Vol. 118: 195–206, 2016
doi: 10.3354/dao02974

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 30

Distribution, prevalence, and pathology of a microsporidian infecting freshwater sculpins

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ABSTRACT: Microsporidian infections are common in many fish species, yet detailed studies of these parasites in ecologically important wild populations are rare. Phylogenetic analysis using rDNA sequence data and parasite morphology indicate that mottled sculpin *Cottus bairdii* and slimy sculpin *C. cognatus* are hosts for *Glugea* sp. microsporidia in the northern USA. *Glugea* sp. is common in the Michigan populations sampled for this study, and prevalence was ≥70% in 4 of 6 infected populations (range ~4 to 80%). *Glugea* sp. infection causes the formation of xenomas associated with the body wall, fat body, gonads, and kidneys. Infections range from mild to very heavy, with variable xenoma numbers and sizes. Female sculpin experience heavier infections and more frequent infection of the gonads relative to males. *Glugea* sp. is transmitted horizontally between hosts through ingestion of spores. Vertical transmission may also be possible, either by spores infecting eggs directly or by spores contaminating the surface of eggs in the ovary or in the nest. The frequency and route of vertical transmission requires further study, but if it occurs, it may partly explain the high prevalence of infection. Our study combined with previous research suggests that additional molecular data and cross-infection experiments should be conducted to clarify species designations in the genus *Glugea*.

KEY WORDS: *Glugea* sp. · Xenoma · Mottled sculpin · *Cottus bairdii* · *C. cognatus* · Transmission · Infection intensity · Phylogeny

INTRODUCTION

The microsporidia are a large and diverse group of obligate intracellular parasites that infect a wide variety of invertebrates and vertebrates. The earliest described microsporidia generated scientific attention because they had strong negative economic consequences. For example, the microsporidian *Nosema bombycis* was a problem in silk worm cultures before it was described in 1857 and continues to cause economic losses (Becnel & Andreadis 1999). Members of the genus *Glugea* have caused substantial mortality in both wild and farmed economically important fish including smelts and flatfish (Canning & Lom 1986, Lee et al. 2004, Kent et al. 2014). *Encephalitozoon cuniculi* has been identified as a cause of chronic secondary illness in immunocompromised humans (Wittner & Weiss 1999). Broadly, the effects of microsporidia on their hosts are context dependent and can range from strongly negative to positive depending on both the environment and host condition (e.g. age, sex, immune status; Wittner & Weiss 1999, Ryan & Kohler 2010, Vavrá & Lukeš 2013).

Microsporidian infections in fish are common and have been associated with negative effects on individuals and populations (Chen & Power 1972, Nepszy & Dechtiar 1972, Olson 1976, Nepszy et al. 1978, Canning & Lom 1986, Vavrá & Lukeš 2013, Phelps et al. 2015). More than 160 species of microsporidia across at least 17 genera infect fish (Lom & Nilsen 2003, Vavrá & Lukeš 2013), and molecular techniques continue to reveal new genera and species while improving phylogenetic resolution (Casal et al. 2008, 2012, Sanders et al. 2012, Su et al. 2014). Common and economically important genera include *Glugea*, *Heterosporis*, *Loma*, *Nucleospora*, *Pleisto-*

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phora, and *Spraguea* (Canning & Lom 1986, Lom 2002, Lom & Nilsen 2003, Phelps et al. 2015).

Fish-infecting microsporidia often cause the formation of xenomas, or spore-filled cysts, in their fish hosts (Fig. 1). Xenomas are host cells that have undergone extreme hypertrophy as the parasite uses host resources to produce massive numbers of spores to infect new hosts (Canning & Lom 1986). Spore production results in the eventual death of infected host cells, while the host itself suffers varying amounts of reduced fitness (Shaw & Kent 1999). The severity of negative effects may depend on how the parasite is transmitted among hosts. Microsporidia can be transmitted horizontally via ingestion of spores or vertically from mother to offspring (Phelps & Goodwin 2008, Vavrá & Lukeš 2013). Typically, horizontal infections are associated with the strongest negative fitness consequences for individuals because large numbers of spores need to be released into the environment for disease spread (Ebert & Herre 1996, Lipsitch et al. 1996). Xenoma-forming microsporidia are known to be spread horizontally but may also use mixed transmission (Canning & Lom 1986, Lee et al. 2004, Phelps & Goodwin 2008). Transmission mechanisms are not fully known for most fish-infecting microsporidia. The microsporidia that produce xenomas in fish are particularly important to study because they cause disease in both ecologically and economically important natural and cultured fish populations.

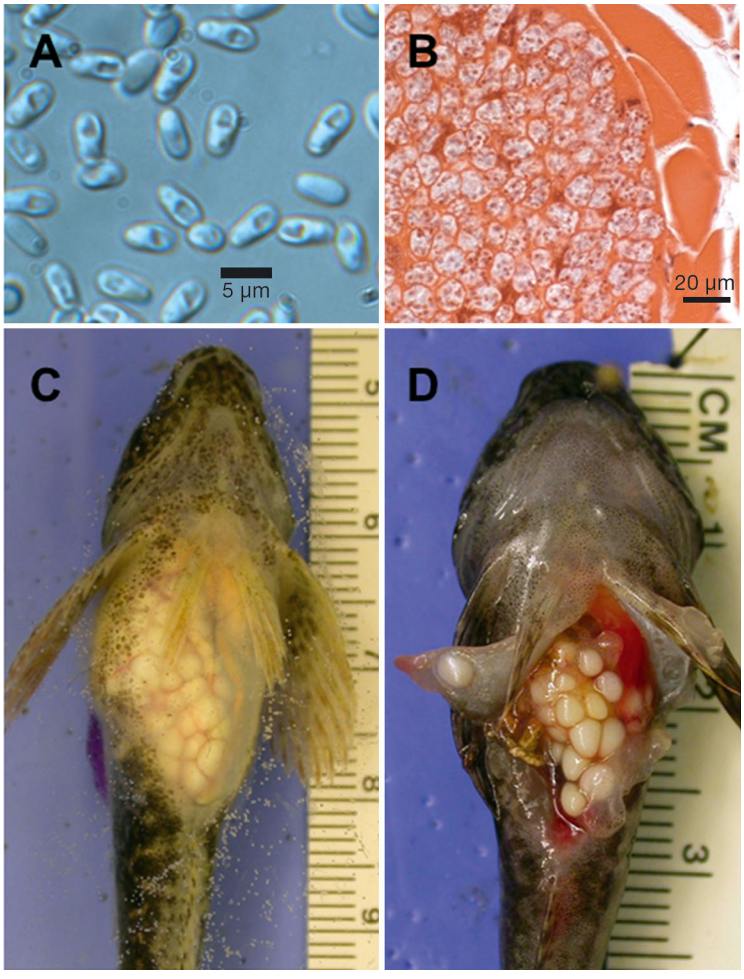

Fig. 1. Characteristics of *Glugea* infecting mottled sculpin *Cottus bairdii*. (A) Spores are pyroform and approximately 5 µm in length with a large posterior vacuole. (B) Spores in sporophorus vesicles embedded in the body wall. (C) Xenomas are often visible through the body wall. (D) Xenomas are variable in size and are primarily associated with the visceral cavity

Microsporidians in the genera *Glugea*, *Loma*, and *Pleistophora* negatively impact dozens of important species including salmonids, smelts, and flatfishes (Canning & Lom 1986, Becker & Speare 2007). For example, *G. hertwigi* reduced reproduction and increased mortality in rainbow smelt populations from both Lake Erie and Lake Ontario (Nepszy & Dechtiar 1972, Scarborough & Weidner 1979). Parasite-induced mortality estimates in Lake Erie are as high as 10 million fish lost per year (Shaw & Kent 1999).

Freshwater sculpins *Cottus* spp. are widely distributed across the northern hemisphere and are a significant part of the fish assemblages of coldwater streams and lakes (Becker 1983, Freeman et al. 1988, Adams & Schmetterling 2007). Sculpin are considered to be excellent indicators of habitat integrity, which makes systems containing sculpins ecologically and economically important (Scott & Crossman 1973, Becker 1983, Adams & Schmetterling 2007). Mottled sculpin *C. bairdii* populations from several southwest Michigan (USA) streams are infected by a microsporidian parasite that causes the formation of xenomas in the body cavity of fish (Fig. 1) (Homola et al. 2014).

Our objectives were to (1) characterize the ribosomal RNA gene (rRNA) sequence of microsporidia infecting multiple sculpin populations for molecular identification and phylogenetic placement, (2) evaluate parasite distribution and prevalence in southwest Michigan sculpin populations, (3) describe the general pathology of infection, and (4) determine the mode(s) of parasite transmission.

MATERIALS AND METHODS

Fish sampling

We sampled mottled sculpin from 11 southwest Michigan streams to examine distribution and prevalence of microsporidian infection between 2007 and 2008. The streams are: Rice Creek, Seven Mile Creek, and Wilder Creek (Calhoun County); Augusta Creek, Castle Creek, Lee Creek, Portage Creek, Sand Creek, Silver Creek, and Spring Brook (Kalamazoo County) and Curtis Creek (St. Joseph County) (Homola et al. 2014). We captured sculpin using a backpack electroshocker and attempted to collect at least 30 fish in each stream. We collected additional fish from Seven Mile Creek and Spring Brook from 2007 to 2011 to look for yearly variation in infection prevalence. Fish were euthanized by an overdose of MS-222, kept on ice during transit, and then stored at −20°C.

Molecular identification

PCR and DNA sequencing have been successfully used to target and amplify the rRNA genes of microsporidia, allowing at least generic-level identification (Vossbrinck et al. 1993, Docker et al. 1997b). We characterized the rRNA genes of this xenoma-forming microsporidian infecting sculpins to explore phylogenetic placement and determine whether the parasite is a unique species. Spores taken from a single xenoma from a mottled sculpin collected from Seven Mile Creek were washed, and DNA was extracted using the Qiagen DNeasy Tissue kit with initial spore disruption by bead beating. We used PCR to target the microsporidian rRNA genes using the universal microsporidian primer pairs ss18f and ss1492r and ss530f and ls580r, which amplify regions including the small subunit ribosomal RNA gene (ssrRNA), internal transcribed spacer (ITS), and partial large subunit ribosomal RNA gene (lsrRNA) (Vossbrinck et al. 1993). PCR was done using Qiagen Fast Cycling PCR Master Mix, 0.5 µM of each primer, and 0.5 to 1 µl of DNA template. The amplified DNA was sequenced using the above primers at the University of Michigan DNA Sequencing Core, and BLAST-searching confirmed it to be a *Glugea* sp. microsporidian. Previous research has shown that morphologically similar microsporidians may not be identical when DNA sequences are examined (Ironside et al. 2003, Haine et al. 2004, Ryan & Kohler 2010). Therefore, we sequenced the rRNA genes from 2 additional infected mottled sculpin populations from Michigan (Augusta Creek and Wilder Creek) in the same manner as above. Additionally, we sequenced microsporidian DNA from a New Hampshire population of slimy sculpin *Cottus cognatus* from Mill Creek (Sullivan County) exhibiting similar infections (D. Ward pers. comm.). For simplicity, hereafter we refer to the microsporidia infecting sculpins as *Glugea* sp. and distinguish populations when appropriate.

The rDNA sequences from *Glugea* sp. infecting sculpins and 36 other microsporidia obtained from GenBank were aligned using MAFFT (Katoh et al. 2005) for phylogenetic analysis (Table 1). The sequences from GenBank were chosen to maximize the number of bases (923–1818 bases) in the alignment and to incorporate microsporidia with similar characteristics (e.g. high BLAST score, fish hosts). The microsporidia *Spraguea* sp. Sdu-2008 (GenBank accession number AB623034) and *Potaspora morhaphis* (EU534408) were used as outgroups. These species infect fish but are genetically distinct from the ingroup taxa. A phylogenetic tree was constructed using maximum parsimony via a heuristic algorithm, and bootstrap values were calculated over 1000 replicates using PAUP (4.0). There were 548 parsimony informative characters over the alignment matrix, and the strict consensus tree is reported. We also compared the *Glugea* sp. parasites from mottled and slimy sculpins to other *Glugea* species sequences available on GenBank using p-distance and percent identity using the Kimura-2 substitution model.

Distribution, prevalence, and pathology

We dissected 1055 fish and recorded fish length, sex, and infection status to see how many of the 11 populations were infected and to estimate infection prevalence within populations. We also looked for differences in infection characteristics between males and females. In addition to field-collected fish, we examined 8 formalin-preserved fish collected from Augusta Creek and Seven Mile Creek in 1999 for microsporidian infection. We examined 354 fish from infected populations to look at the general pathology of infection, including xenoma size and infection location. Infection location was characterized by the proportion of infections involving each tissue type: fat body, gonads, body wall, or kidneys for both males and females. The 'fat body' category included xenomas that were loosely associated with the fat lining the intestine, stomach, and liver. In contrast, xenomas associated with other the tissues were

Table 1. Microsporidian species used in the phylogenetic analysis including host and source information

Microsporidian	Host	GenBank accession number	Aligned bases	Study or source
Glugea sp.	*Cottus bairdii*	KU885381	1373	Present study
Glugea anomala	*Gasterosteus aculeatus*	AF056016	1165	Pomport-Castillon et al. (2000)
Glugea stephani	*Gasterosteus aculeatus*	AF056015	1165	Pomport-Castillon et al. (2000)
Glugea plecoglossi	*Plecoglossus altivelis*	AB623035	1486	Miwa et al. (2011)
Glugea atherinae	*Atherina presbyter*[a]	U15987	1295	GenBank
Glugea sp. LX-2012	*Pagrosomus major*	JX852026	964	Su et al. (2014)
Glugea plecoglossi	*Plecoglossus altivelis*	AJ295326	1405	Bell et al. (2001)
Glugea sp.	*Cottus cognatus*	KU885382	1768	Present study
Glugea hertwigi	*Osmerus mordax*	GQ203287	1794	Lovy et al. (2009)
Pleistophora finisterrensis	*Micromesistius poutassou*	AF044393	1372	Nilsen et al. (1998)
Glugea sp.	*Cottus bairdii*	unpubl.	1319	S. Jones (pers. comm)
Glugea anomala	*Gasterosteus aculeatus*	AF044391	1804	Nilsen et al. (1998)
Pleistophora sp. 3	*Taurulus bubalis*	AF044390	1818	Nilsen et al. (1998)
Glugea anomala	Host unknown	AB923879	1775	GenBank
Pleistophora sp. LM-2014	*Alepes djedaba*	KF830721	1243	GenBank
Loma acerinae	*Gymnocephalus cernuus*	AJ252951	1312	Cheney et al. 2000
Glugea sp.	*Epinephelus awoara*	AY090038	1286	GenBank
Loma acerinae	*Gymnocephalus cernuus*	AF356224	1282	Pekkarinen et al. (2002)
Loma psittaca	*Colomesus psittacus*	FJ843104	1258	Casal et al. (2009)
Pleistophora sp. (PA)	*Penaeus aztecus*	AJ252958	1315	Cheney et al. (2000)
Pleistophora hippoglossoideos	*Hippoglossoides platessoides*	AJ252953	1332	Cheney et al. (2000)
Ovipleistophora mirandellae	*Rutilus rutilus*	AJ252954	1349	Cheney et al. (2000)
Microsporidium sp. STF	*Salmo trutta fario*	AY140647	1432	GenBank
Glugea nagelia	*Cephalopholis hemistiktos*	KJ802012	1752	Abdel-Baki et al. (2015)
Pleistophora mulleri	*Gammarus duebeni celticus*	AJ438985	1483	Terry et al. (2003)
Loma wallae clone P176-6	*Theragra chalcogramma*	HQ157446	949	Brown et al. (2010b)
Heterosporis sp. NBDP-2013a	*Sander vitreus*	KC137548	1806	GenBank
*Loma lingcoda*e clone L16-7	*Ophiodon elongatus*	HQ157509	929	Brown et al. (2010b)
Loma embiotocia clone S103-1	*Cymatogaster aggregata*	HQ157528	923	Brown et al. (2010b)
Pleistophora mulleri	*Gammarus duebeni*	EF119339	1814	Ironside et al. (2008)
Heterosporis anguillarum	*Anguilla japonica*	AF387331	1810	Tsai et al. (2002)
Pleistophora sp. 2	*Zeugopterus punctatus*	AF044389	1790	Nilsen et al. (1998)
Pleistophora typicalis	*Myoxocephalus scorpius*	AF044387	1806	Nilsen et al. (1998)
Loma sp. SVB-PE3 (SV-1)	*Salvelinus fontinalis*	HM626217	1805	Brown et al. (2010b)
Loma salmonae	*Oncorhynchus tshawytscha*	U78736	1392	Docker et al. (1997a)
Ichthyosporidium weissii	*Clevelandia ios*	JQ062988	1783	Sanders et al. (2012)
Loma embiotocia	*Cymatogaster aggregata*	AF320310	1810	GenBank
Spraguea sp. Sdu-2008	*Seriola dumerili*	AB623034	1799	Miwa et al. (2011)
Potaspora morhaphis	*Potamorrhaphis guianensis*	EU534408	1783	Casal et al. (2008)

[a]Reference for this host is unclear

clearly embedded within the body wall, gonads, or kidneys. We tested for differences in the frequency of infection by tissue type between males and females using a *t*-test. Spore size was measured using ImageJ (Schneider et al. 2012) on 25 formalin-fixed spores photographed under differential interference contrast microscopy. We measured fish and xenoma dry mass for 150 of the collected Seven Mile Creek sculpin to quantify infection intensity measured as percent parasite mass ([xenoma mass/fish mass + xenoma mass] × 100). The infection intensity data were non-normal, so we compared infection intensity between females and males using Kolmogorov-Smirnov and Mann-Whitney non-parametric tests.

Parasite transmission

Microparasites employ 2 transmission routes, viz. horizontal (via ingestion) and vertical (from mother to offspring via the egg) or a combination, and each is predicted to affect host fitness differently. We tested for both horizontal and vertical transmission of microsporidia in sculpin. While horizontal transmission has been documented in *Glugea*, the likelihood of vertical transmission has not been tested. In this study, as well as in previous reports (Nepszy et al. 1978, Canning & Lom 1986), xenomas were frequently observed within the gonads, which suggests that vertical transmission may also occur. Vertical

transmission includes both the infection within eggs and on the surface of eggs such that infection is transmitted to offspring at birth (Anderson & May 1981).

We tested for horizontal parasite transmission using a dose-response design. Size-matched fish (n = 24; wet mass = 0.82 ± 0.13 g, total length = 44.6 ± 1.9 mm [mean ± SD and hereafter]) from Spring Brook (*Glugea* sp. infection absent) were randomly assigned to each of 5 spore doses administered by oral intubation, i.e. 0 (n = 4), 20, 200, 2000, or 20 000 (n = 5 for each dose) spores in 0.1 ml of sterilized water (Shaw et al. 1998). Fish were housed in 4 l artificial streams at 18°C under a 12 h light:12 h dark schedule. Live *Gammarus* amphipods were provided daily as food (equal to 10% of mean fish mass adjusted weekly), and 50% water changes were performed weekly.

Starting at 5 wk post exposure, 1 fish from the 20 000 spore treatment was euthanized with an overdose of MS-222 and dissected to determine the earliest point at which xenomas become visible. Fish were examined in the same manner weekly from that point until the experiment was terminated at 9 wk. We used this schedule because it typically takes 6 to 8 wk for xenomas to appear in hosts exposed to other xenoma-forming fish microsporidia (Canning & Lom 1986, Lee et al. 2004).

To test for vertical transmission, brooding females were field-collected from Seven Mile Creek (infected population), euthanized, and dissected to determine infection status. Three to 5 eggs were collected from 10 randomly selected infected females and 1 uninfected female. DNA was extracted from eggs as above, and PCR was used to target parasite rDNA using the above microsporidian-specific primers. The PCR included 14 reactions: 11 fish egg samples, 2 positive controls, and 1 negative control. The products were visualized on a 1.5% agarose gel.

RESULTS

Molecular identification and phylogenetic placement

Microsporidian ribosomal DNA sequences from mottled and slimy sculpin varied by <2% from the majority of previously identified *Glugea* sequences in GenBank (8 of 11), evidence that strongly suggests placement in the genus *Glugea*. The DNA sequences of the *Glugea* sp. parasites showed little variation within the 3 Michigan mottled sculpin populations (0.2%) and compared to the New Hampshire slimy sculpin population (1.1%). There were 17 nucleotide substitutions and 2 gaps over the 1353 bp aligned region between the Michigan and New Hampshire *Glugea* microsporidia based on the consensus sequences. We deposited these sequences as *Glugea* sp. CBG1 (accession no. KU885381) and *Glugea* sp. CCG1 (KU885381) with reference to the source of the isolate (CB: *Cottus bairdii*; CC: *Cottus cognatus*) and the parasite (G1: *Glugea* sp. 1).

Phylogenetic analysis placed 12 species of *Glugea* and *Pleistophora finisterrensis* in a single clade with a 98% bootstrap support (Fig. 2). Two other microsporidian isolates assigned to *Glugea* (*G. nagelia* sp. nov. [KJ802012] and *Glugea* sp. [AY090038]) were grouped separately in their own clade with 100% bootstrap support and differed by >8% from sequences within the primary *Glugea* clade.

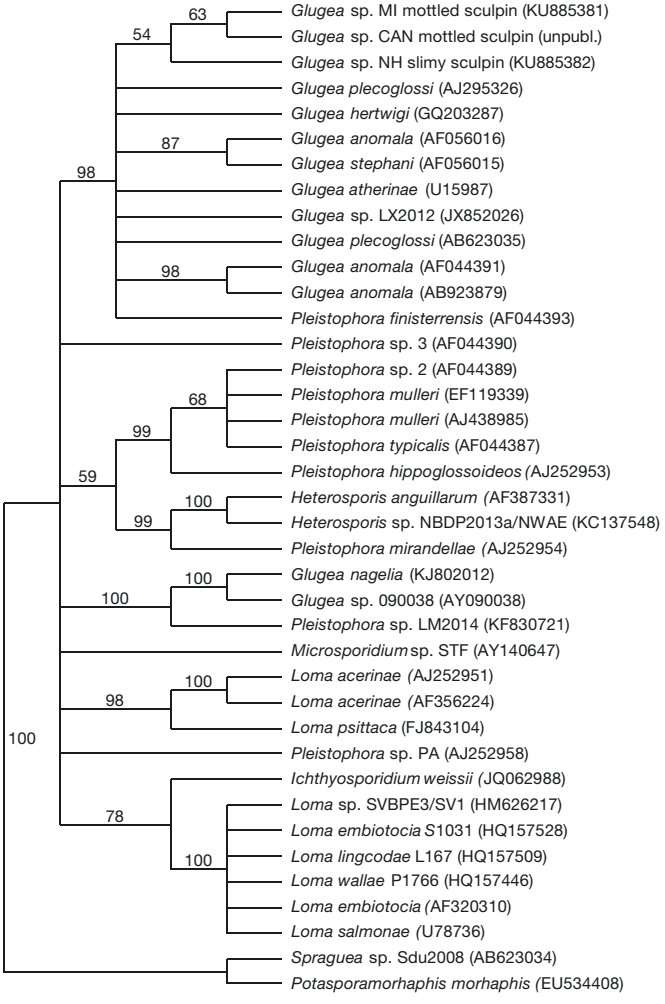

Fig. 2. Phylogenetic relationships of the microsporidian parasites found in sculpins *Cottus* spp. inferred from rRNA gene sequences using the maximum parsimony method. GenBank accession numbers are given in parentheses. Bootstrap probabilities (1000 replicates) are shown on branches (%)

Table 2. Percent identity (below diagonal) and pairwise distance (above diagonal) calculated using the Kimura-2 method for a subset of *Glugea* microsporidians used in the phylogenetic analyses. Species 3 is from mottled sculpin *Cottus bairdii* in Michigan (CB MI) and species 4 is from slimy sculpin *C. cognatus* in New Hampshire (CC NH)

Species	GenBank accession number	1	2	3	4	5	6	7	8	9	10	11	12
1 *G. anomala*	AF044391	–	0.0137	0.0162	0.0177	0.0086	0.0086	0.0135	0.0068	0.0121	0.0438	0.0911	0.1161
2 *G. hertwigi*	GQ203287	98.63	–	0.0125	0.0172	0.0034	0.0034	0.0073	0.0053	0.0106	0.0580	0.0865	0.1090
3 *Glugea* sp.	(CB MI)	98.38	98.76	–	0.0111	0.0045	0.0045	0.0057	0.0058	0.0096	0.0579	0.0849	0.1159
4 *Glugea* sp.	(CC NH)	98.23	98.29	98.89	–	0.0095	0.0095	0.0081	0.0102	0.0153	0.0623	0.0916	0.1156
5 *G. anomala*	AF056016	99.14	99.66	99.55	99.05	–	0.0000	0.0042	0.0053	0.0055	0.0571	0.0793	0.0755
6 *G. stephani*	AF056015	99.14	99.66	99.55	99.05	100.00	–	0.0042	0.0053	0.0055	0.0571	0.0793	0.0755
7 *G. plecoglossi*	AB623035	98.65	99.27	99.43	99.19	99.58	99.58	–	0.0085	0.0064	0.0587	0.0892	0.0981
8 *G. atherinae*	U15987	99.32	99.47	99.42	98.98	99.47	99.47	99.15	–	0.0067	0.0543	0.0868	0.0869
9 *G. plecoglossi*	AJ295326	98.79	98.94	99.04	98.47	99.45	99.45	99.36	99.33	–	0.0535	0.0857	0.1125
10 *G. anomala*	AB923879	95.62	94.20	94.21	93.77	94.29	94.29	94.13	94.57	94.65	–	0.1420	0.1615
11 *Glugea* sp.[a]	AY090038	90.89	91.35	91.51	90.84	92.07	92.07	91.08	91.32	91.43	85.80	–	0.0069
12 *G. nagelia* sp. nov.[a]	KJ802012	88.39	89.10	88.41	88.45	92.45	92.45	90.19	91.31	88.75	83.85	99.31	–

[a]Species are not grouped with other *Glugea* based on phylogenetic analysis

The *Glugea* sp. rDNA isolates from sculpin reported in this study are closely related to multiple *Glugea* species. Nine *Glugea* isolates and *P. finisterrensis* collected from 9 different fish hosts across freshwater and marine habitats around the world differed from the *Glugea* sp. isolates described in this study by <1.6% across the alignment (Table 2).

Distribution and prevalence

Six of the 11 sampled mottled sculpin populations from Michigan were infected by *Glugea* sp. Infection prevalence was very high in 4 of the 6 infected populations (Augusta, Rice, Seven Mile, and Wilder), with 70 to 100% of sexually mature adults having the parasite (Table 3). In contrast, the other 2 populations (Castle and Silver) had infection rates of 5 and 11%, respectively. There was no difference in infection rate between females and males. Prevalence was low in age-0 fish (4.2%; n = 47). Yearly prevalence in adult Seven Mile Creek sculpin ranged from 59% (2010) to 93% (2007) and averaged 74% (n = 441). Prevalence was 0 for Spring Brook in all years (n = 226).

Pathology

We characterized *Glugea* sp. morphology and pathology from the 6 infected mottled sculpin populations. Spores averaged 2.35 ± 0.16 × 5.24 ± 0.20 µm (n = 25), and the posterior vacuole filled approximately 1/3 of the cell (Fig. 1A). Spores occurred in sporophorus vesicles 12.6 ± 1.3 µm (n = 7) in diameter (Fig. 1B). Vesicles generally contained 12 spores, but vesicle size and spore number sometimes varied. Xenomas were frequently visible through the body wall and were primarily concentrated on the left side of the body cavity (ventral view), the same side as the intestines (Fig. 1C). Xenoma size ranged from ~0.5 to 10 mm, but size and number were highly variable (Fig. 1D).

Within the body cavity, xenomas were associated with the viscera and body wall and were covered by layers of host connective tissue. The frequency of xenomas associated with particular host tissues was similar for females (F) and males (M) with the exception of the gonads (Fig. 3). Xenoma frequency in the gonads in females (40%) was nearly double that of

Table 3. Prevalence of *Glugea* sp. in mottled sculpin *Cottus bairdii* in southwest Michigan, USA. Sample size is in parentheses. Dashes (–) indicate fish were not collected

Stream	Prevalence (%)			
	Female	Male	Age-0	Total
Augusta Creek	87.9 (33)	87.5 (24)	–	87.5 (57)
Castle Creek	0.0 (10)	11.1 (9)	0 (4)	4.3 (23)
Curtis Creek	0 (14)	0 (17)	–	0 (31)
Lee Creek	0 (11)	0 (8)	0 (1)	0 (20)
Portage Creek	0 (8)	0 (9)	0 (8)	0 (25)
Rice Creek	100.0 (33)	100.0 (17)	0 (12)	80.6 (62)
Sand Creek	0 (20)	0 (5)	0 (10)	0 (35)
Seven Mile Creek	84.8 (217)	82.9 (193)	6.5 (31)	78.5 (441)
Silver Creek	11.3 (62)	13.8 (29)	0 (8)	11.1 (99)
Spring Brook	0 (103)	0 (88)	0 (35)	0 (226)
Wilder Creek	91.3 (23)	58.8 (17)	0 (4)	70.5 (44)

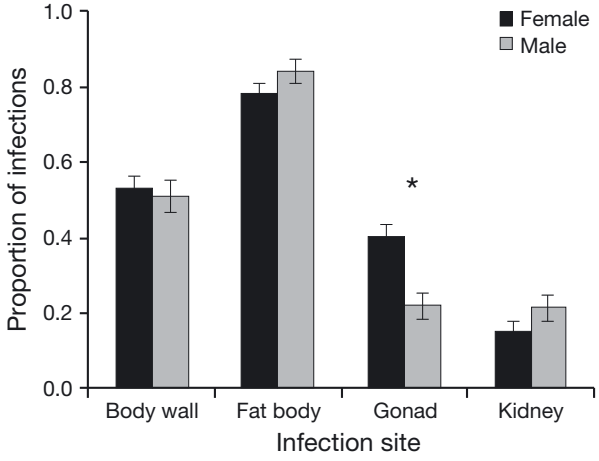

Fig. 3. Proportion of *Glugea* sp. infections in tissues and organs of mottled sculpin *Cottus bairdii* based on dissection of females (n = 209) and males (n = 145). *Statistically significant difference

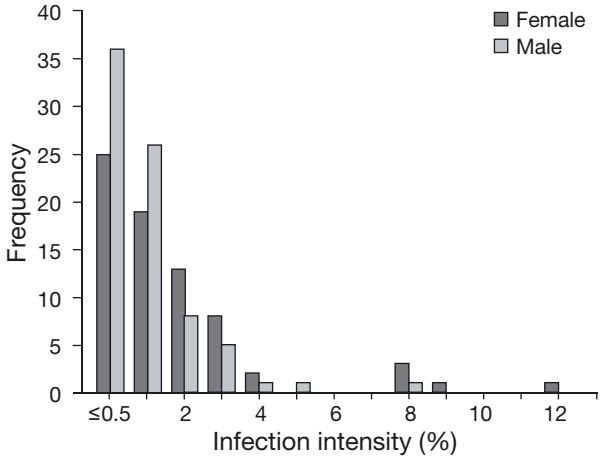

Fig. 4. Infection intensity (parasite mass/fish mass + parasite mass) of *Glugea* sp. in female (n = 72) and male (n = 78) mottled sculpin *Cottus bairdii*

males (22%; t_{338} = 3.74, p < 0.0001). The most common xenoma location was the fat body, with an average of 81% (F = 78%, M = 84%) of infections involving this tissue. The next most common location was the body wall (F = 53%, M = 51%), followed by the gonads (above) and finally the kidneys (F = 15%, M = 21%).

Sculpin infections varied substantially from a single xenoma to hundreds in heavily infected fish. Infection intensity (parasite mass/fish mass + parasite mass) for Seven Mile Creek sculpin was strongly right skewed, with most individuals having light infections and fewer having moderate to heavy infections (Fig. 4, n = 72 females, 78 males). The shape of infection intensity distribution differed significantly between females and males (Kolmogorov-Smirnov = 0.227, p < 0.05). Nearly 40% of females had infections ≥1.5% of their body mass, while only about 20% of males did, and median infection intensity was significantly greater in females (1.06%) relative to males (0.54%; Mann-Whitney: W = 5957, p = 0.0251).

Transmission

Horizontal

Fish exposed to *Glugea* sp. by horizontal transmission became visibly infected after 7 wk at spore doses of ≥2000; 3 of 7 fish examined were infected at this time. After 8 wk, 2 of 3 fish had visible infections, including the single fish examined that was exposed to a 200-spore dose. When the experiment was concluded at 9 wk, no control or 20-spore exposure fish had become visibly infected. The average number of xenomas in infected fish was 2 in the 200 group, 2.3 in the 2000 group, and 8.3 in the 20 000 group (Table 4). Xenomas ranged in size from 0.5 to 2 mm in diameter and were diffuse and pliable. Initial fish size averaged 0.82 ± 0.12 g and final fish size was 1.63 ± 0.12 g, indicating that fish were actively feeding and growing over the course of the experiment.

Vertical

PCR analysis was used to test for vertical transmission of *Glugea* sp. Eggs from 1 of the 10 infected females tested were positive for *Glugea* sp. DNA. The other 9 infected and the 1 uninfected egg samples showed no evidence of *Glugea* sp. DNA. It is not possible to determine whether *Glugea* sp. was on the surface or inside the eggs, although both are consid-

Table 4. *Glugea* sp. spore dose (number of spores per 0.1 ml of water) and xenoma presence at time of dissection. The values indicate the number of mottled sculpin *Cottus bairdii* where xenomas were observed at each week post infection. The number in parentheses is the number of fish examined for xenomas. Dash (–) indicates no fish dissected

Week	Spore dose				
	0	20	200	2000	20 000
5	–	–	–	–	0 (1)
6	–	–	–	–	0 (1)
7	–	0 (2)	0 (2)	1 (3)	2 (2)
8	–	0 (1)	1 (1)	1 (2)	–
9	0 (4)	0 (2)	2 (2)	–	1 (1)

ered vertical transmission. False positive results due to sample contamination have been reported at rates from 0 to 8% in PCR analyses of fungal spores (da Silva et al. 1997, Loeffler et al. 1999). A false positive seems unlikely in this context, because the positive and negative controls were accurate and our false positive rate was 0 during the duration of the study. Additionally, a run test applied to the PCR data, including the positive and negative controls, showed the results to be in a random order, indicating a low probability of systematic contamination ($n = 14$, $p = 0.538$; Balfe 1992).

DISCUSSION

Both parasite morphology and phylogenetic analysis using rDNA sequence data indicate that mottled and slimy sculpin are hosts for *Glugea* sp. microsporidia in the northern USA. Infection causes the formation of variable numbers and sizes of xenomas associated with tissues in the peritoneal cavity, consistent with *Glugea* infections reported from other fish species (Canning & Lom 1986, Lovy et al. 2009). Phylogenetic analysis of *Glugea* sp. rDNA sequences from sculpins places them in a clade with 9 *Glugea* species and 1 *Pleistophora* with 98% bootstrap support.

Comparing *Glugea* sp. rDNA sequences from sculpins and 7 published sequences from *G. anomala* (AF056016, AF044391), *G. stephani* (AF056015), *G. plecoglossi* (AB623035, AJ295326), *G. atherinae* (U15987), and *G. hertwigi* (GQ203287) from 4 unique hosts shows a range of 99.3 to 98.2% sequence identity. We found 0.2% nucleotide variation from mottled sculpin infections in Michigan and 1.1% between Michigan infections and those in slimy sculpin from New Hampshire. This level of sequence divergence is generally within the range seen in microsporidians that have been described as a single species (Brown et al. 2010b, Liu et al. 2013). Studies of the fish-infecting microsporidia *Loma salmonae* and *Heterosporis sutherlandae* showed 1.1 and 1.5% rDNA sequence variability, respectively, and considered these to indicate a single parasite (Docker et al. 1997a, Brown et al. 2010a,b, Phelps et al. 2015). In both cases, the divergence values included multiple host species from multiple locations. However, low genetic variation within *L. salmonae* (HM626203; 0.7%) infecting 5 *Oncorhynchus* spp. led Brown et. al. (2010b) to distinguish it from *Loma* sp. 'SV' (HM626217) infecting *Salvelinus fontinalis* with 2% sequence divergence. In a similar manner to *L. salmonae* and *H. sutherlandae*, *G. anomala*, *G. hertwigi*, and *G. stephani* have been reported from multiple fish hosts (smelts and sticklebacks) from the northern USA and Canada, including areas that overlap the range of sculpins (Scott & Crossman 1973, Becker 1983, Lovy et al. 2009). Overall, the variation in these *Glugea* rDNA sequences could indicate that these parasites represent local isolates of a single species, as has been suggested by multiple authors (Canning & Lom 1986, Pomport-Castillon et al. 2000, Lovy et al. 2009, Liu et al. 2013). Both environment (e.g. local adaptation) and genetics (e.g. paralogs, hybridization) could be contributing to the varying levels of rDNA divergence seen in these *Glugea* (Brown et al. 2010a, Ryan & Kohler 2010). However, we cannot rule out that this is a group of species for which rDNA genes are insufficient to resolve individual species (Docker et al. 1997b). Both additional DNA sequencing (e.g. EF-1α, tubulin, whole genome), and cross-infection studies should be conducted to help resolve the species designations within *Glugea* (Shaw et al. 1997, 2000, Brown et al. 2010a). Given the available data, we are designating the microsporidia in this study as *Glugea* sp. isolate CBG1 and *Glugea* sp. isolate CCG1, without species declarations.

Infection characteristics of *Glugea* sp. infecting mottled sculpin most closely resemble descriptions of *G. hertwigi* from rainbow smelt in North America (Canning & Lom 1986, Lovy et al. 2009). Xenomas ranged in size from barely visible to the naked eye to 1 cm in diameter and are associated with the body wall, fat body, gonads, and kidneys. Our data suggest that the initial site of infection is the fat body, followed by the body wall, the gonads, and finally the kidney. This route seems plausible, as autoinfection proceeds to the nearest tissues and is consistent with studies of *G. hertwigi* in smelt (Delisle 1972). Female sculpin experience heavier infections and more frequent infection of the gonads relative to males. Nearly 40% of females had infections ≥1.5% of their body weight, twice that of males. Infected females also had xenomas in their gonads in 40% of infections compared to 20% in males, despite prevalence being roughly equal. Females having infections in the gonads more often than males were also observed in Lake Erie smelt populations infected by *G. hertwigi* (Nepszy & Dechtiar 1972).

Our data show *Glugea* sp. is common in the Michigan populations sampled for this study; prevalence was ≥70% in 4 of 6 infected populations (range ~4 to 80%). High prevalence can be maintained for multiple years as shown by Seven Mile Creek, where prevalence ranged from 69 to 93% between 2007

and 2011. Prevalence can also fluctuate substantially; in 2007, prevalence in Silver Creek sculpin was 11% but reached 75% in 2011 (Homola et al. 2014). However, prevalence in age-0 fish was very low. Both of the 2 infected age-0 fish were collected from Seven Mile Creek in July 2011 and were heavily infected with large xenomas protruding from the abdomen. Infection in age-0 fish appears to be quite rare, but it is not clear whether juvenile fish carry a latent infection, infection is quickly fatal, or infection is actually rare. Parasite transmission and environmental conditions can affect prevalence.

Horizontal transmission via ingestion of spores is the primary infection mechanism described in previous studies of *Glugea* (Canning & Lom 1986), and our results support this conclusion. Eight of 12 fish exposed to at least 200 spores per os had visible xenomas 7 to 9 wk post exposure. Additionally, the number of xenomas increased with spore dose. It is still not clear how sculpin come to ingest spores in natural flowing water systems. Some authors have suggested that infection is facilitated by a paratenic host (i.e. a prey item that has ingested spores; Scarborough & Weidner 1979, Shaw & Kent 1999), and while that is possible, we did not explicitly test for it and still found high prevalence.

We documented the potential for vertical transmission of *Glugea* sp. within the ovaries of infected female mottled sculpin. Using PCR, we detected *Glugea* sp. DNA associated with the eggs of 1 infected female out of a group of 10. Vertical transmission has not previously been evaluated for *Glugea* parasites, but there are numerous reports of xenomas within the ovaries of females (Nepszy & Dechtiar 1972, Scarborough & Weidner 1979, Canning & Lom 1986). *Glugea* sp. cells could have been within eggs or on the surface; both are considered vertical transmission (Anderson & May 1981). However, we do not know whether infected eggs would have developed into fry or whether those fish would have developed infections. It is notable that in 2 other fish-infecting microsporidia (*Ovipleistophora ovariae* and *Pseudoloma neurophilia*), initial molecular and observational evidence for vertical transmission were doubted, but more sophisticated techniques confirmed that these parasites are vertically transmitted (Phelps & Goodwin 2008, Sanders et al. 2013).

Given the high *Glugea* sp. prevalence in adults and the potential for vertical transmission, the low infection rates we found in juveniles were unexpected. It is common for xenomas to be observed in the ovaries of mature females infected by *G. hertwigi*, and smelt have been reported to expel xenomas from ovaries into the nest during spawning (Delisle 1972, Nepszy & Dechtiar 1972, Scarborough & Weidner 1979). Parasite spores are certainly deposited in the nest by infected females and males through spawn and feces, which makes it likely that fry have sufficient contact with spores in the nest such that many infections would be effectively vertical (i.e. transmitted at hatching from an infected parent to offspring). While this idea conflicts with the low prevalence we found in juvenile fish, it is congruent with a latent infection period in juveniles. In fact, Nepszy et al. (1978) found a 4 mo lag period after hatching until prevalence increased rapidly in juveniles, and the highest prevalence was observed in late summer and early fall. We did not sample adequately during the late summer and fall to detect this type of lag or observe heavy infections in juvenile fish. Yet *Glugea* sp. prevalence in age-1 sculpin born the previous year is similar to older fish, and low water temperatures over winter are known to inhibit microsporidian growth in fish (Canning & Lom 1986). Collectively, this information suggests that early infection should be investigated more carefully, and monthly or seasonal sampling should be done to understand infection and prevalence of fish-infecting microsporidia. Certainly, spawning females and nest-guarding males releasing billions of spores into the nest could explain the high prevalence we observed in our study, and if this exposure results in infection, it blurs the line between horizontal and vertical transmission.

More research is needed to examine the effect *Glugea* sp. has on sculpin, but it may impact behavior, reproduction, and mortality. Moderate to heavily infected individuals have visibly less fat body than uninfected fish, so they may have decreased fitness. Additionally, any negative effects on individuals could be significant at the population level given the higher infection intensity in females and the high prevalence in general. However, work on these populations by Homola et al. (2014) suggests that individual- and population-level effects are weak. While studies of rainbow smelt populations from multiple locations, including Lakes Erie and Ontario, showed that *G. hertwigi* reduced reproduction and increased mortality (Chen & Power 1972, Nepszy & Dechtiar 1972, Scarborough & Weidner 1979), models indicated no correlation between infection prevalence and smelt recruitment in Lake Erie over a 20 yr study (Henderson & Nepszy 1989). Compensatory mortality could explain weak population level effects even in populations with high infection prevalence and negative fitness effects (Gulland 1995, Kistner & Belovsky 2014).

DNA sequence analysis indicates that species boundaries are not well defined in some groups of microsporidia such as *Glugea*, which infect a broad range of marine and freshwater hosts. More molecular work and cross-infectivity studies should be conducted to clarify whether species designations are valid. Molecular techniques can also be used to look at the recent evolutionary history of fish microsporidians to determine whether their host range is expanding or whether they have been introduced into new regions. Microsporidian infection characteristics vary widely but are often associated with reduced individual fitness or lost economic value of infected fish. More research is needed to understand whether the negative effects of microsporidians translate to ecologically important changes in host populations.

Acknowledgements. Simon Jones and Darren Ward provided valuable information and assistance that improved this study. The Brown family generously allowed us access to Seven Mile Creek. Several undergraduate students contributed to this research, including S. Curry, R. Heenan, E. Piller, E. Cooper, C. Huston, and E. Helderop. T. Barkman, M. Buchalski, J. Landosky, and 2 anonymous reviewers provided many useful comments on the manuscript. Funding was provided by the George L. Disborough Trout Unlimited Research Grant, the Gwen Frostic Doctoral Fellowship, and the WMU Graduate College and Department of Biological Sciences.

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Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: November 2, 2015; Accepted: February 2, 2016
Proofs received from author(s): March 10, 2016