

Validation of a quantitative PCR diagnostic method for detection of the microsporidian *Ovipleistophora ovariae* in the cyprinid fish *Notemigonus crysoleucas*

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ABSTRACT: Microsporidian parasites are easily detected by light microscopy when infections are heavy and spores are present. However, early infections without spores, or light infections with low numbers of spores, are easily missed. This limitation has made it difficult to conduct investigations into microsporidian prevalence and transmission. In this study, we developed a quantitative TaqMan polymerase chain reaction assay to assess the presence of *Ovipleistophora ovariae* in the tissues of the cyprinid fish *Notemigonus crysoleucas* (golden shiner). The efficiency of the primer set was 100.8%, with a correlation coefficient of threshold position to copy number of 0.997 over 9 logs using a plasmid containing the cloned reaction product. No product was produced from other closely related microsporidian species (*Nucleospora salmonis*, *Pseudoloma neurophila*, *Glugea stephani*, *Heterosporis* sp., and *O. mirandella*). The coefficient of variation for replicate assays done on different days was 12.4%. The assay detects *O. ovariae* reliably at less than 10 genomic copies and 0.14 spores per reaction, but maximum sensitivity is only achieved when sonication is included as part of the DNA purification step. Using the assay, we found 4.44×10^1 to 7.91×10^6 copies μg^{-1} host DNA in female golden shiners, with the spore density increasing during the spawning season. The parasite was also detected for the first time in the testes of male golden shiners at 2.60×10^1 to 8.62×10^2 copies μg^{-1} host DNA.

KEY WORDS: Microsporidia · PCR · Cyprinid

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INTRODUCTION

Microsporidians infect a wide range of hosts, from single-celled protozoa to higher vertebrates, including humans. The first microsporidian recorded in vertebrates was *Glugea anomala*, producing subcutaneous cysts in the three-spined stickleback *Gasterosteus aculeatus* (Moniez 1887). More than 100 species of microsporidians in 14 genera occur in fishes (Canning & Lom 1986, Shaw & Kent 1999).

Many of the microsporidians that infect fish are important parasites. A *Heterosporis* sp. in Minnesota and Wisconsin, USA, causes mortality in yellow perch *Perca flavescens*, and in milder cases, muscle is partially replaced by parasite spores that render the meat unpalatable (Sutherland et al. 2000). The North Amer-

ican ocean pout *Macrozoarces americanus* fishery collapsed as a result of heavy infections by the microsporidian *Pleistophora macrozoarcides* (Fischthal 1944, Sandholzer et al. 1945, Sheehy et al. 1974). The collapse of a rainbow smelt *Osmerus mordax* fishery was in part attributed to infections of *Glugea hertwigi* (Haley 1954). Sublethal infections cause reduced growth, anorexia (Matthews & Matthews 1980, Figueras et al. 1992), impairment of swimming ability (Sprengel & Luchtenberg 1991), reduced reproductive success (Summerfelt 1964, Wiklund et al. 1996), and liquefaction of muscle tissue (Nigrelli 1946, Grabda 1978, Egidius & Soleim 1986, Pulsford & Matthews 1991). Members of the genera *Glugea*, *Nucleospora*, *Loma*, and *Pleistophora* also have significant effects on farm-raised fishes (Chilmonczyk et al. 1991, Shaw &

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Kent 1999). Most morbidity from microsporidians occurs when the formation of large numbers of mature spores impacts organ function.

Spore formation is the final step in the microsporidian life cycle. The development of mature spores is complex and diverse across the microsporidians. In the early stages, if horizontally transmitted, mature spores contact a suitable fish host either through water or through food. The microsporidian sporoplasm and nucleic acid are then discharged from the spore through a polar tube directly into a host cell. If the spore enters its host fish with food and a suitable host cell is not within range of the polar tube, the sporoplasm is extruded and migrates through the animal's digestive tract and into the blood stream, traveling to other parts of the host's body until a suitable cell is found and infected (Lom & Dykova 1992). Once in the host cell, a proliferative phase occurs, and the sporoplasm undergoes nuclear division (Cali & Takvorian 1999). These multinucleate cells separate, forming uni-, di-, or tetra-nucleate cells. Next in the sporogonic phase, these cells undergo division of either the cytoplasm (plasmotomy) or nucleus (fission) into multinucleate cells. Following this division, the cells divide yet again, with the plasmalemma pulling away from the sporophorous vesicle (SPOV) forming what eventually becomes the mature mono- or diplokaryotic spores (Vavra & Larsson 1999). The mature spores are typically oval and generally vary in length from 1 to 12 μm (Lom & Dykova 1992, Cali & Takvorian 1999).

Mature spores associated with heavy infections by microsporidians are easily detected by microscopy, but the small size of the spores can make light infections difficult to recognize. During the early stages of infections when mature spores are not present, detection by standard microscopy is even more difficult. Because of these limitations, there are large gaps in our knowledge of the life cycles, host range, and transmission of many microsporidians. In this study we developed a quantitative real-time polymerase chain reaction (qPCR) assay to detect the microsporidian *Ovipleistophora ovariae* (formerly *Pleistophora ovariae*; Pekkarinen et al. 2002) in the tissues of infected fish. The *O. ovariae* parasite is widespread in the US and impacts the fertility of golden shiners *Notemigonus crysoleucas* (Summerfelt 1964), an important baitfish species. Our PCR assay will enable the detection of low-level infections and the detection of infection in hosts when spores have not yet developed. This is a critical tool in efforts to control or eradicate this parasite.

MATERIALS AND METHODS

Sample collection and DNA extraction from fish. At 1 wk intervals during the 5 wk golden shiner *Notem-*

igonus crysoleucas spawning season, spawning broodfish were collected from a hatchery. Ovaries and testes from golden shiners were preserved, with half of each organ in 10% neutral buffered formalin and the other half in 70% ethanol. Formalin-fixed tissues were then embedded in paraffin, sectioned at 5 μm , stained with hematoxylin and eosin or acid fast stain (Stevens & Francis 1996), and used to confirm the presence of *Ovipleistophora ovariae* spores. Ethanol-preserved ovary and testis tissues from a minimum of 15 female and 5 male broodfish wk^{-1} were pooled into groups of 5, and DNA was extracted using a 200 μl elution volume (Qiagen Dneasy tissue kit). Linear regression statistics using Microsoft EXCEL ($\alpha = 0.05$) were used to compare numbers of *O. ovariae* genomes μg^{-1} host DNA during the spawning season.

DNA extraction from purified spores. *Ovipleistophora ovariae* spores were purified from adult female golden shiners. Ovarian tissue was removed from infected fish and placed in a stomacher (Tekmar) with 10 ml saline with penicillin, streptomycin-L-glutamine (PSG; Invitrogen) and homogenized for 120 s at high speed. The slurry was then passed through consecutively smaller syringe needles (18, 21, and 25 gauge), filtered through a 70 μm mesh screen, then diluted in 9 parts of distilled water (dH_2O), and held 1 h at room temperature to lyse any remaining intact fish cells. The lysate was centrifuged at $1500 \times g$ for 20 min, then the pellet was suspended in 15 ml dH_2O and layered on top of a 50% Percoll (Sigma Chemical) solution (prepared with dH_2O) and centrifuged at $1500 \times g$ for 45 min. The pellet of purified spores was resuspended in 1 ml PSG. This method of purifying the spores was modified from a protocol kindly provided by Drs. V. Waltral, J. D. Ferguson, and C. Whipps (Oregon State University). Spores were counted using a hemocytometer.

To compare extraction efficiency, the DNA from purified spores was purified by 2 methods. The first was a commercial kit and the manufacturer's protocol for cultured animal cells (Qiagen DNeasy tissue kit), except that the concentration of proteinase K was doubled and initial incubation was for 3 h rather than the recommended 10 min. In a second method designed to ensure opening of the spores, 100 μl of purified spores ($3000 \text{ spores } \mu\text{l}^{-1}$ PSG) were sonicated (Sonic Dismembrator 60, Thermo Fisher Scientific) for 10 s at 5 W at the beginning of the proteinase K digestion. Extracted DNA template concentrations and purity were determined using spectrophotometry (Nanodrop ND-1000).

Primers and probe selection. Primers and TaqMan probe (Table 1) were designed using published sequences of *Ovipleistophora ovariae* (GenBank AJ252955) and selected to maximize mismatches with the closely related microsporidians *O. mirandella*

Table 1. Primer and locked nucleic acid (LNA) probe sequences for the detection of *Ovipleistophora ovariae* (GenBank AJ252955) by quantitative PCR. +: location of LNA bases

Primer	T_m (50 mM NaCl)	Sequence
Forward	57.9°C	5'-CTC AAC GTG GGA CAG CTT ACC-3'
Reverse	56.1°C	5'-TCC TGT CAC AAC AAC CGT AAG TAA-3'
LNA probe	67.3°C	5'-FAM-CGA +CGA +CCG CAC G+AG-BHQ1-3'

(GenBank AJ295327), *Heterosporis* sp. (GenBank AF356225), *Glugea stephani* (GenBank AF056015), and *Pseudoloma neurophila* (GenBank AF322654), using commercial software (Beacon Designer 4.0; PREMIER Biosoft International). The TaqMan probe contained 3 'locked nucleic acids' (LNA), first described by Singh et al. (1998), to raise the melting temperature and increase the sensitivity and specificity of real time PCR (Reynisson et al. 2005, Karkare & Bhatnagar 2006). The probe was labeled with FAM fluorescent dye on the 5' end and the quencher BHQ-1 on the 3' end (Integrated DNA Technologies). The PCR product had a length of 165 bp (Fig. 1), which was confirmed using an ethidium bromide-stained agarose gel.

Real-time PCR. The real-time PCR reactions for *Ovipleistophora ovariae* were prepared in 12.5 μ l volumes consisting of 2.5 μ l of DNA template, 0.5 μ l of 10 μ M forward and reverse primer, 0.5 μ l of 10 μ M FAM-labeled probe, 6.25 μ l of 2 \times Supermix (Bio-Rad Laboratories), and 2.25 μ l dH₂O. The PCR was carried out on a real-time PCR machine (iCycler; Bio-Rad Laboratories) using the manufacturer's software (iQ Real-Time Detection System). The cycling protocol was 2 min at 94°C, followed by 40 cycles of 45 s at 59°C, 45 s at 72°C, and 30 s at 95°C, with a final extension phase for 10 min at 72°C.

Standard curve/dynamic range. The *Ovipleistophora ovariae* PCR product was purified (GFX PCR DNA & Gel Band Purification Kit; Amersham Biosciences) and cloned into *Escherichia coli* (TOPO-TA cloning kit for sequencing; Invitrogen) according to the manufacturer's protocol. Recombinant plasmid DNA was then purified using a commercial kit (QIAprep Spin Miniprep Kit and a microcentrifuge; Amersham Biosciences). The copy number per volume for the purified plasmid was calculated from the concentration of extracted DNA quantified by spectrophotometry.

A standard curve was produced by serial dilution of purified plasmid in TE buffer (10 mM Tris, 1 mM EDTA solution, pH 8.0 \pm 0.1) to produce template concentrations of 10⁹ to 10⁻¹ copies per reaction. An additional standard curve was developed using 250 ng μ l⁻¹ common carp *Cyprinus carpio* spleen DNA (purified using the Qiagen DNeasy tissue kit; Bio-Rad Laboratories) in

all tubes, but spiked with known concentrations of the template plasmid DNA. All reactions were run in triplicate using the above PCR protocol. From the standard curves, the PCR efficiency and correlation coefficient were calculated (iQ Real-Time Detection System; Bio-Rad Laboratories). Common carp DNA was used because the samples collected were negative for *Ovipleistophora ovariae* with this

assay. The negative control used in all assays was goldfish *Carassius auratus auratus* kidney DNA.

Minimum detection limit. The detection limit using the plasmid template was defined as the lowest plasmid copy number producing a threshold cycle (C_T) significantly different from reactions with no template DNA. The detection limit for purified spores was determined from both serial dilutions of DNA extracted from known numbers of purified spores using the commercial kit (assuming 100% DNA extraction efficiency) and DNA from spores that had gone through the sonication procedure.

Precision. DNA samples from 5 known positive fish were tested for *Ovipleistophora ovariae* using the PCR assay described above. The reactions were run in triplicate on 6 different days, and the mean and coefficient of variation (CV) were calculated for each sample.

Specificity. PCR reactions as described above were run using the *Ovipleistophora ovariae* and other closely related microsporidians with the primer set for *O. ovariae*. *Nucleospora salmonis*, *Pseudoloma neurophila*, and *Glugea stephani* were provided by Dr. C. Whipps (Oregon State University) as extracted DNA from tissue, and *Heterosporis* sp. was provided by Dr. D. Cloutman (Bemidji State University) as infected yellow perch *Perca flavescens* tissue. *Ovipleistophora mirandella* was provided by Dr. F. Nilsen (Havforsknings Instituttet—Institute of Marine Research, Norway) as purified DNA and pure spores. The DNA was purified following the methods described for *O. ovariae*.

RESULTS

Standard curve/dynamic range. The standard curve of the plasmid in TE Buffer was linear over 9 logs of plasmid dilutions. The efficiency for the PCR reaction was 100.8%, and the correlation coefficient of threshold position to copy number was 0.997 (Fig. 2). The standard curve for plasmid spiked into 250 ng μ l⁻¹ common carp DNA was also linear over 9 logs. The PCR efficiency for this reaction was 99.4%, and the correlation coefficient was 1.000.

Minimum detection limit. The minimum copy number of purified plasmid detected using our quantitative PCR assay was less than 10 copies per reaction (9.00×10^0 copies per reaction; Fig. 2). Using the standard proteinase K digestion with purified spores and assuming no loss of *Ovipleistophora ovariae* DNA during purification, the calculated sensitivity of the assay was 72 spores per reaction. However, with the sonicated spore

DNA preparations, the detection limit was 0.14 spores per reaction.

Precision. Five *Ovipleistophora ovariae* positive ovary samples tested with our assay on 6 different days had a CV in copy number ranging from 8.75 to 16.22% with an average of 12.43% (Table 2).

Specificity. The primers and LNA probe were specific for *Ovipleistophora ovariae*. There were no

<i>Ovipleistophora ovariae</i>	AATTGACGGAAGAACCACCAAGGAGTGGAGTGTGCGGCTTAATTC <u>AACT</u>	898
<i>Ovipleistophora mirandella</i>	AATTGACGGAAGAACCACCAAGGAGTGGAGTGTGCGGCTTAATTC <u>AACT</u>	899
<i>Heterosporis</i> sp.	AATTGACGGAAGAACCACCAAGGAGTGGAGTGTGCGGCTTAATTTGACT	882
<i>Glugea stephani</i>	AATTGACGGAAGAACCACCAAGGAGTGGAGTGTGCGGCTTAATTTGACT	692
<i>Pseudoloma neurophilia</i>	AATTGACGGATGAACACCTCAAATAGTGGAGTGTGCGGCTTAATTTGACT	876
	***** **	
<i>Ovipleistophora ovariae</i>	<u>CAACGTGGGACAGCTTACCAGGCC</u> CGACGACCGCACGAG <u>TGTAGTACACG</u>	948
<i>Ovipleistophora mirandella</i>	CAACGTGGGACAGCTTACCAGGCCGACGACCGCACGAGTGTAGTACACG	949
<i>Heterosporis</i> sp.	CAACGCGGGACAGCTTACCAGGCCGACGACCGCAGGAGTGTGTACACG	932
<i>Glugea stephani</i>	CAACGCGGGACAGCTTACCAGGCCGACGCGCGGACGAGTGTGTACACG	742
<i>Pseudoloma neurophilia</i>	CAACGCGGGACATCTTACCAGGCCGACGACCGGACGAGCGTGACACGCG	926
	***** **	
<i>Ovipleistophora ovariae</i>	ATAGGTTGAAGAGTGGTGCATGGCCGTTAACGACAAGTGGGGTGACCTTT	998
<i>Ovipleistophora mirandella</i>	ATAGGTTGAAGAGTGGTGCATGGCCGTTAACGACAAGTGGGGTGACCTTT	999
<i>Heterosporis</i> sp.	ATAGGTCGAAGAGTGGTGCATGGCCGTTAACGACAAGTGGGGTGACCTTT	982
<i>Glugea stephani</i>	ATAGGTCGAAGAGTGGTGCATGGCCGTTAACGACGAGTGGGTGACCTTT	792
<i>Pseudoloma neurophilia</i>	ATAGGTCGAGAGTGGTGCATGGCCGTTAACGACGAGTGGAGTGATCTTT	976
	***** * ***** **	
<i>Ovipleistophora ovariae</i>	GGGTTAAGTCCGGGAAGTAGTGAGACCCCTGCTGTTACTTTACTTACGGT	1048
<i>Ovipleistophora mirandella</i>	GGGTTAAGTCCGGGAAGTAGTGAGACCCCTGCTGTTACT-----AGT	1041
<i>Heterosporis</i> sp.	GGGTTAAGTCCGGGAAGTAGTGAGACCCCTGCTGTTTTT-----T	1022
<i>Glugea stephani</i>	GGGTTAATCCGGGAAGTAGTGAGACCCCTACCG-----	826
<i>Pseudoloma neurophilia</i>	GGGTTAAGTCCGTAATTAGTGAGACCCAGC-----	1008
	***** ** ***** *	
<i>Ovipleistophora ovariae</i>	<u>TGTTGTGACAGGA</u> CAGGTGCTTAAAGCACAGGAAGGAAGGGTCAAGAACA	1098
<i>Ovipleistophora mirandella</i>	AATTGTGACAGGACAGGTGCTCAAAGCACAGGAAGGAAGGGTCAAGAACA	1091
<i>Heterosporis</i> sp.	TTTTG-GACAGGACAGGTGCTCAAAGCACAGGAAGGAAGGGTCAAGAACA	1071
<i>Glugea stephani</i>	-----AAAGGGACAGGTGCCGAAAGCACAGGAAGGAAGGGTCAAGAACA	870
<i>Pseudoloma neurophilia</i>	-----GGAAGGACAGGTGCCGCAACGCACAGGAAGGATGGGTCAAGGACA	1052
	***** ** ***** **	

Fig. 1. Partial sequences of *Ovipleistophora ovariae* (GenBank AJ252955), *Ovipleistophora mirandella* (GenBank AJ295327), *Heterosporis* sp. (GenBank AF356225), *Glugea stephani* (GenBank AF056015), and *Pseudoloma neurophilia* (GenBank AF322654) showing area of heterogeneity. Asterisks indicate sequence homology and dashes indicate gaps in the sequence. Primer locations are underlined and probe site is boxed. Numbers on the right represent the partial sequence location in the entire 16S rDNA sequence provided by GenBank

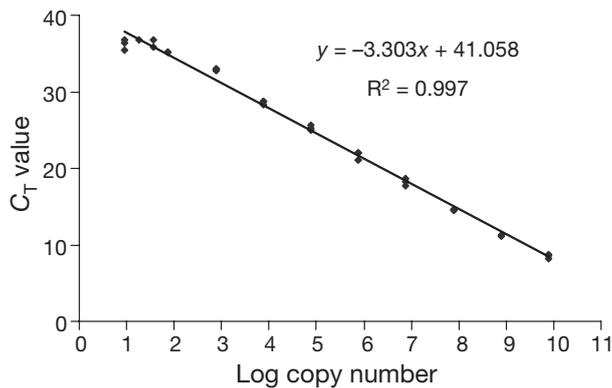


Fig. 2. Quantitative PCR standard curve with known quantities of *Ovipleistophora ovariae* plasmid diluted into buffer. The PCR efficiency was 100.8%. C_T : threshold cycle (detection limit)

increases in fluorescence through 40 cycles using *Nucleospora salmonis*, *Pseudoloma neurophila*, *Glugea stephani*, *Heterosporis* sp., or *O. mirandella* as the template.

Detection of *Ovipleistophora ovariae* in fish. All female golden shiners tested were positive for *Ovipleistophora ovariae* by histology and by PCR. The copies of *O. ovariae* μg^{-1} host DNA (prepared by standard proteinase K digestion) in golden shiner ovaries increased from 4.40×10^1 in April to 7.91×10^6 in the fourth week of sampling (May; $F = 12.42$, $df = 1, 12$, $p = 0.0042$). No *O. ovariae* spores were apparent by histology in the testes of male golden shiners, but they tested positive by PCR at a mean (SD) of 2.72×10^2 (3.49×10^2) copies μg^{-1} host DNA with no seasonal trends ($F = 0.58$, $df = 1, 3$, $p = 0.5021$; Table 3). Mean numbers of spores in males was significantly lower than the mean (SD) in female fish of 1.70×10^6 (3.47×10^6) copies μg^{-1} host DNA ($F = 8.94$, $df = 1, 8$, $p = 0.017$).

DISCUSSION

Our qPCR assay is sensitive, precise, and specific for *Ovipleistophora ovariae* in golden shiners. This holds true for purified plasmid DNA, fish DNA spiked with

Table 3. Copies of *Ovipleistophora ovariae* DNA μg^{-1} host DNA in female and male golden shiners *Notemigonus crysoleucus* during the spawning season of 13 April to 5 May 2006

Date	Ovary	Testis
13 April 2006	4.44×10^1	2.60×10^1
17 April 2006	9.98×10^4	4.35×10^1
26 April 2006	1.06×10^5	8.62×10^2
1 May 2006	7.91×10^6	3.12×10^2
5 May 2006	3.91×10^5	1.15×10^2

plasmid template, and DNA purified from spores of *O. ovariae*. We chose to report our pathogen densities as 'per μg host DNA.' It would also be possible to report it as 'per host genomic equivalents' by running a parallel quantitative reaction using primers to a host gene like glucokinase (Gilad et al. 2004). These glucokinase primers cross-react with golden shiner and fathead minnow *Pimephales promelas* glucokinase genes (GenBank AF053332; data not shown), but we have not found the benefits of 'per host genomic equivalent' units to outweigh the additional costs involved in doubling the number of required PCR reactions.

The specificity of the assay was tested against a variety of closely related microsporidians and was found not to cross-react. This is not surprising given that the primers and probe have mismatches with the published sequences of other microsporidians (Fig. 1). Performing the assay with uninfected golden shiners as a negative control would have been desirable, but all fish that we tested were strongly positive. Therefore, DNA from species that tested negative (common carp and goldfish) was used.

The sensitivity of the assay reliably detected fewer than 10 copies of plasmid template per reaction and was comparable to other microsporidian qPCR assays (Docker et al. 1997, Whipps & Kent 2006, Whipps et al. 2006). The detection limit for *Ovipleistophora ovariae* spores was initially calculated to be 72 spores per reaction, but microscopic examination of pure spore DNA preparations at the end of the proteinase K digestion revealed many unopened spores. This was surprising given that this same methodology is

Table 2. *Ovipleistophora ovariae* copy number per reaction by a quantitative PCR assay. Results from 5 golden shiner *Notemigonus crysoleucus* ovary samples tested on 6 different days. Mean coefficient of variation (CV) was 12.43%

Sample	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Mean	SD	CV (%)
1	2.80×10^6	4.07×10^6	3.95×10^6	3.93×10^6	3.46×10^6	3.32×10^6	3.59×10^6	4.88×10^5	13.59
2	1.48×10^6	2.05×10^6	2.10×10^6	2.30×10^6	2.04×10^6	1.81×10^6	1.96×10^6	2.84×10^5	14.45
3	8.53×10^5	1.03×10^6	1.08×10^6	9.37×10^5	9.12×10^5	1.05×10^6	9.77×10^5	8.94×10^4	9.15
4	4.58×10^5	5.93×10^5	5.42×10^5	5.78×10^5	6.39×10^5	4.09×10^5	5.37×10^5	8.70×10^4	16.22
5	4.38×10^6	4.59×10^6	5.02×10^6	4.96×10^6	4.56×10^6	3.93×10^6	4.57×10^6	4.00×10^5	8.75

commonly used for other microsporidians (Docker et al. 1997). When we briefly sonicated spore preparations during proteinase K digestion, intact spores were no longer seen, and the sensitivity of the assay improved to just 0.14 spores per reaction. Thus, the assay is over 500× more sensitive when the sonication step is used. The sensitivity is less than 1 spore per reaction, because the spores contain multiple copies of the small subunit rDNA, which our primers target (Whipps & Kent 2006).

The seasonal change in female golden shiners agrees with research showing a positive correlation between seasonal infection levels and temperature for *Nucleospora salmonis* and *Loma salmonae* (Georgiadis et al. 1998, Beaman et al. 1999, respectively). The synchronization of spore production with the peak spawning season in golden shiners may be entirely temperature dependent, but the parasite may respond to hormonal cues also involved in egg maturation. Given that our DNA purification without sonication was very inefficient for mature spores, it may be that the *Ovipleistophora ovariae* DNA detected in our tissue samples was mostly from immature life stages where the resistant spore wall was not yet formed. As a result, the seasonal variation in females may have been due to the proliferation of immature forms during the spawning season.

The discovery of *Ovipleistophora ovariae* in male fish proves the benefits of using qPCR as a diagnostic tool. With the low levels of infection, and perhaps because mature spores were not present, spores were not found in testes by histological methods. It is not known why males are not infected at the same severity as females. It has been suggested that this is due to hormonal or nutritional factors; however, Summerfelt (1964) speculated that heavy infections may be lethal to males, and as a result, only low infections are observed. It is also possible that *O. ovariae* specifically targets the ovary, and infections of other organs (i.e. male testes) do not provide suitable conditions for proliferation, hence the low infections observed. We also expect the assay to be helpful in detecting the presence of *O. ovariae* in fish species other than golden shiners and fathead minnows, the only known hosts (Nagel & Hoffman 1977, Nagel & Summerfelt 1977, Ruehl-Fehlert et al. 2005).

The quantitative assay that we describe has proven useful in establishing the prevalence of *Ovipleistophora ovariae* in a population of golden shiners, and it has shown for the first time that male golden shiners carry the parasite. We expect that the sensitivity and specificity of this assay will be valuable in future investigations of microsporidian biology and epidemiology, especially questions of transmission and susceptible hosts.

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