

Thesis Proposal

For

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Candidate for the Degree of

Master of Science in Aquaculture/Fisheries

TITLE: Validation of a Quantitative PCR Diagnostic Method for *Ovipleistophora ovariae* in Golden Shiners and Determination of Vertical Transmission from Broodstock.

OBJECTIVES:

- (1) To validate a quantitative Taqman PCR assay for the detection of *Ovipleistophora ovariae* in golden shiner eggs, fry, and adults.
- (2) To use that assay to determine if vertical transmission of *Ovipleistophora ovariae* occurs in golden shiners.
- (3) To determine if vertical transmission (if it occurs) is within golden shiner eggs or on their surface

APPROVAL:

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## INTRODUCTION

Arkansas farmers produce more than 60% (by value) of farm-raised baitfish in the United States, with the primary product being the golden shiner, *Notemigonus crysoleucus*. Arkansas farm gate sales of golden shiners are nearly \$15 million and the economic impact of the industry is very important to the economy of the delta region (U.S. Department of Agriculture 2000).

One of the factors currently limiting further efficiency gains in golden shiner production is the prevalence of infection by the microsporidian parasite *Ovipleistophora ovariae* (formally known as *Pleistophora ovariae*) (Pekkarienen et al. 2002). The *O. ovariae* parasite was first described from farm ponds located in southern Illinois but was later found to be widespread in Kentucky, Missouri and in Arkansas (Summerfelt 1964, Wilhelm 1964, Tucker 1967). *Ovipleistophora ovariae* infects the ovaries and renders older fish sterile (Summerfelt 1994) forcing golden shiner farmers to use 1-year-old fish as breeders rather than more mature broodfish.

The use of 1-year-old broodfish has been associated with several problems. Small broodfish may decrease farm efficiency because they produce fewer eggs and are less reliable in their spawning (Warner 1972, Munro 1990). On the other hand, due to the fractional spawning behavior, this may not be the case. In addition, the prevalence of *O. ovariae* is associated with other important problems.

Some states prohibit or restrict the importation of baitfish based on purported fish health concerns that may actually be a guise for trade protection. (Maine vs. Taylor 1990, Gunderson and Tucker 2000). *Ovipleistophora ovariae* was listed as one of three species of concern during the Maine vs. Taylor case in the United States Supreme Court,

which resulted in the ban of imported baitfish to the state of Maine. The presence of *O. ovariae* in cultured golden shiners limits the freedom of farmers to market their product.

Another problem, associated with the sterilization of mature fish by *O. ovariae*, is that the parasite prevents farmers from selectively breeding golden shiners for optimal market size, fecundity, disease resistance, and handling characteristics. The *O. ovariae* parasite limits the number of spawning seasons that desirable fish could be used and would force breeders to replace all brood stock on an annual basis.

Due to the limitations that *O. ovariae* puts on shiner culture, there is a great deal of interest in eradicating the parasite from the industry. In order to achieve this goal, we must first determine the life cycle of the parasite and understand how it is transmitted from generation to generation. The presence of parasite spores in the ovary makes vertical transmission a likely mode.

Vertical transmission is seen in other microsporidian species, for example *Nosema granulosis* in crustaceans (Terry et al. 1999) and several other species infecting insects including *Amblyospora* spp., *Culicospora* spp. and *Edhazardia* spp. (Bencel and Andreadis 1999). The fish-parasitizing microsporidia *Pleistophora mulleri* and another undescribed *Microsporidium* sp. are more closely related to *O. ovariae* and have been found to be vertically transmitted (Terry et al. 2004). Some researchers are confident that vertical transmission exists for *O. ovariae*, however they are unsure if it is transovarial (in the egg) or transovum (on the egg) (J. E. Smith, University of Leeds, personal communication). The parasite has been found in 5% of 38 golden shiner blastulas examined after infection of female golden shiner broodfish per os with *O. ovariae*

(Summerfelt 1972) but other experts have argued that this is not conclusive evidence, as cited in Shaw and Kent (1999) according to Canning and Lom (1986).

In this project, I will determine the mechanism of intergenerational transmission by developing and validating a quantitative PCR method for *O. ovariae*. I will use the assay to test eggs, fry, and adult golden shiners by qPCR to determine if *O. ovariae* is vertically transmitted and if that transmission occurs within eggs or on their surfaces. Once the mode of transmission is known, plans can be developed to raise parasite free broodfish for producing fish for sale and for selective breeding programs. Determination of the mode of vertical transmission will be accomplished by the completion of the following three objectives.

Specifically,

- (1) To validate a quantitative TaqMan assay used for the detection of *Ovipleistophora ovariae* in golden shiner eggs, fry, and adults.
- (2) To use the assay to determine if vertical transmission of *Ovipleistophora ovariae* occurs in golden shiners.
- (3) To determine if vertical transmission (if it occurs) is within golden shiner eggs or on their surface.

This will reduce hatchery costs, increase fecundity, and possibly allow the expansion of the baitfish industry into states that currently prohibit importation. The methods

established will be useful in this and other *Ovipleistophora ovariae* research in the future and will provide broader insights into the biology of microsporidians.

## LITERATURE REVIEW

*Ovipleistophora ovariae* (formerly *Pleistophora ovariae*) is a parasite of the class Microsporea. This parasite infects the ovaries of golden shiners reducing fecundity by more than 40% (Lom and Dykova 1992). As fish age increases from 1 to 4 years the incidence of infection increases from 30 to 75% and fecundity is reduced to levels that render the fish functionally sterile (Lom and Dykova 1992, Summerfelt and Warner 1970a). After age four, the incidence of *O. ovariae* infection decreases, possibly as a result of selective mortality (Lom and Dykova 1992, Shaw and Kent 1999). The severity of the infection varies with season, the highest levels of observable infection corresponding with the spawning seasons of April through June (Lom and Dykova 1992, Summerfelt 1994).

### *Distribution*

*Ovipleistophora ovariae* is widespread, affecting golden shiners in 12 southeastern states and California in both the wild and in commercial hatcheries. According to a 1994 review, the areas where the parasite has been reported include: Alabama, Arkansas, California, Illinois, Kansas, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, Tennessee and Texas (Summerfelt 1994). In populations that carry the parasite prevalence of infection is 46% (Summerfelt and Warner 1970a), although incidence levels may be as high as 100% on some commercial farms (Lom and Dykova 1992).

### *Morphology/Development*

Microsporidians have been classified within the kingdom Protozoa. It has also been postulated that they may fit better into the Kingdom Archezoa (ancient deep-branching eukaryotes) because of their unicellular structure and lack of mitochondria (Vossbrink et al. 1987; Vavra and Larsson 1999). However, the recent discovery in microsporidians of simple mitochondrial organelles (Katinka et al. 2001), combined with revised phylogenetic analysis suggests that they are better classified as a highly derived sister group of the fungi (Hirt et al. 1999, Baldauf 2003).

Classification of microsporidian parasites has been traditionally based on morphology of the spore stage because other developmental stages are extremely difficult to classify. Spores have a strong resistant membrane (0.17-0.3  $\mu\text{m}$  thick) that consists of three layers: a thin outer proteinaceous layer (exospore) which is smooth or finely corrugated, a thick inner chitinous layer (endospore), and a simple cell membrane on the innermost portion of the wall (Egusa 1991, Lom and Dykova 1992). In general, spores are about 5  $\mu\text{m}$  in length and round to oval shaped. The spores of *O. ovariae* are egg-shaped and about 8.4  $\mu\text{m}$  x 4.2  $\mu\text{m}$  in size. Microsporidian spores lack the normal eukaryotic mitochondria and typical Golgi apparatus but have microbody-like organelles and ribosomes that resemble those of prokaryotic organisms (Curgy et al. 1980, Ishihara and Hayashi 1968). Spores do contain an extrusion or “hatching” apparatus with a polar tube, a polaroplast, and a posterior vacuole (Lom and Dykova 1992). The remaining space within the spore consists of the sporoplasm, which contains the endoplasmic reticulum and free ribosomes. Tightly wound spirals of DNA form from the compacted

polyribosomes and create a single nucleus or a diplokaryon (depending on genus) (1992). These spirals and the sporoplasm are released to achieve infection.

The sporoplasm and nucleic acid of microsporidians are discharged through an extended polar tube directly into the host cell. If a host cell is not within range of the polar tube, the extruded sporoplasm can flow through the digestive tract and into the blood stream traveling to other parts of the host's body where it then enters a host cell.

Once the sporoplasm has been released, the developmental cycle begins. There are two phases to the development of microsporidians, a proliferative phase, merogony (also known as schizogony (Vavra and Sprague 1976)), and sporogony (Lom and Dykova 1992).

Proliferative Phase – After infection of a host cell, microsporidian sporoplasms undergo nuclear division, either binary fission, plasmotomy or multiple fission, depending on genera (Cali and Takvorian 1999). In the genus *Pleistophora*, relatively few new cells are produced in this phase. Most of the reproduction is done during the next developmental stage, sporogony (Cali and Takvorian 1999).

Sporogony – Sporonts are created when sporoplasms undergo a process of plasmalemmal thickening that progresses to a uniformly dense covering that serves as the exospore coat of the spore stage. Membrane thickening was first demonstrated in *Nosema* (Cali 1971) and occurs in many genera. *Pleistophora* has not been reported as being abnormal. There are exceptions such as, *Nosema algerae* and *Brachiola vesicularum* where the thickening occurs prior to sporogony. The opposite has been seen in *Enterocytozoon bieneusi* where the thickening is delayed until right before the spore formation (Cali and Takvorian 1999). After plasmalemmal thickening, internal cell

division creates the multicellular sporoblast. Sporoblasts decrease in size as they mature and develop ER, ribosomes, a posterior vacuole and an endospore (1999). When development of these organelles is complete, the mature sporoblast becomes a spore. The number of spores resulting from one sporont can range from 16 (Egusa 1991) to more than 100 (Cali and Takvorian 1999).

### *Specificity*

Microsporidians infect a wide range of animal groups from microscopic organisms to humans. More than 100 species in 20 genera occur in fishes (Canning and Lom 1986, Shaw and Lent 1999), but species that cause a great deal of harm to fish are limited in number and may not be entirely host specific (Egusa 1992, Lom and Dykova 1992). Recently, *Heterosporis* sp. has been discovered in Minnesota and Wisconsin and found to cause mortality of yellow perch (Sutherland 2000) as well as sex ratio distortion (Hodgins, personal communication). A few members of the genus *Glugea*, *Loma*, and *Pleistophora* have been under investigation for years due to their destructive effects on both wild and farm raised fish species.

In the genus *Pleistophora* (and now *Ovipleistophora*) there are 30 species that occur in fishes, eight of which are of economic importance: *P. ehrenbaumi* in wolffish (*Anarhichas* spp.), *P. finisterrensis* in blue whiting (*Micromesistius poutassou*), *P. hippoglossoides* in plaice (*Hippoglossoides platessoides*), *P. hypheobryconis* in ornamental fishes, *P. macrozoarcides* in ocean pout, *P. mirandellae* in European cyprinids, *P. senegalensis* in sea bream, and *O. ovariae* in baitfish (family Cyprinidae) (Shaw and Lent 1999).

*Ovipleistophora ovariae* is not entirely host specific to golden shiners. Fathead minnows (*Pimephales promelas*) have also been infected by the parasite but at a lower severity and incidence than golden shiners (Nagel and Hoffman 1977, Ruehl-Fehlert et al. 2005). *Ovipleistophora ovariae* could not be detected in goldfish (*Carassius auratus*) exposed in ponds to infected golden shiners and fathead minnows (Nagel and Summerfelt 1977). No other hosts are known. Most microsporidians infect only a narrow host range and *Pleistophora tahoensis*, a microsporidian that parasitizes the Piute sculpin, *Cottus beldingi*, (Summerfelt and Ebert 1969), has not been found in lake trout, *Salvelinus namaycush*, that prey on infected sculpins (Hoffman 1967, Canning and Lom 1986).

#### *Detection*

*Ovipleistophora ovariae* infects developing ova creating conspicuous white marbling or translucent opaque spots where oocytes have been destroyed (Summerfelt 1994, Lom and Dykova 1992). No behavioral or external signs of the disease are seen, but infected adult females are often bigger than similar uninfected females within the same population because the nutrients normally used for egg production are directed towards body growth (Warner 1972, Lom and Dykova 1992, Summerfelt 1994).

Infections by *O. ovariae* are usually detected by preparing wet mounts from fresh, frozen, or preserved ovaries. This is more effective during the pre and post spawning seasons because during the fall through the winter months obvious spores are rare or absent in both juveniles and adults (Summerfelt 1994). Embedding the ovary in paraffin and staining the slides allows for easier detection of low spore counts. It is important to note that the spores will be slightly smaller (3.6  $\mu\text{m}$  X 6.5  $\mu\text{m}$ ) once they have been fixed and stained (Parker and Warner 1970). The effectiveness of both histology and light or

electron microscopy is limited because young fish may be carrying the parasite in early stages of development that are difficult to detect by these methods (Lom and Dykova 1992).

### *Vertical Transmission*

Vertical transmission is defined as the direct transfer of pathogens from parent to progeny (Fine 1975). In various species of microsporidians, vertical transmission has been described both as supplementary to horizontal transmission and as the sole method of infection (Anderson and May 1981, Terry et al. 1999a). The microsporidian parasite, *Nosema granulosis*, displays only vertical transmission and feminizes its crustacean host (Terry et al. 1999a, Ironside et al. 2003a). In species infecting insects, it has been noted that vertical transmission is the single most important adaptation for parasite survival (Lucarotti and Andreadis 1995).

There are two distinct methods of vertical transmission, transovarial and transovum. In transovarial transmission, the parasite gains entry to the egg via the ovaries of the female host. This is thought to be the most common form of vertical transmission, however it is not fully understood how the parasite enters the egg (Bencel and Andreadis 1999). The second method, transovum, is where the parasite attaches to the outside of the egg or surrounding tissue and is consumed by the host larvae upon hatching. Transovum is far less likely than transovarial transmission, but has been documented with *Nosema algerae* in mosquitoes (*Anopheles stephensi*) (Alger and Undeen 1970, Canning and Hulls 1970) and *Nosema pyrausta* in the European corn bearer (*Ostrinia Nubilalis*) (Kramer 1959).

The 13 species of microsporidians known to utilize vertical transmission occur sporadically in many genera including *Encephalitozoon*, *Amblyospora*, *Edhazardia*, *Pleistophora*, and *Flabelliforma* (Smith and Dunn 2001, Terry et al. 2004). Since vertical transmission occurs sporadically throughout the microsporidians, the trait appears to have evolved either independently in several lineages or as an ancestral trait and been lost in some species (Terry et al. 2004). Vertical transmission in *O. ovariae* has not been reported, but the infection of oocytes by this parasite makes transovarial transmission likely (Terry et al. 1997).

It has been thought that cases of vertical transmission may be highly underestimated. Studies have shown that when *Nosema granulosis* is combined with bacterial endosymbionts, methods of transovarial transmission exist and do not cause any pathological effects to the host (Terry et al. 1997, 1998, 1999b). This suggests that vertically transmitted parasites may be cryptic and are more prevalent than previously expected. Therefore, those that displayed the cryptic behavior would not be detected by normal disease-associated screening methods (Dunn and Smith 2001). This can be achieved with our quantitative PCR test.

## **METHODS**

### PCR METHODS FOR DETECTING *Ovipleistophora ovariae*

#### *Development of a TaqMan PCR assay.*

A quantitative PCR method will be developed for *O. ovariae*. Three primer sets and a fluorescent Taqman probe will be designed using *O. ovariae* sequence from GenBank and the primer design software, (Beakon Designer, PREMIER Biosoft

International, Palo Alto, CA). Real-time PCR will be performed using the primer sets with DNA extracted from the ovaries of *O. ovariae* positive fish, negative fish, and reagent blanks. Positive fish will be sampled from the UAPB station and confirmed by histology and H&E staining. Negative fish will be of other fish species that do not sustain infection by the parasite. The PCR reactions will be run in triplicate without the TaqMan probe but including cyber green, a fluorescent dye that fluoresces only when bound to double stranded DNA. The PCR reactions will be run in triplicate using a range of annealing temperatures. A single primer pair will be chosen based on sensitivity (detection of target DNA in serial dilutions and primer efficiency), specificity (no product with negative controls), and production of a single product (determined by melting point). Once the best primer set is identified, it will be tested in real-time quantitative PCR using the matching fluorescent TaqMan probe.

*Validation of the TaqMan PCR assay.*

Specificity - A panel of 10 *O. ovariae* positive fish and 10 negative fish will be tested by the assay. The assay will also be tested using the closely related fish microsporeans, *Pleistophora mirandellae*, *Kudoa* and *Heterosporis* that will be obtained from Oregon State University, Bemidji State University, the University of Wisconsin, and Dr. Frank Nilsen of Norway. The assay will be judged specific if it produces the appropriate product only with *O. ovariae* positive fish.

Sensitivity - To test for sensitivity, the PCR product will be cloned into *E. coli* cells (TOPO TA cloning kit for sequencing, Invitrogen, Carlsbad CA). The purified plasmid will be serially diluted for use as the template in TaqMan PCR reactions.

Sensitivity will be defined as the minimum copy number that produces a threshold cycle significantly lower than that of the negative controls. We will use the threshold cycle data to perform an ANOVA statistical test to compare the differences. This experiment will be performed once as above, then repeated using the plasmid diluted in golden shiner DNA at 250ng/ul.

To make sure that our DNA extraction method is optimal for *O. ovariae* spores, we will homogenize the ovaries or ovary tissue from 3 known positive fish, then extract half of each homogenate using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and half using TRIzol reagent (Invitrogen, Carlsbad, CA)). The threshold cycles for the two types of preparation will be compared to determine the optimal DNA purification method.

Quantitative/Accuracy - To determine the range of the Taqman assay we will use the cloned and purified PCR product. The concentration of the plasmid preparation will be established by spectrophotometry then serial dilutions from  $10^8$  down to one copy per reaction will be tested by PCR. The PCR machine will produce a standard curve and display the efficiency of the reaction. The assay will be judged quantitative and accurate if it produces a high primer efficiency (between 95 and 105%) and the correlation coefficient of the line describing the relationship between copy number and threshold cycle is greater than 0.9.

Precision - A group of DNA samples extracted from ten positive and two negative fish will be tested on three different days using the quantitative assay. The standard error for all of the assays will be determined and the assay judged precise if the standard error is less than 15%.

## VERTICAL TRANSMISSION

On a total of nine occasions during the early, middle, and late parts of the spawning season, infected populations of golden shiner brood fish will be identified by collecting 25 female and 25 male brood fish and performing histology and PCR (as previously described) to verify *O. ovariae* infection. Two thousand five hundred fertilized eggs resulting from the spawning of each group of broodfish will be collected immediately, then an additional 1000 will be collected after the eggs are disinfected with formalin by the farmer (Figure 1). The eggs will be removed from the spawning substrate using two different methods, sulfite and spawning directly from the mat. The 1500 non-disinfected eggs removed by sulfite will be used for the control, bleach and RNase AWAY (Molecular Bioproducts, San Diego, CA) treatments described below. Five hundred of the non-disinfected eggs will be spawned from the mat to compare fry hatched from sulfite removal. The eggs removed with sulfite, after disinfection, will be used as controls to compare standard farm procedures with our results. In addition, eggs will be scraped after disinfection to compare removal strategies. A subset of 100 eggs from each group will be saved for *O. ovariae* PCR. The remaining eggs will be hatched in the laboratory and the fry tested for *O. ovariae* by PCR, with the exception of 1000 eggs that will be reserved for the study described below.

To verify whether or not the *O. ovariae* spores reside inside or outside of the egg, six sub samples of 100 eggs each from the non-disinfected sulfite group will be 'cleaned' to eliminate genetic material on the outside of the egg. The DNA from *O. ovariae* associated with the exterior of the eggs will be removed either by ten minute bath

treatments in 1%, 5%, and 10% dilutions of commercial bleach or in similar concentrations of RNase AWAY. The DNA then extracted from treated and untreated eggs will be used as the template for TaqMan *O. ovariae* PCR. To control for possible accidental destruction of DNA within the eggs, additional PCR reactions will be run using a set of primers for the golden shiner glucokinase gene (Gilad et al. 2004). If the glucokinase PCR is negative, it will show that the DNA template from treated eggs is no longer suitable for PCR.

I will consider *O. ovariae* to be vertically transmitted if we find evidence that the parasite exists on fry hatched from eggs of infected broodfish. If vertical transmission occurs, it will be considered to be on the outside of the egg (transovum) if we detect *O. ovariae* before disinfection but not after the eggs have been cleaned. It will be concluded to be inside the eggs (transovarial) if after all disinfection process DNA from eggs with intact template DNA still test positive.

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Figure 1.

