaluri*, the cause of PGD (Pote et al. 2000; Griffin et al. 2009). For unknown reasons, these morphologically and genetically similar myxozoans produce dissimilar changes in the channel catfish, with vastly different tissue

tropism. Infection and maturation of *H. adiposa* results in subclinical cutaneous lesions in the adipose fin, while *H. ictaluri* is known to cause severe gill damage during entry and is the most significant parasitic disease affecting channel catfish aquaculture (Wise et al. 2008). Comparatively, tissue damage and immune response to encysted mature *H. ictaluri* myxospores in the gills are negligible (Pote et al. 2012). While descriptions of *H. adiposa* infection have been previously limited (Minchew 1977; Griffin et al. 2009), this report confirmed *H. adiposa* within intact plasmodia by excision of myxospores using LCM and sequencing of the 18S rRNA gene, as well as details histopathologic changes associated with the presence of mature plasmodia. Unfortunately, early stages of infection and processes leading to the final maturation site were not observed and are unlikely to be revealed until the actinospore stage is identified and controlled developmental studies can be performed.

Compliance with ethical standards

All animal procedures (fish handling) carried out during the study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC, PRN# 2015–2774).

Conflict of interest The authors declare that they have no conflict of interest.

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