# 3.2.3 Ovipleistophoriasis: A Microsporidian Disease of the Golden Shiner Ovary

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## A. Name of Disease or Etiological Agent

*Ovipleistophora ovariae*, initially *Plistophora ovariae* Summerfelt, 1964, is the etiological agent for ovipleistophoriasis (microsporidiosis) of the golden shiner *Notemigonus crysoleucas*. The genus *Plistophora* in the original species description was claimed a junior synonym to *Pleistophora* (Sprague 1971). Based on ultrastructure and subunit ribosomal DNA, Pekkarinen et al. (2002) established the genus *Ovipleistophora* for *Pleistophora mirandellae*-like microsporidia, and included *P. ovariae* in the new genus. *Ovipleistophora* is one of 14 genera and *O. ovariae* one of about 156 recorded species of microsporidians of fishes (Lom and Nilsen 2003).

*Ovipleistophora ovariae* is a microscopic, obligate intracellular (cytozoic), but non-xenoma-forming microsporidian parasite of the developing oocytes of its host. Spores have not been found in the testes of broodfish (Summerfelt 1964; Summerfelt and Warner 1970a; Tucker 1967; Phelps and Goodwin 2008). Although mechanical vectors that serve as food for its fish host may be involved in spore transmission, its entire life cycle, both an asexual multiplicative stage (merogony) and spore formation (sporogony) are completed in a single host (monogenic).

### 1. Economic Importance

The parasite is important to the extent that it reduces fecundity or sterilizes (parasitic castration) the host. Mean ovary weight from fish with infection was nearly half (55%) that of fish without an infection (Summerfelt 1964). The intensity of infection averaged 45% of 473 fish examined in histological sections of collections from 24 fish farms in 12 states (Summerfelt and Warner 1970a). The golden shiner is an intermittent spawner; at spawning, the cross sectional area of an uninfected ovary is dominated by advanced egg stages that are filled with extravesicular (secondary) yolk (Fig. 1). In comparison, both ovaries of a fish with intense infection are filled with a dense stroma containing masses of spores, atretic ova, phagocytes and other cellular debris (Figure 2).

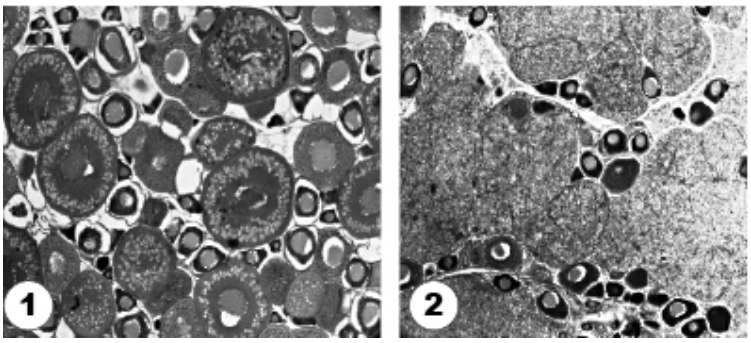



**Figure 1-2.** Histological sections of golden shiner ovary: **1**, normal ovary of spawning golden shiner, which has a full range of oocyte stages including previtellogenic oocytes, ova with peripheral intravesicular (clear vacuoles), and mature ova that have a thickened, bilaminar follicular epithelium and a mass of coalesced extravesicular (darker, solid stain), secondary yolk in perinuclear location; **2**, section through ovary of golden shiner with heavy *O. ovariae* infection. This ovary had no vitellogenic ova and the bulk of the ovarian volume was filled with dense stroma composed of masses of spores, cellular debris from atretic ova, and phagocytic cells. Early previtellogenic oocytes are limited to the margins of the ovigerous lamellae.

In 2005, fish farmers reported production of 560 million golden shiners, with a farm gate value of $17.1 million, or 45% of the total value for baitfish culture in the United States in 2005 (USDA 2006). Unlike aquaculture of food fish, where the market size is 0.5 kg or more, baitfish culture requires production of large numbers of small fish (circa 2 g). In the past, *O. ovariae* was considered a potential threat to golden shiner culture (Meyer 1967) because the consequence of atresia or infertility of infected eggs would force use of more broodstock and a greater number mats in the egg transfer method of culture that was the practice of the majority of producers (Stone et al. 1997). Producers unaware of the infection reported that older fish have poor reproduction (Stone et al. 1997). Because the incidence of *O. ovariae* increases with age of the fish — 30, 40, and 65% in age classes 0, 1, and 2, respectively (Summerfelt and Warner 1970b) — it was recommended that producers use yearling shiners for brood fish (Dorman 1993). Farmers using yearling broodfish no longer regard *O. ovariae* as important (A. Goodwin, personal communication). Furthermore, because golden shiners are so fecund, a reduction in fecundity is not really a constraint, especially with the use of yearling broodfish (N. Stone, University of Arkansas Fisheries/Aquaculture Center, personal communication).

Infection causes ovum atresia in the ovary, and field observations indicate a high percentage of dead eggs on spawning mats from heavily infected fish (Summerfelt and Warner 1970a). There is no experimental evidence that *O. ovariae* causes mortality of the fish. Histologically, host reaction is minimal until oocyte atresia takes place when an influx of macrophages occurs, eventually digesting the spores and other cellular debris of the atretic oocytes. Lack of host response may be of the parasite's influence, as some microsporidia are able to suppress the host's inflammatory response (Dyková and Lom 1980); however, this could compromise the ability of young fish to ward off other infectious agents. Within typical culture conditions, the survival of golden shiners from fry to yearlings is only 25-35%, an extremely high rate of loss compared to other cultured species (Stone and Rowan 1993). Opinion on their impact on survival has changed, the problem is now regarded to be the result of bird predation and poor over-winter survival of small fish that are deliberately stunted by reduced feeding to keep them small for spring bait sales (N. Stone, University of Arkansas at Pine Bluff, personal communication).

**2. Classification**.—Microsporidia have a diverse systematic history. Once grouped with all spore-

forming protozoans, they were placed in the subphylum Cnidospora with the Myxosporidia (Honiberg 1964). Kudo (1966) classified them as Order Microsporia in the Class Cnidosporidia. The polar filament of microsporidia is totally different in structure and function from that of the myxosporidia, and because of this, the microsporidia and related taxa were upgraded to the phylum Microspora (Sprague 1977, 1992), Class Microsporidea (Hoffman 1999), Order Microsporidia (Lom and Dyková 1992).

Although microsporidia—also used as the common name of the group—lack most cytoplasmic organelles of eukaryotic cells (mitochondria and Golgi) and their ribosomes are of a prokaryotic nature (lacking 5.8S ribosomal RNA), they do show a distinct nuclear membrane and division with an intranuclear mitotic spindle characteristic of eukaryotic cells. They have undergone severe selective reduction of their biology from loss of mitochondria to gene structure due to their specialized intracellular parasitic adaptation (Keeling et al. 2000; Keeling and Fast 2002). Recent studies in molecular phylogeny, as well as details of spore structure, provide evidence for considering microsporidia as a highly specialized monophyletic taxon more closely related to fungi than to other protists (Vávra and Larsson 1999).

Several classifications of the Phylum Microspora have been proposed, they were originally based on spore morphology, sporogonial stages, number of spores produced per sporont, arrangement of nuclei, nuclear cycle, life cycle and type of host reaction, but now include input from molecular biology. A complete taxonomic hierarchy has not been described but partial lists have been reported (Canning 1990, Sprague 1992), and (Lom and Nilsen 2003).

Phylum Microspora Sprague 1977 (aka Microsporidia - Sprague and Becnel 1999; Shaw and Kent 1999).
Class Microsporea (aka Microsporidea by Hoffman 1999).
Order Microsporida (aka Microsporidia, Lom and Dyková 1992).
Suborder Pansporoblastina (spores are produced within sporophorous vesicles).
Group 1
Family Pleistophoridae
*Ovipleistophora ovariae*

There are approximately 144 to 150 described genera of microsporidia, and 1,200 to 1,300 individual species (Sprague et al. 1992; Sprague and Becnel 1999; Desportes-Livage 2000). The number of genera and species of microsporidia described from fishes has grown from 14 genera and 100 species enumerated by Shaw and Kent (1999) to 14 genera and 156 recorded species by Lom and Nilsen (2003).

## B. Geographical Range and Host Species of the Disease

### 1. Geographical Range

The geographical range of *O. ovariae* is related to its known host, foremost is the golden shiner, which has a large natural geographic range (39 states) and a nonindiginous extension to 7 additional states (Fuller et al. 1999). The widespread distribution of golden shiners is related to its popularity as a bait fish and bait bucket introductions, but it has been stocked and used in hatcheries by some fishery agencies as a forage fish (Fuller et al. 1999). Summerfelt and Warner (1970b) documented *O. ovariae* from 45 of 49 fish farms in 12 states: Alabama, Arkansas, California, Kansas, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, Tennessee and Texas (Summerfelt

and Warner 1970b). Tucker (1967) observed *O. ovariae* infection in golden shiners from 8 of 9 Arkansas fish farms in five counties in Arkansas. Given the nearly ubiquitous occurrence of *O. ovariae* in cultured golden shiners, it is reasonable to assume that its geographical range in North America will include nearly all fish farms producing golden shiners, which in 2005, totaled 76 farms in 22 states USDA 2006), but the geographical range should include the untold number of baitfish vendors of golden shiners. The only report of *O. ovariae* in a feral population of golden shiners was from an Oklahoma creek, and a pond in the same watershed (Summerfelt and Warner 1970a). Ruehl-Fehlert et al. (2005) observed *O. ovariae* in a laboratory population in Wuppertal, Germany.

### 2. Host Species

Five species of microsporidia have been described in ovaries of fishes (Canning and Lom 1986), but only *O. ovariae*, has been reported in oocytes of a North American fish. The type host for *O. ovariae* is the golden shiner, and fathead minnows are an incidental host. Nagel and Hoffman (1977) reported *O. ovariae* in one fathead minnow from an Arkansas source. Ruehl-Fehlert et al. (2005) suggested *O. ovariae* as the microsporidian in an infestation in the ovary of adult fathead minnows held under laboratory conditions for ecotoxicological studies in Wuppertal, Germany. These fish were obtained from a commercial source in the United States.

Although a few species of microsporidia of fish (e.g., *Glugea stephani*) show broad host specificity, the majority of species are host specific (Shaw and Kent 1999; Nilsen 2000). Nagel and Summerfelt (1977a) did not find *O. ovariae* in goldfish *Carassius auratus* reared in the same hatchery ponds used for golden shiners, and their efforts to experimentally transmit the parasite by intraperitoneal or intramuscular injections of spores from infected shiners to goldfish were unsuccessful, although this technique has been used to infect goldfish and other cyprinid fish with *Pleistophora hypessobryconis*.

A frequently expressed concern is whether a predatory fish would acquire an infection after consuming an infected golden shiner. There is no evidence to support that concern for any species of microsporidia, in fact, *Pleistophora tahoenis*, which parasitizes intramuscular connective tissue of the Piute sculpin *Cottus beldingi* (Summerfelt and Ebert 1969), has not been described as a parasite of lake trout *Salvelinus namaycush* in Lake Tahoe, Nevada-California even though the sculpin is their major prey (Canning and Lom 1986; Hoffman 1999).

## C. Epizootiology and Life cycle

### 1. Transmission

Transmission of *O. ovariae* (Figure 3) is both horizontal (per oral) and vertical (transovarian; Summerfelt 1972, 1974; Phelps and Goodwin 2008). Intrahost transmission, which is from one oocyte to another, increases the intensity of infection within the same host (Summerfelt and Warner 1974a). The life cycle includes a sequence of a proliferative process called merogony that is followed by spore formation (sporogony).

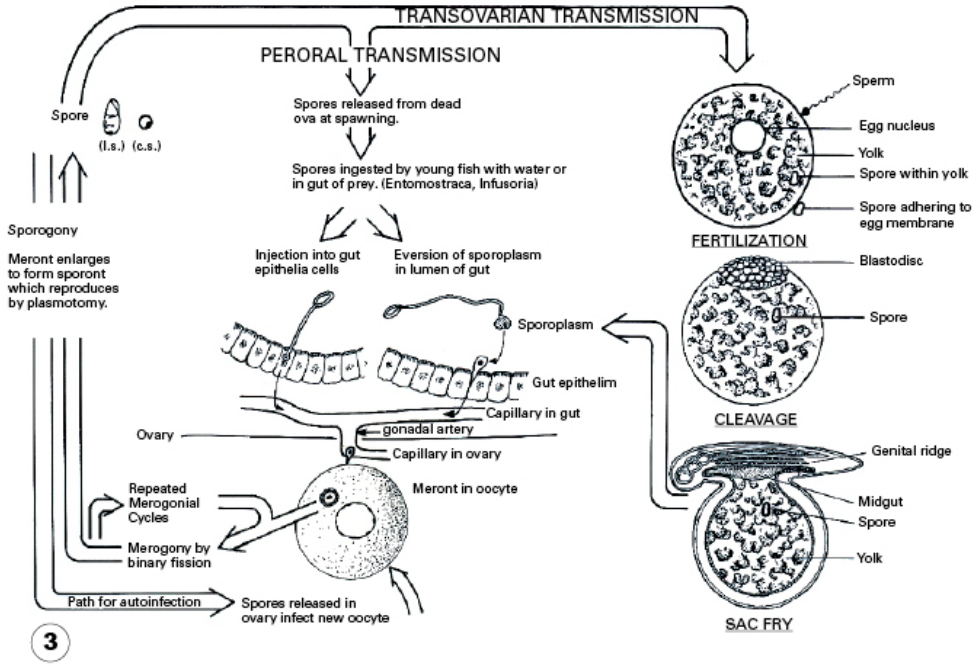



**Figure 3.** Life cycle of *O. ovariae* showing both horizontal (peroral) and vertical (transovarian) transmission, two options for vertical transmission, and a hypothetical pathway for autoinfection (see text for details).

Horizontal transmission begins when a fish ingests viable spores that were discharged from spawning of infected fish on mats used in the egg transfer method of culture (Summerfelt 1974) and as well as on vegetation in the "wild" or free spawning method when eggs are allowed to hatch in the same pond (Stone et al. 1997). Although young fish may be exposed to large numbers of waterborne spores in pond culture, given that freshwater fish are hyperosmotic regulators, that is, they do not swallow much water (Eddy 2006), per oral transmission may not occur by fish swallowing water contaminated with spores.

Organisms that serve as food (e.g., zooplankton or infusoria) for the host fish may serve as mechanical vectors; e.g., spores consumed by cladocera or copepod nauplii would end up in the fish's gut after the organism is digested. Some investigators suspect that an invertebrate intermediate host is needed for the fish-infecting microsporidia, but only a few studies have been published that demonstrate this mode of transmission (Lom and Nilsen 2003). *Amblyospora* sp., a parasite of *Culex annulirostris* mosquitoes, is one such example of the use of an intermediate host; it requires a copepod to complete the life cycle (Sweeney et al., 1985; Andreadis 1985). Further research is needed to determine whether a true intermediate host is involved in survival and transmission of *O. ovariae*, but direct per oral transmission of *O. ovariae* without the need for an intermediate host was experimentally demonstrated by feeding spore suspensions adsorbed to the feed to one-month-old fingerlings every other day for four weeks (Summerfelt 1974).

After an ingested spore reaches the gut of a golden shiner, the spore is activated to extrude the polar filament, which becomes a hollow tube through which sporoplasm is injected into a gut epithelia cell. Larsson (1986) states that ultrastructual studies of microsporidia show direct penetration and injection of the sporoplasm into the gut epithelium cell, "refuting the theory that sporoplasms are release in the gut lumen." Putz (1971) achieved experimental transmission of *O. ovariae* by

intraperitoneal injection of both sonicated and non-sonicated spores, but sonicated and filtered preparations were noninfective. Such *in vivo* transmission of spores in the body cavity implies polar filament extrusion, sporoplasm emergence and transport to the ovary. Lom (1969) used intramuscular transplantation to achieve transmission of the microsporidian, *Pleistophora hyphessobryconis*.

Although "little is known about the transport" of microsporidia from the gut epithelium to specific host cells (Canning and Lom 1986), it is assumed that the sporoplasm of *O. ovariae* uses the circulatory pathway to reach the ovary. The first microscopic evidence of infection of *O. ovariae* in golden shiners is the presence of meront in the cytoplasm of a pre-vitellogenic oocyte. This occurs before thickening of the follicular epithelium and formation of the zona radiata of the chorion as that structure may prevent penetration of sporoplasm (Figures 4 and 5).

In spite efforts to define and distinguish the terminology, merogony and meront are used in current literature rather albeit often without rationale for distinguishing the difference.

Transovarian (or transovum) transmission—maternal parent to offspring—requires ovulation of infected ova followed by successful fertilization and development. Heavily infected ova are fragile and probably cannot be fertilized, or if they are fertilized, they are probably incapable of development. Lightly infected ova can be fertilized with embryological development. Summerfelt (1974) observed spores in histological sections of the blastula stage of fertilized eggs derived from golden shiners from two culture ponds. Phelps and Goodwin (2008) provided evidence for transovarian transmission with positive PCR for *O. ovariae* in fertilized eggs and in fry produced from infected broodfish.

Vertical transmission by spores attached to the surface of the egg was reported in *Amplyospora salinaria*, a microsporidian parasite of the mosquito *Culex salinarius* (Becnel and Andreadis 1998). Summerfelt and Warner (1970a) observed *O. ovariae* spores on live golden shiner eggs removed from spawning mats, but spores have not been observed on the chorion of dead ova or live zygotes picked off spawning mats that contained an abundance of spores (Summerfelt 1974). Thus, transovarian transmission must be accomplished by successful fertilization and development of ova that contain meronts, sporogonial plasmodia, or spores within their cytoplasm.

A latent period, in which the parasite cannot be observed in histological sections, occurs between first exposure to the sporoplasm and the first appearance of meronts in the oocytes. The presence of *O. ovariae* DNA in eggs and fry from infected broodstock is evidence for vertical transmission (Phelps and Goodwin 2008), yet, the parasite is not detectable microscopically until meronts or spores are visible. Study of the seasonal cycle of oogenesis and parasitism in golden shiners demonstrated that sporogony does not take place until vitellogenesis commences (Warner 1972) , which is stimulated by either the surge in GnRH, gonadotropin, estrogen or some combination, or in response to vitellogenesis itself, which takes place as a response to the hormonal cascade.

## 2. Merogony and Sporogony

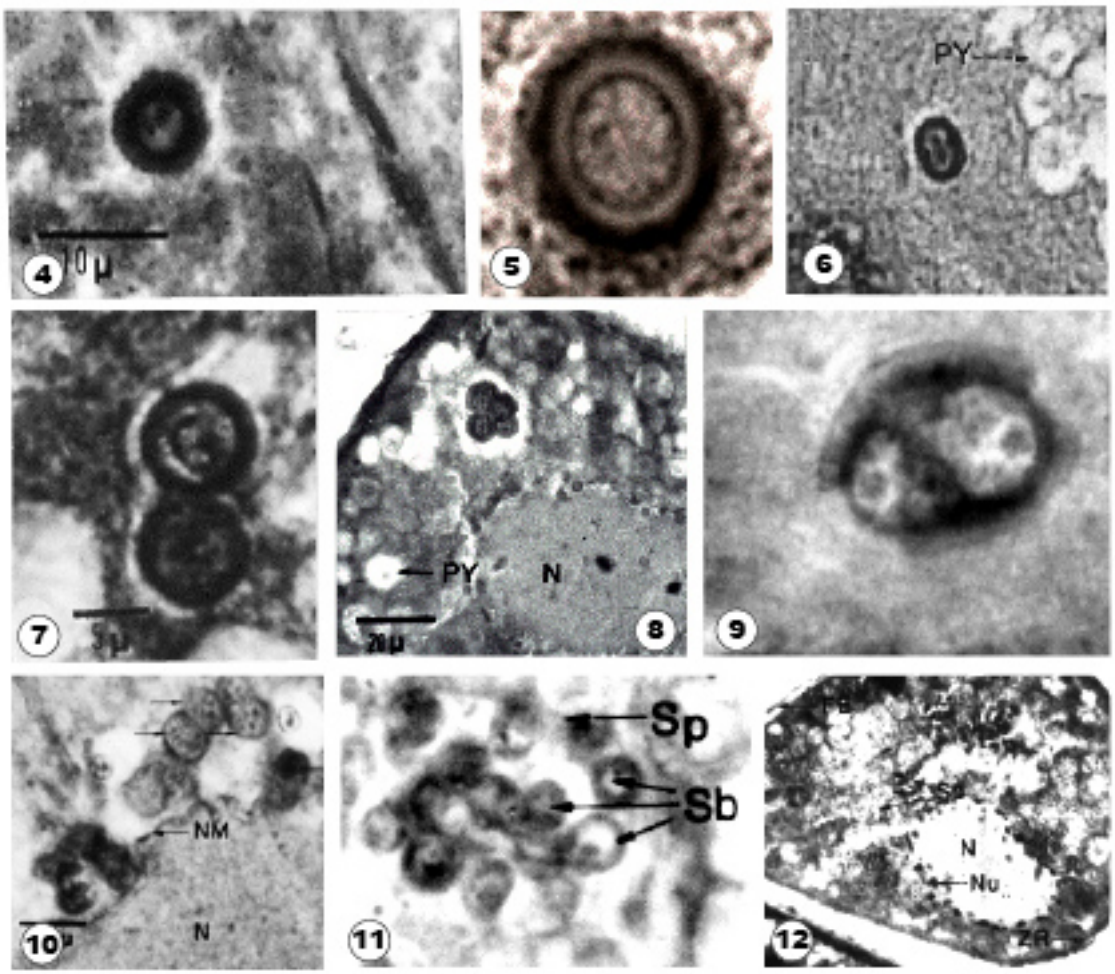
Merogony.— The proliferative phase of the life cycle of microsporidia is merogony (aka schizogony), and the daughter cells are called meronts (aka schizonts). In the original species description, Summerfelt (1964) did not discuss this phase of the life cycle, but Summerfelt and Warner (1974a and 1974b) characterized aspects of schizogony, a term used for merogony and schizont for meront. Lom and Dykova (1992:127) state, "The term merogony is used in microsporidia to describe cell replication by multiple and binary fission, although the term should be restricted to multiple fission only". Sprague and Becnel (1999) state that merogony is "an

indeterminate series of binary divisions of diplokaryotic cells (meronts)...". They also state that schizogony should be restricted to division by haplophasic individuals, which have haploid (unpaired) nuclei. A species with a diplokaryon has a pair of haploid nuclei. Sprague and Becnel (1999) state, "It is now logical and expedient to restrict this term (i.e., meronts) to diplokaryotic cells." Although *O. ovariae* is monokaryotic, it is not known whether they are haplophasic. Yet, contrary to logic of these definitions, nowadays, distinguished authorities commonly use merogony to describe the proliferative stage of all microsporidians and schizogony is no longer used (Cali and Takvorian 1999; Sprague and Becnel 1999; Lom and Nilsen 2003). Vávra and Larsson (1999), however, describe schizogony as a process where spores are formed by budding of the multinucleate sporogonial plasmodium by a three-stage process.

The first recognizable stage of *O. ovariae* in histological sections is a uninucleate meront (Figures 4 and 5). Merogony in *O. ovariae* begins with binary fission often with a conspicuous mitotic spindle (Figure 6). The meront (circa 7-8 mμ) is enclosed by a thick (1.5 μm), darkly stained, amorphous envelope (Figure 5) that may provide the parasite with an expanded surface for absorption (Canning et al. 1986). The merogonial envelope, which is in direct contact with host cell cytoplasm, may be the product of the meront plasmalemma or induced by the oocyte (Lom and Nielsen 2003). The only observable cytoplasmic reaction to the infection is a halo, or clear area around a cluster of meronts (Figure 7). Merogony consists of an indeterminate number of cycles of binary fission (Figures 6, 7 and 8).

The proliferative phase is followed by a sporulation phase termed sporogony. Sporogony begins when a meront undergoes a series of nuclear divisions (i.e., karyogomy) without cytokinesis. Several multinucleate plasmodia are released into the host cell cytoplasm from rupture of the merogonial envelope (Figure 9). In the cytoplasm, the plasmodia are identified as small, rounded or somewhat elliptical cells (i.e., parasitic cells within the ova) with numerous punctate nuclei (Figure 10). The plasmodium produces divides by fragmentation (or segmented) into sporoblasts that mature into spores (Figure 11). The egg cytoplasm is completely replaced with sporophorous vesicles filled with spores (Figure 11). Atretic oocytes are filled with spores, cellular debris, fibrin and macrophages that have phagocytized and lysed the spores (Leida 1978).

The high intensity of ovarian infection typical of *O. ovariae*, forces one to wonder whether each infected oocyte results from a sporoplasm derived from an individual spore, or whether the sporoplasm undergoes a multiplicative stage during the latent period that allows it to invade many oocytes. The intensity of infection of an individual oocyte undoubtedly results from the successive merogonic divisions and the numerous sporogonic plasmodia.

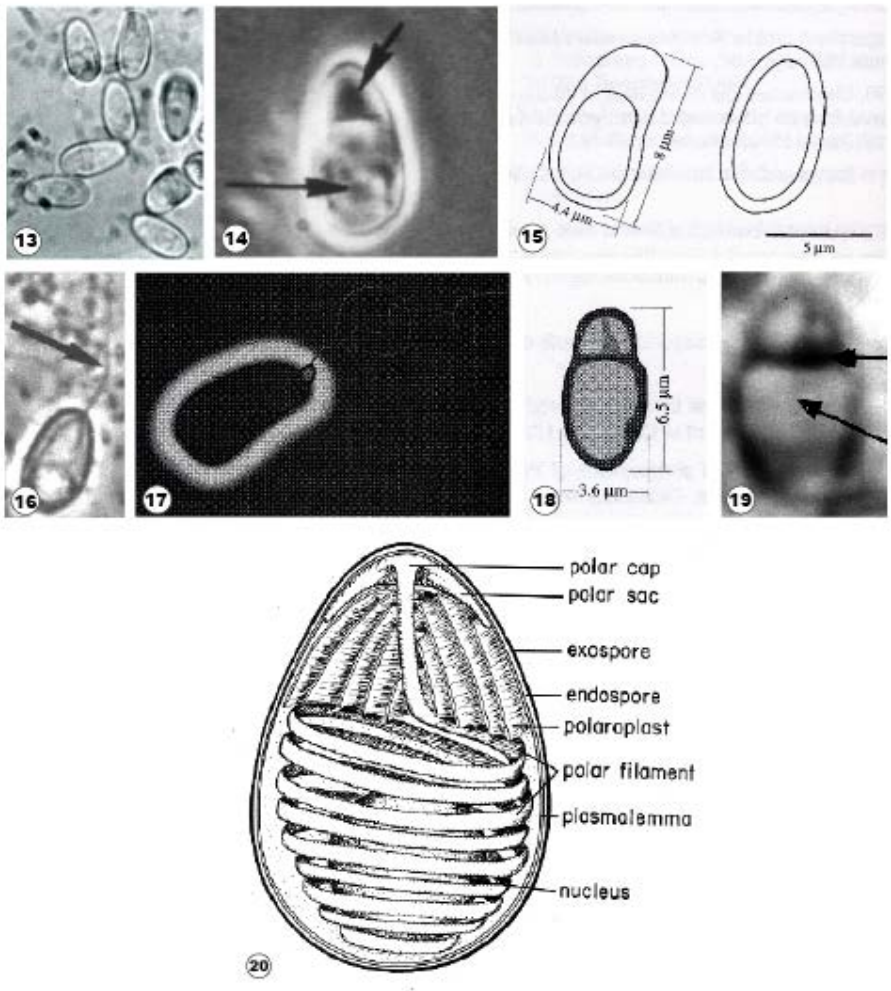



**Figures 4-8.** Merogony of *Ovipleistophora ovariae*: **4**, a uninucleate meront in cytoplasm; **5**, higher magnification shows darker outer and lighter inner rings; **6** shows a meront in telophase stage of mitosis and onset of cytokinesis; **7**, two daughter meronts; **8**, a cluster of meronts imbedded in the cytoplasm of an ovum with primary yolk (PY), N is the host nucleus, and NM the nuclear membrane.

**Figures 9-12.** Sporogony of *Ovipleistophora ovariae*: **9**, the merogonial envelope breaks open; **10**, the sporogonial plasmodium has a thin sporophilous membrane (arrows); **11**, the plasmodium becomes transformed into a sporophorous vesicle (Sp) that segments into a family of 8-16, 12 sporoblasts (Sb), that mature into spores; **12**, heavily infected ova contain numerous sporophilous vesicles and free spores.

### 3. Morphology of the spore

The spore is the most obvious indicator of infection and spore characteristics have been paramount to species identification. Observed with light microscopy, the dominant spore features are the anterior and posterior vacuoles (Figure 13).




**Figures 13-20.** Spore characteristics of *O. ovariae:* ***13***, cluster of fresh spores in wet mount of a squash preparation as seen in brightfield microscopy showing an abundance of ovoid- or ellipsoidal-shaped spores; **14,** refractile nature of spore in dark-field, showing thick exospore and typical halo, anterior polaroplast (upper arrow) and a large posterior vacuole (clear area) that is about 60% of spore length; **15**, schematic with dimensions of fresh spores (contrast with fixed and stained spores in **18**); **16,** fresh spore after extrusion of the polar filament (110-160 µm long), the sporoplasm (infective unit) is injected into a gut cell of the host by passing through the length of the polar tube; **17**, negative stain of spore with extruded polar filament; **18,** dimensions of fixed and stained spores (H&E); **19**, fixed and stained (H&E) spore showing girdle with nucleus (upper arrow) and posterior vacuole (lower arrow); **20**, schematic representation of spore morphology based on electron microscopic studies (Nagel 1978, with permission of author).

The spore of *O. ovariae* has an individual nucleus (monokaryon) and is of one size, therefore it is classed as monomorphic, in contrast to the dimorphic spores of *O. mirandellae*, which produces a bimodal frequency distribution of spore measurements (Pekkarinen et al. 2002). Fixed and stained spores are smaller than fresh spores (Parker and Warner 1970; Summerfelt and Warner 1970a). In fixed and stained slides of the spores, the nucleus appears as a dark band between the anterior and

posterior vacuoles (Figure 18-19). The spore has a rigid spore coat (shell), with an outer protein layer (exospore) and inner chitin layer (endospore), the latter a characteristic of fungi.

Electron microscopy discloses the complex extrusion apparatus that allows the parasite to inject the sporoplasm (cell cytoplasm and nucleus) into the epithelia cell of the host gut (Figure 20). The extrusion apparatus consists of an anchoring apparatus (polar cap and polar sac), a laminar structure (the polaroplast) in the anterior vacuole, occupying about one third of the spore length, and the polar filament. Most of the length of the polar filament is coiled around the walls of the lower half of the spore (Figure 20). The extruded polar filament becomes a hollow tube (polar tube) through which the sporoplasm passes. Although the chitinous spore shell is impermeable, the polar cap is sensitive to digestion in the host's gut, allowing intake of water by the polaroplast, which causes an explosive extrusion of the hollow polar filament. The sporoplasm, with the nucleus, is injected through the polar filament? into the host cell.

Development of *O. ovariae* is synchronized with the reproductive cycle of the host, an adaptation that connects peak spore formation to ovulation. Chen and Power (1972) noted a similar host-parasite relationship of *Glugea hertwigi* in American smelt *Osmerus mordax* where highest parasite load corresponded with the onset of gonadal maturation in both male and female fish. Golden shiners are fractional spawners, females may spawn several times during the spawning season. Consequentially, multiple egg stages are commonplace, ranging from tiny oogonia to mature oocytes filled with secondary yolk (Figure 1). Single meronts are earliest stage of the parasite seen in histological sections of pre-vitellogenic oocytes of (Figure 4). Because meront and merogony is uncommonly observed, it is assumed that this proliferative stage is short, and that the parasite rapidly moves on to sporogony (Summerfelt and Warner 1970a).

## D. Disease Signs.

### 1. Behavioral Changes Associated with the Disease.

Behavioral changes of infected fish have not described.

### 2. External Gross Signs

No external gross signs of infection have been described.

### 3. Internal Gross Signs

When a mature, uninfected ovary from a freshly sacrificed Golden Shiner is viewed with the unaided eye, it appears as a uniformly colored translucent mass. Ovaries of fish infected with *O. ovariae* have a conspicuously white marbling or whitish opaque streaks (Figure 21), which are areas of amorphous masses of atretic oocytes. *O. ovariae* infects only the developing ova (oocytes). The parasite is a non-xenoma-forming microsporidian; i.e., the infected oocytes are not hypertrophied into tumor like swellings.

**Figure 21.** Cutaway of body cavity to reveal mottled appearance of infected ovary due to heavy infection of ova with *O. ovariae* (Photograph by N. Phelps).

Procedures for staining slides with ovary smears are described by Kudo (1966) and Canning and Lom (1986). Kudo (1966) describes the Heidenhain's iron haematoxylin and Feulgen's nuclear reaction for nuclear staining. Canning and Lom (1986) noted that microsporidian spores are unique among other protozoan spores in that they are Gram-positive. Histological sections of the ovary may be stained with H&E.

## E. Disease Diagnostic Procedures.

### 1. Presumptive Diagnosis

An experienced diagnostician may detect an infection from observation of conspicuous white marbling through fresh ovary (Figure 13), but presumptive diagnosis is determined from observation of spores in a wet mount from fresh chilled, frozen, or preserved ovaries. Presence of spores in a wet mount of macerated fresh ovary tissue is indicative of a patent infection. Summerfelt and Warner (1970a) detected an infection in 31% of 472 fish by observations of wet mounts, and in 46% of these same fish by examination of histological sections from paired ovary samples, a statistically significant difference.

Failure to find spores in the ovaries of young-of-year (YOY) or mature fish collected in late fall or winter spring is not proof that the female is uninfected. Meronts, the first phase of the life cycle, are only seen in histological sections of oocytes with primary yolk. A real-time polymerase chain reaction (qPCR) assay has been developed that can detect *O. ovariae* in eggs, fry or subadults before spores are formed (Phelps and Goodwin 2007). The PCR assay detected nuclear material of *O. ovariae* in male fish in which no spores were observed by histology.

A wet mount preparation begins by macerating an ovary in a mortar with 1 or 2 ml saline (0.85%), but it is usually sufficient to thoroughly dice a piece of ovary with a razor blade on a glass slide with a drop of water. A sample of fluid from the macerated ovary should be examined as a wet mount under a coverslip with brightfield light microscopy at 950X magnification (Figure 2A) or with dark phase microscopy. Spores in wet mounts are refractile when examined by dark phase microscopy (Figures 10-13).

Spore measurement should be done using an ocular micrometer that is calibrated against a stage micrometer. Summerfelt (1964) reported 8.4 µm long by 4.2 µm wide for fresh spores, but less for fixed and stained spores. The dimensions were essentially the same in Schaudinn-fixed spores (6.4 by 3.4 µm), and Carnoy-fixed spores (6.6 by 3.3 µm). Parker and Warner (1970) reported a mean

length and width of 8.3 by 4.4 µm, respectively, for fresh spores, and compared the effects of fixation (7 fixatives), dehydration, and staining (5 stains). All seven fixatives produced shrinkage in spore length and width, 10% formalin caused the least shrinkage, Schaudinn's, and ethyl alcohol caused the most reduction in length and width. Further reduction in both length and width was caused by dehydration, but the effects of staining were negligible. Due to shrinkage caused by fixation and staining, spores are best observed in a monolayer from fresh specimens (Canning and Lom 1986). Spore measurement is not required for examination of golden shiners, however, if a host species other than golden shiner is examined, spore measurements may be useful to differentiate if different species of *Ovipleistophora* or *Pleistophora* are suspected.

In fresh material, the refractile portion of the *O. ovariae* spore is about 40% of the total spore length, followed by a less refractile or clear area occupying the rest of the spore. The coiled polar tube is visible in the clear area. The polar tube may be extruded (Figure 16), by applying mechanical pressure to the coverslip, which is done by applying pressure to the top of the coverslip of wet mount (R. R. Kudo, pers. com.) For microsporidia of insects, high pH conditions in the presence of alkali metal ions is effective for polar tube extrusion. Canning and Lom (1986) state that 5 to 10% hydrogen peroxide an be used in difficult cases but may result in incomplete extrusion of the tube."

There are several methods to concentrate the spores. One is to homogenize the ovary such as with a stomacher, and filter through muslin to remove larger debris. This step is followed by repeated centrifugation, resuspension, and filtration steps (Canning and Lom 1986). The ether separation method (Landolt 1973), without enzyme digestion, was recommended by Leida (1978) for preparation of a relatively pure suspension of *O. ovariae* spores. The triangulation technique (Cole 1970), or a sucrose density gradient technique is also effective for concentration of spores.

Stained smears may be prepared from the suspension used for the wet mount. General procedures for the preparation and staining of protozoan are given by Kudo (1966). Canning and Lom (1986) describe staining methods specifically for microsporidia. Leida (1978) reported that spores stained well with toluidine blue- the polar cap stained a dark blue-purple, while the polar filament stained a less intensive blue. Results with PAS were variable, as the polar cap and polar filament stained faintly. Haematoxylin-eosin stained spores have a dark blue or purple girdle-like ring, sometimes with a dark nuclear spot (as in Figure 12), while preparations stained with Giemsa resulted in the polar cap intensely blue and the nucleus less intensely purple.

**2. Confirmatory Diagnosis**

Histological sections are not needed when spores are found in wet mounts, however, histological confirmation is required to establish "no infection" in ovaries of prespawning fish to ascertain presence of life stages (meronts and sporoblasts) other than spores. Ovary samples from late summer through early spring, should be fixed in 10% neutral (or buffered) formalin or Bouin's fixative for histological examination. As previously stated, wet mounts or stained smears are not as reliable as histological sections to detect the parasite (Summerfelt and Warner 1970a).

Paraffin sections should be prepared following standard histological procedures (Luna 1968). Ovary may be sectioned longitudinally or transversely at 6 to 10 µm, with 3 to 5 sections per slide. Stain one slide with metachromatic blue stain containing 0.1% toluidine blue (Summerfelt and Warner 1970a), another with Mallory's aniline blue-collagen stain (Biological Staining Commission 1960:52-53), and a third with haematoxylin-eosin (H&E). The metachromatic blue stain is useful for a quick overview of a section to identify spores. Spores stain light to dark blue, with a dark band in the middle and dark blue at the end. This stain is poor for distinguishing cytological details of the

developing oocytes. Mallory's stain is highly differential for primary and secondary yolk, connective tissue, nucleoli, and the follicle. It is also excellent for differentiating meronts, sporoblasts and spores from the cytoplasm and yolk of the oocytes. The intensity of infection can be estimated from the percentage of the cross-sectional area of the ovarian sections occupied by infected oocytes or atretic eggs.

Staging of oocyte maturation is based on a numerical scale of 1 to 7, where stage 7 is the mature ovum. The first developmental stage that can be detected in histology sections are the meronts, which are first evident in stage 2-4 oocytes (Summerfelt and Warner 1970a). Spores are most abundant in stage 6 and 7 oocytes as the fish are ready to spawn (i.e., May or approximately 25°C water temperature).

Confirmation can also be made using quantitative PCR (Phelps and Goodwin 2007).

## F. Procedures for Detecting Subclinical Infections.

When examining a juvenile fish in the fall or winter, when the spores are uncommon, the only evidence of infection will be the presence of meronts in histological sections. Meronts can be seen in the cytoplasm of the stage 2-4 oocytes. As the season progresses and late-stage oocytes develop, sporoblasts will develop within sporophorous vesicles. At the present time, no serological tests have been developed to detect asymptomatic infections i.e., the premeront latent stage. A quantitative PCR assay can be used to detect subclinical infections for O. ovariae (GenBank AJ252955; Phelps and Goodwin 2007).

## G. Procedures for Determining Prior Exposure to the Etiological Agent.

Currently no immunological test is available to determine prior exposure to *O. ovariae*.

## H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent.

Viability and survival is not needed to determine presence of spores from the ovary of golden shiners. Samples may consist of living or moribund fish, which may be frozen or preserved in Bouin's solution or 10%, buffered formalin.

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