THE SEASONAL CYCLE OF OOGENESIS AND PARASITISM IN GOLDEN SHINERS, *NOTEMIGONUS CRYSOLEUCAS* (MITCHILL), INFECTED WITH THE MICROSPORIDAN *PLEISTOPHORA OVARIAE* SUMMERFELT

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THE SEASONAL CYCLE OF OOGENESIS AND PARASITISM

IN GOLDEN SHINERS, NOTEMIGONUS CRYSOLEUCAS

(MITCHILL), INFECTED WITH THE

MICROSPORIDAN PLEISTOPHORA

OVARIAE SUMMERFELT

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CHAPTER I

INTRODUCTION

The protozoan Pleistophora ovariae Summerfelt (1964) (Microsporida, Nosematidae), undergoes schizogony and sporogony within developing oocytes of the golden shiner Notemigonus crysoleucas (Mitchill). In a survey of the incidence of the parasite in the United States in commercial fish farms (Summerfelt and Warner, 1970a, 1970b), P. ovariae was found in 46 of 49 sources in 12 states.

Schizonts, sporonts, and spores appear in a large percentage of the developing oocytes in parasitized fish. As a possible result of this infection, fish farmers report that their brood fish have not produced as many young fish (i.e., viable eggs) as in the past but they compensate for reduced fecundity by using more brood fish. Ten years ago the common stocking density for brood fish was 11.4 kg/ha water; now many producers stock brooders as densely as 45 kg/ha water to get the same number of offspring as they obtained before (Martin 1970, personal communication).[1] In order to manage around the problem with the parasite, farmers use larger numbers of younger brood stock which are less heavily infected than older brood stock. These younger fish are smaller, produce less eggs, and are less reliable in their spawning; therefore, the higher stocking densities are necessary to ensure

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[1]Mayo Martin, BSFW, Fish Farming Research Station, Stuttgart, Arkansas.

adequate number of eggs (Meyer, personal communication).[2] Presumably, higher stocking density is necessary because P. ovariae induces atresia of developing oocytes, reducing fecundity. Malone (1970) stated that this parasite has cost the minnow industry more in production than any other problem.

The literature on P. ovariae, while more abundant than for most microsporidan infections of fish, lacks information on transmission, description, seasonal variation in intraovarian life stages, and effect on fecundity of the host.

The life stages of P. ovariae in the ovary were not described by Summerfelt (1964) but are characterized in a thesis by Wilhelm (1964). However, in the course of a study on geographic distribution of the parasite (Summerfelt and Warner, 1970a), observations on the morphology of spore development indicated that Wilhelm had misinterpreted certain aspects of schizogony. He misidentified the early sporant calling it a multinucleate schizont. His description of sporogony omitted the dyad stage.

Wilhelm (1964) attempted to correlate life stages of the parasite with developmental stages of oocytes. But, because of an inadequate description of oogenesis, small sample size, lack of quantification and mis-identification of sporont, he found no correlation between egg size and a given stage of the parasite. He stated that "the occurrence of the various developmental stages is not seasonal in nature." However, on the basis of the present study this seems to be an error.

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[2]F. P. Meyer, BSFW, Fish Farming Research Station, Stuttgart, Arkansas.

This study is presented to clarify description of the life stages and provide more detail on the interrelationship between the development of the parasite and oogenesis in the minnow. The specific objectives of this study were:

1. to describe the intraovarian stages of *Pleistophora ovariae* in the golden shiner;
2. to test the hypothesis that the occurrence and abundance of life stages of *P. ovariae* are correlated with the reproductive cycle of the host.

CHAPTER II

LITERATURE REVIEW

Pleistophora[1] ovariae (Class Cnidosporidea, Order Microsporida) is a protozoan parasite found in the ovaries of the golden shiner.

Kudo (1966) characterized species of Pleistophora as follows: each spore contains a single polar filament; spores are oval, ellipsoid, pyriform, or subcylindrical (length less than four times the width); sporont develops into variable number of sporoblasts (often more than 16) each of which becomes a spore. When P. ovariae (Summerfelt, 1964) was described it was distinguished from the 13 other species in fish hosts on the basis of a combination of features; host, site of infection, geographical location of host, habitat of host, spore size, spore morphology, and certain features of sporogony.

Twenty-five species of Microsporida in North American freshwater and euryhaline fishes were listed by Putz and McLaughlin (1970). A cosmopolitan list of 52 species of Microsporida from freshwater, euryhaline, and marine habitats was given by Summerfelt (1971).

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[1]Spelling of the genus has been altered several times (Pleistophora and Plistophora). Sprague and Vernick (1968) and Sprague (1971) reviewed this question and concluded that Pleistophora (Gurley, 1893) is the correct spelling.

Distribution of *Pleistophora ovariae*

Summerfelt (1964) found *P. ovariae* in fish from Kentucky, Missouri, Arkansas, and Illinois. Tucker (1967) found *P. ovariae* in fish in eight of nine minnow farms in five Arkansas counties. Meyer (1967) observed *P. ovariae* to be of frequent occurrence in the fish of commercial minnow producers in Arkansas and surrounding states and regarded it as a potential threat to the golden shiner industry. Summerfelt and Warner (1970b) found infected golden shiner in 46 of 49 fish farms checked in 12 states (Alabama, Arkansas, California, Kansas, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, Tennessee, and Texas) and from two "wild" sources, i.e., two farm ponds near Stillwater, Oklahoma.

During the summer months, commercial producers transport golden shiners to bait dealers throughout the United States and Canada, and exchanging of fish among minnow producers is commonplace. Together, these factors contribute to the widespread distribution of infected shiners in fish farms. However, aside from the two "wild" sources, the distribution of this parasite in naturally reproducing populations is unknown.

Site of Infection

Microsporida are obligate intracellular parasites. Most species have been described from insects (Thomson, 1960) and fish (Summerfelt, 1971). Their tissue specificity in fish hosts was recently reviewed by Summerfelt (1971). He found their tissue specificity to vary, but *Pleistophora* species were generally very tissue specific. Van Duijn (1967) showed *P. hyphessobryconis* to be a common pathogen in the neon

tetra (Hyphessobrycon innesi) and several other aquarium fishes. Lom (1969) experimentally introduced P. hyphessobryconis into goldfish (Carassius auratus) where it was said to have developed in the muscles and connective tissue. Van Duijn reported it in muscles of the head, body, and ovaries. However, no histological preparations were presented showing the parasite in muscle, connective tissue, or ova.

The most common tissue infected by species in the genus Pleistophora was muscle (Canning et al., 1964; Wilhelm, 1964; Putz et al., 1965; Summerfelt and Ebert, 1969; and Summerfelt, 1971) and gonads, primarily ovaries. P. mirandellae (Wilhelm, 1964), P. ovariae (Summerfelt, 1964), and P. sciaenae (Weiser, 1949) were reported in fish ovaries. P. longifilis occurred in the testes of Barbus flavialitis (Kudo, 1924).

Summerfelt (1964) found spores of P. ovariae in wet-mount preparations from the ovaries, liver, and kidneys. However, tissue specificity of P. ovariae was not evaluated from examination of histological sections. Spores found in the liver and kidney were probably contaminated during sample preparation (Summerfelt, personal communication). Wilhelm (1964) and Summerfelt and Warner (1970a, 1970b) found P. ovariae ovariae only in golden shiner ovaries.

Effect of Pleistophora ovariae on Host

Pleistophora ovariae has never been implicated as the cause of mortality among golden shiners, but piscine infections of other microsporidans are often fatal.

Putz et al. (1965) reported that infection by P. cepedianae killed gizzard shad, Dorosoma cepedianum, in an Ohio reservoir. Wales

and Wolf (1955) reported epizootics from Pleistophora sp., probably synonymous with P. salmonae (Putz and Hoffman, 1964 in Putz et al., 1965), caused high mortality in fingerling California steelheads Salmo gairdneri. Lethal infections of Glugea hertwigi have occurred regularly in young-of-the-year smelt, Osmerus eperlanus mordax (Delisle, 1969).

Mean body lengths of fish infected with P. ovariae, stratified by month of collection, were significantly larger than the corresponding mean lengths of uninfected fish in 19 out of 24 comparisons (Summerfelt and Warner, 1970a), and in no case was the uninfected fish significantly larger. Similarly, monthly means of body weights of infected fish were greater in 21 of 24 comparisons, none of the means of uninfected fish being significantly larger. The differences in lengths and weights were much greater in the first summer of life than in other years, especially in August and September when infected fish were 2.5 and 3.0 times larger than the uninfected fish.

The larger size of infected fish may have resulted from a reduced need for lipoproteins and phospholipids in yolk formation due to partial parasitic castration which enhanced growth rates. Atz (1957) stated that both male and female fish usually showed reduced growth with the onset of sexual maturity. He explained this as a result of greater diversion of energies into production of gametes. Summerfelt and Warner (1970b) suggested that parasitic lesions may influence production of sex steroids, or affect the estrogen-gonadotropin interrelationships. Fisher and Sanborn (1962) demonstrated that certain Nosema species induced juvenile insect hosts to produce hormone which resulted in larger

adults, shorter pupation periods, and adults that retained many pupal characters.

Microsporidan Spore Morphology

Lom and Corliss (1967) systematized the nomenclature of the basic components of the microsporidan spore; a shell, a polaroplast, a coiled polar filament, a fluid-filled vacuole at the posterior end, and a sporoplasm (germ).

Shells examined by numerous workers were all trilaminar, either smooth or rough (Dissanaike, 1957; Huger, 1960; Canning, 1962; and Sprague and Vernick, 1968) and were composed of chitin (Erickson and Blanquet, 1969).

The polaroplast is located in the anterior one-third of the spore and varies in structure with the physiological state of the spore (Lom and Vavra, 1961). Huger (1960) called it the anterior vacuole. Lom and Vavra (1963) described the polaroplast as tubular or granular and capable of enormous swelling for eversion of the polar filament. Kudo and Daniels (1963) and Lom and Corliss (1967) described the polaroplast as a laminated structure, originating possibly by transformation of endoplasmic reticulum. These authors described the sequence the polaroplast followed in causing eversion of the polar filament: it quickly imbibed water from the environment, caused considerable intrasporal pressure, which induced polar filament eversion. Sprague and Vernick (1969) believed part of the Golgi apparatus transformed directly into the polaroplast complex.

The polar cap (McMannus positive cap, polar granules, polar mass) surrounds the polar filament at its base (Huger, 1960; Kudo and Daniels,

1963) and forms the apical part of polaroplast to which the polar filament adhered (Lom and Vavra, 1963; Lom and Corliss, 1967). Sprague and Vernick (1969) said that the polar cap was derived from the Golgi apparatus.

The polar filament is an organelle for attachment or injection of the sporoplasm into the host cell. Its basal portion is joined with the polar cap at the anterior part of the spore. The polar filament extends from the polar cap obliquely and posteriorly to where it coils around the periphery of the posterior vacuole. Huger (1960) described the ultrastructure of the polar filament as solid with nine small fibrils surrounding two larger fibrils (modified flagella). However, Lom and Vavra (1961, 1963), Kudo and Daniels (1963), Petri and Schiodt (1966), Lom and Corliss (1967), Erickson et al. (1968), Ishihara (1968), Sprague and Vernick (1968), and Stanier et al. (1968) maintained that although the polar filament was filled with an electron dense material, it was, nonetheless, hollow and lacked fibrils characteristic of flagella.

Excellent descriptions of polar filament eversion were given by Lom and Vavra (1961) and Ishihara (1968). Gibbs (1953) said that violently extruded polar filaments could penetrate tissues and serve to attach spores to cells or conducted the "sporozoite" (amoebula) to a distinct part of the tissue. Ishihara (1968) demonstrated the polar filament piercing into both the wall and cytoplasm of the silk gland in *Bombyx mori*. He stated that enlarged parts of the polar filaments, which contained electron dense material, probably showed the sporoplasm being forced out of the cell. Sprague and Vernick (1968) stated, "We have all but conclusive proof that the polar filament functions as an inoculating needle."

The sporoplasm has one or two nuclei, is surrounded by a single smooth membrane, and lies within the coiled polar filament in the posterior portion of the spore (Lom and Vavra, 1961; Kudo and Daniels, 1963; Petri and Schiodt, 1966; Lom and Corliss, 1967; and Sprague and Vernick, 1968). Lom and Vavra (1963) said it was situated between the polaroplast and the fluid-filled posterior vacuole.

Sprague and Vernick (1967) described sporogonic karyokinesis. The nucleus became "dumbell" shaped, broke near the center of the isthmus, and each half persisted. They postulated that the nuclear extension (isthmus portion), because of its position and structure, was the polar filament primordium. They further postulated that the nuclear vesicle maintained a connection with the posterior end of the tubular polar filament. In a follow up study, Erickson et al. (1968) demonstrated that the everted polar filament terminated in either a cup-shaped or a saccate enlargement. They postulated that the terminal sac on the polar filament, the nuclear vesicle, and the posterior vacuole of the spore were all three the same structure.

Sprague and Vernick (1968), using light and electron microscopy, demonstrated in Glugea sp. that when the nuclear isthmus broke at karyokinesis each half remained polarized and persisted as a tubular prolongation and was, in fact, the polar filament primordium. They concluded that the sporoplasm among the coils of the polar filament was residual and of no further consequence. They believed the nuclei were located in the posterior vacuole, but not in the cytoplasm. Thus, when the polar filament was everted, the extruded content would be primarily DNA and nucleoplasm. For this reason they preferred the use of "germ" rather than sporoplasm to denote the infective body.

Most of the typical cell organelles are found in Microsporida, with the exception of mitochondria. Sprague and Vernick (1968) suggested that the host cell mitochondria also served the microsporidan component. They believed that when the germ left the spore to enter a new host it was a simple subcellular structure consisting mostly of DNA and a likely kinetic element. They suggested apparent structural and physiological analogies with certain viruses (phages) in which the genome passed into the host cell to live and reproduce with absolute dependence on the host cell.

The Golgi complex transforms directly into several spore organelles: the polaroplast, certain membraneous investments of the polar filament, the terminal sac on the polar filament, and perhaps part of the posterior vacuolar system (Sprague and Vernick, 1969).

Summerfelt (1964) described the spores of P. ovariae as ovoid or ellipsiodal with a large posterior vacuole. The major portion of the polar filament was believed to occur in the posterior vacuole. Although only three windings of the filament were reported (Summerfelt, 1964), the average length of extruded polar filaments was about 135 microns. A girdle-like ring, visible in stained specimens just anterior to the center of the spore, was presumably the sporoplasm containing one nucleus.

Length and width of fresh spores reported by Summerfelt (1964) averaged 8.42 by 4.24 microns from fish from an Illinois source and 8.62 by 3.61 microns from fish from a Kentucky source. Wilhelm (1964) reported dimensions of 8.5 by 4.6 microns for fresh spores from the same Illinois source as studies by Summerfelt. Parker and Warner (1970) found mean fresh spore lengths and widths of 8.30 by 4.45 microns,

respectively. Also, fixation, dehydration, and staining of spores always reduced the mean spore length, but the type of fixative determined whether mean spore width increased or decreased (Parker and Warner, 1970). Spore dimensions and modal (two or more distinct spore sizes) spore size frequency distribution were widely used in distinguishing species characteristics. Summerfelt and Warner (1970a) observed in P. ovariae a reduced spore length of approximately one micron in Bouin's or 10% formalin fixation, and an additional one micron after dehydration and staining. Kudo (1921), Blunck (1954), Walters (1958), Thomson (1960), and Parker and Warner (1970) advised that similar fixation and staining be used when spore dimensions serve as taxonomic characteristics. Age of the host in six age classes of golden shiners was not a significant factor affecting spore size (Summerfelt and Warner, 1970a).

CHAPTER III

MATERIALS AND METHODS

Golden shiners, Notemigonus crysoleucas, were obtained from an Oklahoma source (code 05, Summerfelt and Warner, 1970a) in February 1969 to establish a population of shiners in a pond near campus. The fish were approximately nine months old and were the offspring of female shiners heavily infected with Pleistophora ovariae (23 of 23 checked were heavily infected). Young fish, which had never spawned, were chosen to ensure that all fish were the same age and to examine if intensity of infection would be greater in the second spawning (1970) than in the first (1969).

On February 20, 1969, the fish were placed in a newly renovated, 1/20-acre, mud-bottom pond. The pond had not been used for fish production for approximately 25 years. Lake Carl Blackwell, Payne County, Oklahoma, served as the water source. Surface water temperatures of the pond were taken at random intervals throughout the sampling period.

Forty fish were collected at biweekly intervals from February 20, 1969, through May 18, 1970. Two exceptions to the regular biweekly collections were: (1) samples were taken on April 17, 1969, following the regular sampling on April 10, 1969, and sampling continued at biweekly intervals from the 17th; and (2) no samples were taken between December 18, 1969, and January 26, 1970.

Immediately after collection, fish were fixed in 10% formalin and taken to the laboratory. In the laboratory, the weight, total length, and gonadal weight were recorded; and gonads were placed in a "Kaformacet" fixative (212.5 ml water; 6.0 g potassium dichromate; 25 ml conc. formaldehyde; 12.5 ml acetic acid) to improve staining differentiation. Specimens were refrigerated for 24 hours in the fixative, then dehydrated with 2-propanol and toluene, and infiltrated and embedded in Paraplast[1] using standard methods (Humason, 1967). Sectioning, staining, clearing, and mounting (Permount)[2] were done following procedures recommended by Humason (1967) and the Biological Stain Commission (1960). Two slides, each with 4 to 10 serial sections (7 microns thick), were made from each ovary. One slide was stained with Mallory's analine blue collagen stain (Biological Stain Commission, 1960) and the other with Delafield's hematoxylin and eosin (Humason, 1967).

Using these histological preparations, the maturity of ovaries of ten fish from each sampling period was described by obtaining the percentages of the ovary occupied by each of the different oocyte stages during a given sampling period. This was done by classifying and counting all oocytes within several randomly selected fields until 100 total oocytes had been counted for each ovary. Seven developmental stages of oocytes were classified on the basis of morphology and staining reactions described for the stickleback (*Eucalia inconstans*) by Braekevelt and McMillan (1967).

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[1]W. H. Curtin Company, Tulsa, Oklahoma.

[2]Fisher Scientific Company, Fairlawn, New Jersey.

The average area occupied by an oocyte in each stage of development was multiplied by the number of oocytes of that stage. The total area occupied by the 100 oocytes counted was summed. The percentage of the total area occupied by each oocyte stage was then calculated.

Two estimates of the percentage of the ovary affected were made. First, each of the counted oocytes was inspected for the presence of recognizable parasitic stages and the percentage of infected oocytes was computed. Second, an estimation of the percentage of each ovary affected by the parasite was made using an imaging screen[3] mounted on the photographic tube of the microscope's trinocular head. A positive overlay made from standard 5-lines-per-inch graph paper was attached to the screen surface. An area on the screen containing 300 squares was selected for use in estimating per cent affected. The 10X objective and 10X eyepiece projected the image of the ovary to the screen. A count was made of the number of squares containing intact but infected oocytes and areas of the ovary filled with spores derived from atresia of infected oocytes. If infected oocytes occupied more than 50% of the square, the entire square was counted as infected, otherwise, the square was counted as uninfected. The percentage of ovary affected was derived as an average from examination of four microscope fields of one ovary of each fish.

The mean number of sporonts per unit volume of an affected oocyte was determined using a modification of Haug's (1967) equation:

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[3]Hudson Photographic Industries, Inc., Irvington on Hudson, New York.

$$N_v \cong \frac{N_A \times V_1}{A\ (D + T - 2K)}$$

where

N_v = estimated number of sporonts per unit volume of oocyte,

N_A = actual number of sporont profiles counted per volume of egg profile examined,

V_1 = thickness of section times A; or, volume of egg profile,

D = diameter of structure being counted (sporont),

A = area of section (oocyte profile),

T = thickness of section (oocyte profile) (7μ), and

K = factor which tells how thick a section of schizont or sporont must be in the oocyte profile before the structure (sporont) can be observed and identified. Two microns was the estimated value used here.

An estimate of the mean number of sporonts in each egg stage was calculated multiplying the obtained mean number of sporonts per unit volume of a given egg stage by the mean volume of that egg stage.

A mean volume of each egg stage was computed in sections of ovaries fixed in Bouin's, Kaformacet, and frozen. Different egg stages were not differentially affected by shrinkage, and shrinkage was judged proportional to egg size enabling the use of the equation above without further modification.

CHAPTER IV

RESULTS AND DISCUSSION

Morphology and Dynamics of Oogenesis

Emphasis of this investigation is concerned with the seasonal dynamics of Pleistophora ovariae in the ovary of the golden shiner, especially a morphological description of its life stages. This emphasis necessitated a morphological description of oogenesis and the reproductive cycle of the host.

Production of mature ova involves a series of sequential changes beginning with multiplication of the small oogonia and culminating with formation of secondary oocytes capable of being spawned and fertilized. The process is divided into stages to facilitate description of seasonal dynamics. Beach (1959) placed developing oocytes into one of three groups; immature ova, maturing ova, and mature ova. Three groups of ova were used by Hurley and Fisher (1966): recruitment stock, maturing oocytes, and atretic oocytes. Malone and Hisaoka (1963) recognized five stages of development; Braekevelt and McMillan (1967) seven stages. The general characteristics used by Braekevelt and McMillan (1967) to separate ova of the stickleback into stages of development are used to recognize seven stages of development of golden shiner oogenesis (Table I). Golden shiner oocytes are generally larger than those of the stickleback of the same stage, otherwise ova characteristics are similar.

TABLE I

CRITERIA USED TO DISTINGUISH THE DEVELOPMENTAL STAGES OF THE EGGS IN THE GOLDEN SHINER, NOTEMIGONUS CRYSOLEUCAS*

Egg Stage	Diameter (microns)	Nucleus	Cytoplasm	Membranes
0	6-7	Spherical, vesicular, one prominent nucleolus.	Thin margin around nucleus.	Nuclear membrane distinct.
I	17-46 mean 32	16-19μ Spherical, vesicular, and centrally located; 1 or 2 prominent nucleoli; chromatin conspicuous.	Homogeneous and basophilic.	Nuclear membrane prominent; cell membrane indistinct.
II	50-99 mean 74	37-45μ 3-5 basophilic (dark red) nucleoli.	Homogeneous and basophilic.	Nuclear membrane present with smooth margin; flattened, follicular cell nuclei around periphery.
III	110-240 mean 175	74-96μ Spherical, 20-35 nucleoli, neucleoplasm acidophilic, much paler than cytoplasm.	Homogeneous becoming frothy, less basophilic.	Nuclear membrane well developed and irregular; follicular epithelium thickening but monolaminar, zona radiata forming.
IV	180-430 mean 273	63-118μ Nucleus becoming irregular in shape; nucleoplasm acidophilic, with 50-65 nucleoli.	Vesicles of primary yolk, light blue, migrating inward.	Nuclear membrane more irregular; vitelline membrane visible.

TABLE I (Continued)

Egg Stage	Diameter (microns)	Nucleus	Cytoplasm	Membranes
V	200-530 mean 355	48-130μ Nucleus is irregular and accentric; nucleoplasm acidophilic.	Vesicles of primary yolk (intravesicular) fill cytoplasm.	Zona radiata blue changing to red; vitelline membrane larger than IV; follicular layer bilaminar.
VI	310-820 mean 523	76-128μ Strongly acidophilic nucleoplasm, nucleus irregular and located peripherally.	Secondary yolk (extravisicular) forming a red to yellow band at periphery of nucleus; primary yolk (blue) around periphery of cytoplasm.	Zona radiata well striated; follicular layer thickened; vitelline membrane well developed.
VII	470-950 mean 617	86-121μ Nucleus irregular and peripheral.	Secondary yolk globules consolidate, filling most of cytoplasm; primary yolk forms thin marginal layer.	All membranes prominent; zona radiata stains red.

*Seven micron sections stained with Mallory's Analine Blue Collagen Stain (Biol. Stain Comm., 1960).

The dimensions of oocyte diameters in Table I are from fixed, dehydrated, and stained oocytes, and are about 10% smaller than fresh oocytes (Table IV).

Gross Ovary Morphology

Golden shiner ovaries are paired, elongate organs lying ventral and lateral to the air bladder, and dorsal to the intestine. They are completely enclosed by a thin layer of visceral peritoneum which fuses posteriorly to form a single, delicate oviduct. Beneath this peritoneum is a thin elastic connective tissue layer called the tunica albuginea (Figure 1). Ovigerous lamellae are folds of the tunica albuginea which project into the lumen of the ovary. The ovigerous lamellae contain oogonia and oocytes in various stages of development, connective tissue, and capillaries. Ripe ova, released from the ovigerous lamellae, drop into the ovocoel (lumen of the ovary), and during spawning are forced out the urogenital pore by muscular contractions of the oviduct.

The early germ cells (oogonia) are first located on the edge of the ovigerous lamellae bordering the ovocoel. As the oocytes mature and enlarge, all stages are pushed deeper into the stroma.

Morphology and Dynamics of Oogenesis

The ovarian cycle of the golden shiner, like that of most oviparous teleosts, begins with proliferation of oogonia (stage 0) in the summer. The oogonia develop into primary oocytes (stages I and II) by September. The first polar body is visible in stage III and persists in stage IV oocytes (secondary oocytes). The eggs mature to stages IV and V during the fall and "over-winter" in these stages. In late March, when the

water temperatures approach 10° C, stage VI eggs become very plentiful. The stage VII egg, which is spawned, is apparently still a secondary oocyte because the second polar body is not observed. Presumably, the second meiotic division is stimulated by fertilization. Spawning begins in early May when water temperatures are 20-22° C. Shiners are intermittent spawners and will spawn several times during the spring and summer. After spawning, all unspawned eggs over stage III are resorbed, oocyte debris is removed and another ovarian cycle begins with proliferation of the oogonia and appearance of numerous stage I oocytes.

Stage 0. Residual oogonia in the ovigerous lamellae give rise to each successive years oocytes by mitotic division (Figure 2). The oogonia are small (6-7 u), somewhat spherical to cuboidal cells, with a slight, indistinct rim of lightly basophilic cytoplasm, and a large vesicular nucleus, containing a thick ring of peripheral chromatin and a single, large, centrally-located nucleolus. Oogonia often appear in nests along the edge of the ovigerous lamellae. The percentage of total area occupied by stage 0 and the percentage of total number of eggs represented by stage 0 were not calculated.

Stage I. As the oogonia begin to grow, the amount of cytoplasm increases, nuclear changes occur, and it becomes a primary oocyte (Figures 3 and 4). The cytoplasm is homogeneous and basophilic. The nucleus (16-19 u) occupies a large portion of the cell and is basophilic and located centrally. One or two prominent nucleoli are present lying adjacent to the nuclear membrane. Stained chromatin material appears as threads (lampbrush chromosomes) and clumps. Mean egg diameter is

32 microns. The cytoplasmic membrane is not visible, but the oocyte is bounded by the ovigerous lamellae.

The percentage of total eggs represented by stage I oocytes was less than 10% from February 20, 1969 until June 5, 1969 (Figure 17). The percentage then increased to 20% by July 17, 1969. Stage I oocytes reached a maximum of 8% of total area and 39% of total number of viable oocytes in the August 28, 1969 sample (Figure 17). About 55% of the total area of the ovaries was occupied by spore filled atretic follicles at this same collection date (Figure 43).

Seasonal variation in stage I indicates that atresia is near completion when oogenesis commences and that many of the progenitors of the ova to be spawned in May and June are formed within a few months after the previous spawning season.

Stage II. Cytoplasmic basophilia is maximum (Figures 5 and 6). Mean cell diameter is 74 microns and a seven micron section of the nucleus contains three to five visible nucleoli. Several flattened follicular cell nuclei denote a thin monolayered follicular epithelium around the periphery of the oocyte. The zona radiata is not visible.

The maximum percentage of total egg area was 92.6% and maximum percentage of total egg number was 76%. Seasonal trends in fluctuation of percentage of total area and total number of stage II eggs are similar to those for stage I eggs. The seasonal maximum for stage II eggs occurred the next sampling period (September 11, 1969) after stage I reached a maximum. Maturation to stage II from stage I required approximately two weeks.

The number of, and area occupied by, eggs reaching stage II was approximately the same in February of both years (Figure 17). However,

during any given sampling period after February, the percentage of stage II eggs was about 15% higher in 1970. This increase in oocyte production in 1970 suggested that fecundity would be higher that year, as would be expected in older fish. However, all oocyte stages larger than stage II decreased in number in 1970 apparently as the result of parasitism.

Stage III. Occurrence of a polar body in this stage indicates that this large (175 μ) germ cell is a secondary oocyte. Its cytoplasm is frothy and less basophilic than in stage II (Table I). Chemical changes in the cytoplasm result in more affinity for acid stains and the lipids are dissolved out by alcohols in the dehydration process, giving the cytoplasm a frothy appearance.

In a seven micron section, 20-35 nucleoli are present on the inner margin of the irregular nuclear membrane; otherwise, the nucleoplasm is pale and acidophilic (Figure 7). The nuclear membrane in oocytes of stage I and II, in contrast, is very regular in outline. The follicular epithelium of stage III oocytes is a distinct monolayer with a thickened cytoplasm and a elongated nucleus. The zona radiata is first visible in stage III eggs, but is not yet striated.

The number and relative area of the ovary occuped by stage III eggs declined each biweekly collection between February 20 and April 24, 1969 (early spring), while stage IV and V were increasing.

The July-August peak followed a sharp increase in stage II eggs (Figure 17). The maturation apparently is very rapid as an increase in stage VI oocytes occurred within four weeks (Figure 17). The absence of changes in relative abundance of stages IV and V corresponding to

this particular change is probably due to the speed of oogenesis and to sampling error.

A fall increase in stage III oocytes was observed during late September and early October 1969. It coincided with the decline in number of stage II oocytes. The percentage of total area occupied by stage III decreased slightly in late October and then the percentage of area and of total eggs stayed quite constant (between 20 and 35%) throughout the remainder of the sampling time. This pattern of increase and stabilization, which varies from that observed in the spring of 1969, may be due to the effects of P. ovariae, or age (the fish had not spawned prior to 1969). In either case, the relative seasonal abundance of certain egg stages changed following the first spawning season. From October 9, 1969, until the end of sample on May 18, 1970, there was less than 12% fluctuation in the percentage of total eggs present. The maximum percentage of area ever occupied by stage III eggs was down from 50% in 1969 to 34% in 1970. The lack of uninfected controls makes it impossible to determine if these reductions are "normal" or the result of parasitism.

Stage IV. The mean oocyte diameter is 273 microns (Figures 8 and 9). About 60 nucleoli are visible per nucleus in a seven micron section. Like stage III, the nucleus is acidophilic and the nuclear membrane very irregular in shape. Zona radiata and follicular epithelium are thicker than in the stage III oocyte. The stage IV eggs have vacuoles containing light blue staining (Mallory's stain) yolk (primary yolk, intravesicular yolk) nuclei. These vacuoles appear abundantly around the periphery of the cytoplasm.

Stage IV comprised 15% of total number in February 1969, but the percentage was 40% in March and April 1969. The 25% increase between late February and early April coincided with a 30% decrease in the same interval in stage III oocytes. The rate of conversion of stage III to IV is six weeks as measured from the mode of stage III in February until the mode obtained by stage IV in mid-April (Figure 17). The change from stage III to IV involves enlargement in mean oocyte diameter (36%) and formation of the intravesicular yolk. The relative abundance of stage IV oocytes was less than 10% from mid-April until early October 1969. The October increase corresponds to a maturation (decrease in number) of stage III eggs (Figure 17). The fairly high percentage of area occupied by stage IV oocytes from October 1969 until March 1970 is evidence that the majority of the maturing ova "overwintered" as stage IV eggs. In addition, the decrease in stage IV oocytes in March 1970 corresponded to an increase in stage V oocytes.

Again, as with stage III, the number of stage IV oocytes was less in 1970 than in 1969. It is assumed that this reduction is the result of parasitism rather than age. However, it is not possible to support this assumption at this time because of lack of information from uninfected controls.

Stage V. The mean stage V oocyte diameter is 355 microns (Figure 10). The nucleus is acidophilic, the follicular epithelium bilaminate, the zona radiata and vitelline membrane thickened, and the nucleoli distinct (Figure 11). During stage V, the zona radiata thickens and becomes visibly striated. This stage is conspicuous because the primary (intravesicular) yolk fills the entire cytoplasm (Figure 12). Prior to development of striations, the zona radiata stains basophilic, but after

Figure 1. Longitudinal Section Through Ovary Showing Ovigerous Lamella (OL), Tunica Albuginea (TA), Spacious Ovocoel (OC), Mesovarium (M), and Abdominal Fat (FB); Dorsal Surface to the Right, Ventral to the Left

Figure 2. Ovigerous Lamellae With Numerous Oogonia. Oogonia are Characterized by a Single Vesicular Nucleus Containing a Broad Ring of Peripheral Chromatin (PC) and a Single Nucleolus (Nu)

Figure 3. Early Stage I Oocyte With Large, Single Nucleolus and Lampbrush Chromosomes (Cr)

Figure 4. Late Stage I Oocyte With Two Nucleoli, More Granular Chromatin, Lampbrush Chromosomes Less Prominent, and Distinct Nuclear Membrane (Arrow)

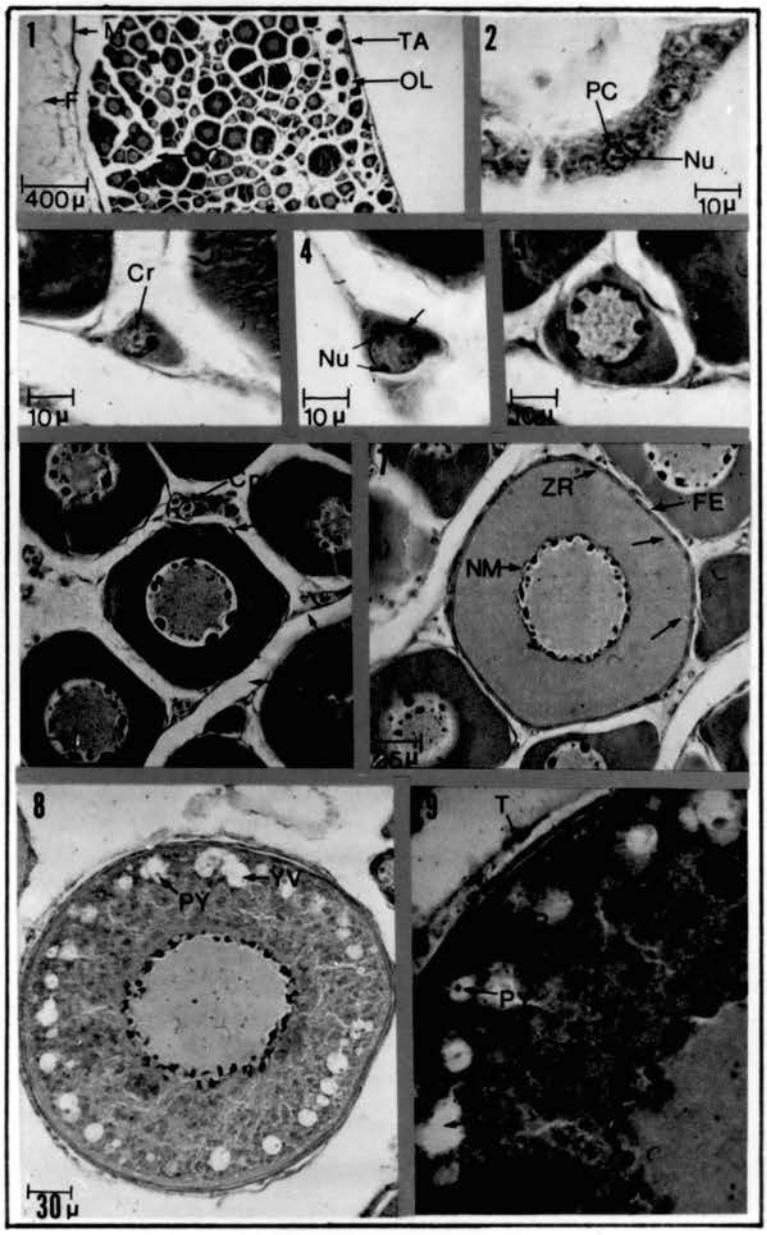
Figure 5. Early Stage II Oocyte With Seven Nucleoli and Coarsely Granulated Nucleoplasm

Figure 6. Stage II Oocyte With Numerous Nucleoli, Follicular Epithelium Containing Scattered Flattened Nuclei (N), Basophilic Cytoplasm and Finely Granulated Nucleoplasm. Capillaries (Cp) are Numerous Between Adjacent Oocytes

Figure 7. Stage III Oocyte With Numerous Nucleoli, an Irregular Nuclear Membrane (NM), Conspicuous and Thickened Follicular Epithelium (FE) Containing Numerous Ovoid Nuclei, a Dark, but Thin Zona Radiata (ZR), and a Frothy Periphery (Arrow)

Figure 8. Stage IV Oocyte With Peripherally Located Yolk (YV) Vacuoles Containing Primary Yolk (PY)

Figure 9. Stage IV Oocyte Showing Thickened Follicular Epithelium (FE), a Superficial Covering of Theca Cells (T), a Thin Zona Radiata (ZR) (Without Striations), Yolk Vacuoles (YV), Primary Yolk (PY), and an Irregular Nuclear Membrane


1
TA
OL
400µ
2
PC
Nu
10µ
Cr
10µ
4
Nu
10µ
ZR
FE
NM
8
PY
VV
30µ
9
T
P

striations appear it stains acidophilic as did the secondary (extravesicular) yolk appearing in stage VI.

Stage V oocytes appeared first in April 1969. At this time and throughout the entire sampling period, increases in the relative abundance of this oocyte stage corresponded to a decrease in stage IV oocytes. This suggests that stage IV oocytes are maturing into stage V oocytes.

Very few stage V oocytes are present during August and September, perhaps because of maturation to stage VI and VII or because of parasitism. Stage V oocytes occupied a highly variable percentage of the ovary, representing 15% on February 9, 1970, 5% on March 23, 1970, then 24% on April 6, 1970.

A low percentage of stage V oocytes were present at any given time (Figure 17) because this egg stage occurs primarily just prior to spawning when growth and development are very rapid. Possibly, the time required for maturation from IV to VI is so rapid that at any given time only a few oocytes can be observed in stage V. Furthermore, late stage IV and some early stage VI oocytes are difficult to distinguish from stage V oocytes. Therefore, some stage V could be classified in either of these stages. It may be advisable to combine stage IV and V oocytes as a single class.

Stage V oocytes showed an increase in maximum area but a decrease in percentage of number of eggs in 1970 compared to 1969. These changes, however, are less than 10% and are probably not significant when the previously mentioned identification problems are considered.

Stage VI. Mean oocyte diameter is 523 microns (Figure 12). The nuclei are strongly acidophilic, sometimes acentrically displaced and

irregularly shaped (Table I). Secondary (extravesicular) yolk, which stains red with Mallory's stain (acidophilic), appears first in the perinuclear region and migrates outward to fill the cytoplasm (Figure 12).

The presence of large numbers of stage VI oocytes generally corresponds to the spawning season. Fish spawned primarily during a four-week interval from mid-April to mid-May 1969 and again during the similar period in 1970. The fecundity in 1970 is less than in 1969 as indicated by a reduced number of stage VI eggs. It seems that the reduction in numbers and area of eggs in histological sections are a result of increased incidence and intensity of P. ovariae. The maximum percentage of total area occupied by stage VI eggs was 16% less in 1970 compared to in 1969, and the maximum percentage of total eggs decreased 24% in 1970 (Figure 18).

The fall and winter peaks for stage VI eggs (Figure 17) were the result of one or two fish ovaries in the 10-fish sample that have stage VI oocytes present. Because of the large size of the stage VI oocytes in relation to that of stage III and IV oocytes, the total area of this stage showed a sharp increase. However, the curve of the percentage of the total eggs showed little variation.

Stage VII. Mean oocyte diameter is 617 microns (Table I). The zona radiata attains maximum thickness, stains red, and the striations are prominent. The follicular epithelium is bilaminar (Figure 14). The nucleus has a rough margin, sometimes is located eccentrically, and often is surrounded by a mass of coalesced secondary yolk (Figure 15 and 16). Secondary yolk fills most of the cytoplasm except for extreme cortical areas where one or two layers of primary (intravesicular) yolk

Figure 10. Stage V Oocyte Filled With Yolk Vacuoles and Primary Yolk

Figure 11. Stage V Oocyte Showing Abundant Primary Yolk Vacuoles (YV), Vitelline Membrane (V), Thickened Zona Radiata (ZR), Bilaminar Follicular Epithelium (FE), and Nucleus With Numerous Nucleoli (Nu).

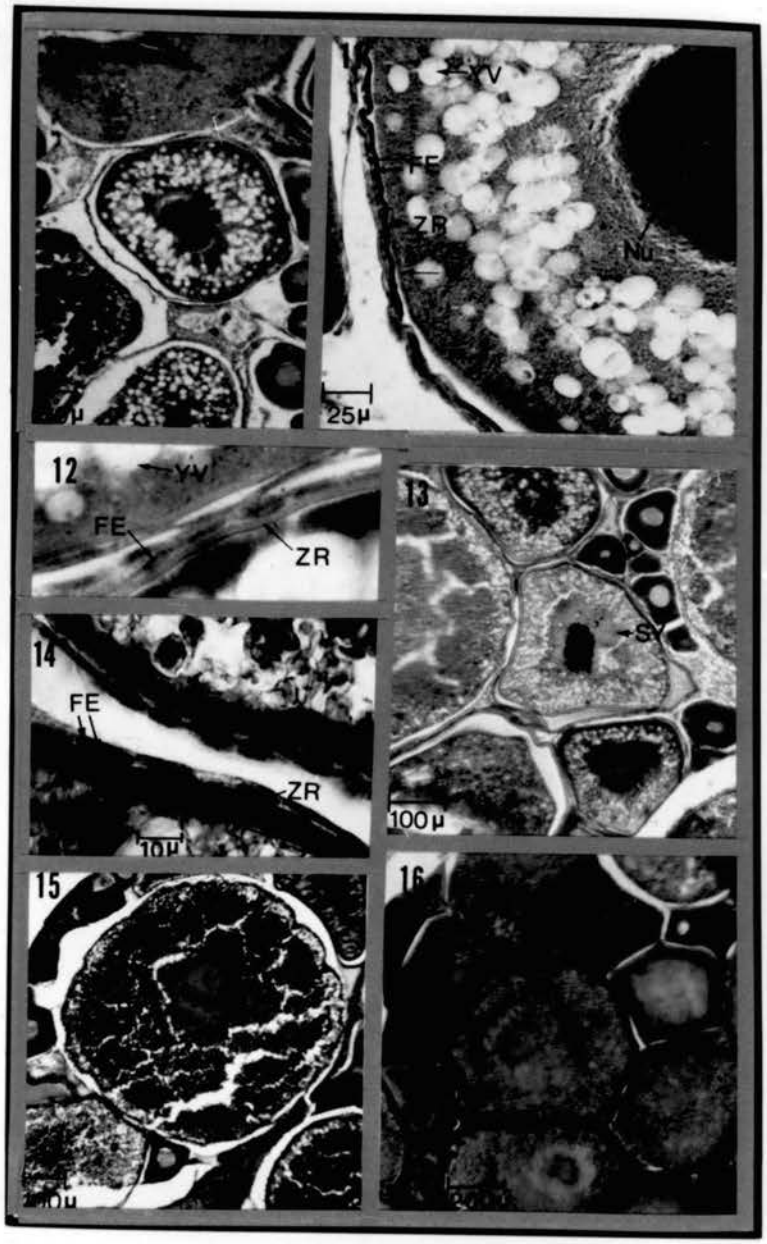
Figure 12. Margin of Stage VI Oocyte Showing Primary Yolk Vacuoles (YV), Thickened Zona Radiata (ZR), and Bilaminar Follicular Epithelium (FE)

Figure 13. Stage VI Oocyte Showing Perinuclear Accumulation of Secondary (Extravesicular) Yolk (SY), Peripheral Location of Primary Yolk, Irregular Outline of the Nucleus, and Thickened Follicular Epithelium

Figure 14. Margin of Stage VII Oocyte Showing a Striated Zona Radiata (ZR) and a Thickened Bilaminar (Double Arrows) Follicular Epithelium (FE)

Figure 15. Stage VII Oocyte Showing Peripheral Displacement of Primary Yolk (PY), Predominance of Secondary Yolk (SY), and Coalescence of Secondary Yolk Vacuoles Near the Nucleus.

Figure 16. Mature Ovary in Spawning Condition Showing Expansive Nature of Ovigerous Lamellae Filled With Enlarged Stage VII Oocytes, Which Obliterate the Ovocoel


YV
FE
ZR
Nu
25μ
12
YV
FE
ZR
13
SY
100μ
14
FE
ZR
10μ
15
16

vesicles and vacuoles are found. Secondary yolk globules may coalesce and form larger globules.

Only a few stage VII oocytes were found. Three factors probably contributing to this are: the relatively short interval stage VII oocytes exist within the ovary before spawning; the destruction of early egg stages before they fully mature; and the difficulty of preparing stage VII for histological sectioning. The alcohol hardens the yolk materials and sections disintegrate during sectioning.

Unlike the problems of recognizing stage V oocytes, stage VII oocytes can readily be recognized. However, stage VII oocytes are extremely difficult to keep in good condition through the cutting, mounting, and staining processes.

Stage VII oocytes did not exceed 2% of the total eggs present. The maximum per cent of total area occupied by stage VII was 26% on July 17, 1969 (Figure 17). However, stage VII oocytes were found in only 2 of the 10 fish in that sample. This peak occurred when stage VI oocytes were declining in prevalence (Figure 17). The data on percentage of stage VII oocytes infected for the periods on July 2, 1969, July 17, 1969, July 31, 1969, and February 9, 1970 are derived from small samplings: one fish and three eggs, two fish and 18 eggs, two fish and three eggs, and one fish and nine eggs, respectively.

Spawning occurs during late April and May 1969. The fact that stage VII oocytes represented less than 2% of the total, both in area and number, is indicative of the problems associated with finding eggs of this stage.

The rate of maturation varies with the season of the year. During August and September, the rate of maturation is very rapid, requiring

Figure 17. Comparison of Percentage of Total Area and Total Number of Oocyte Stages I Through VII Throughout the Entire Collection Period

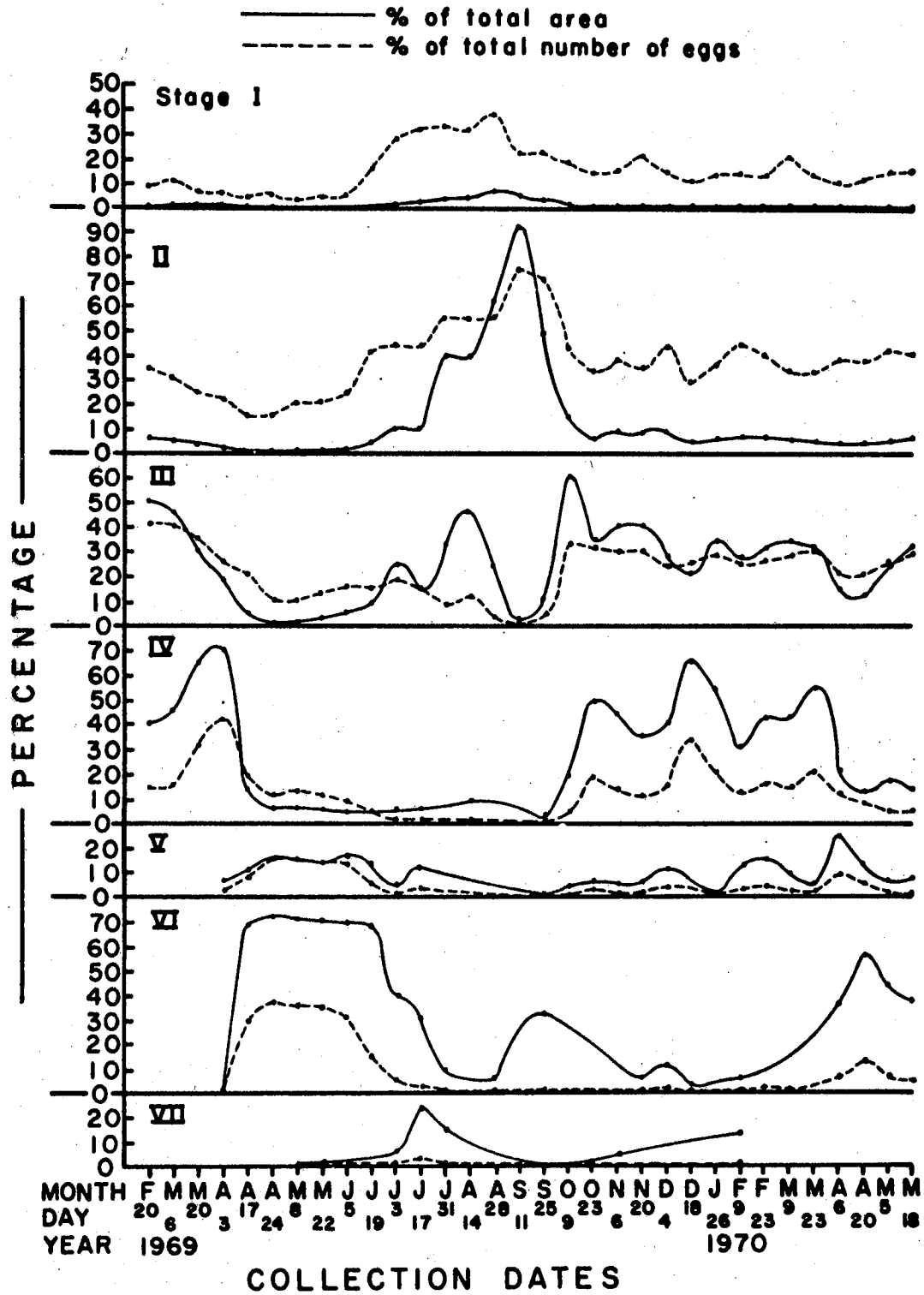
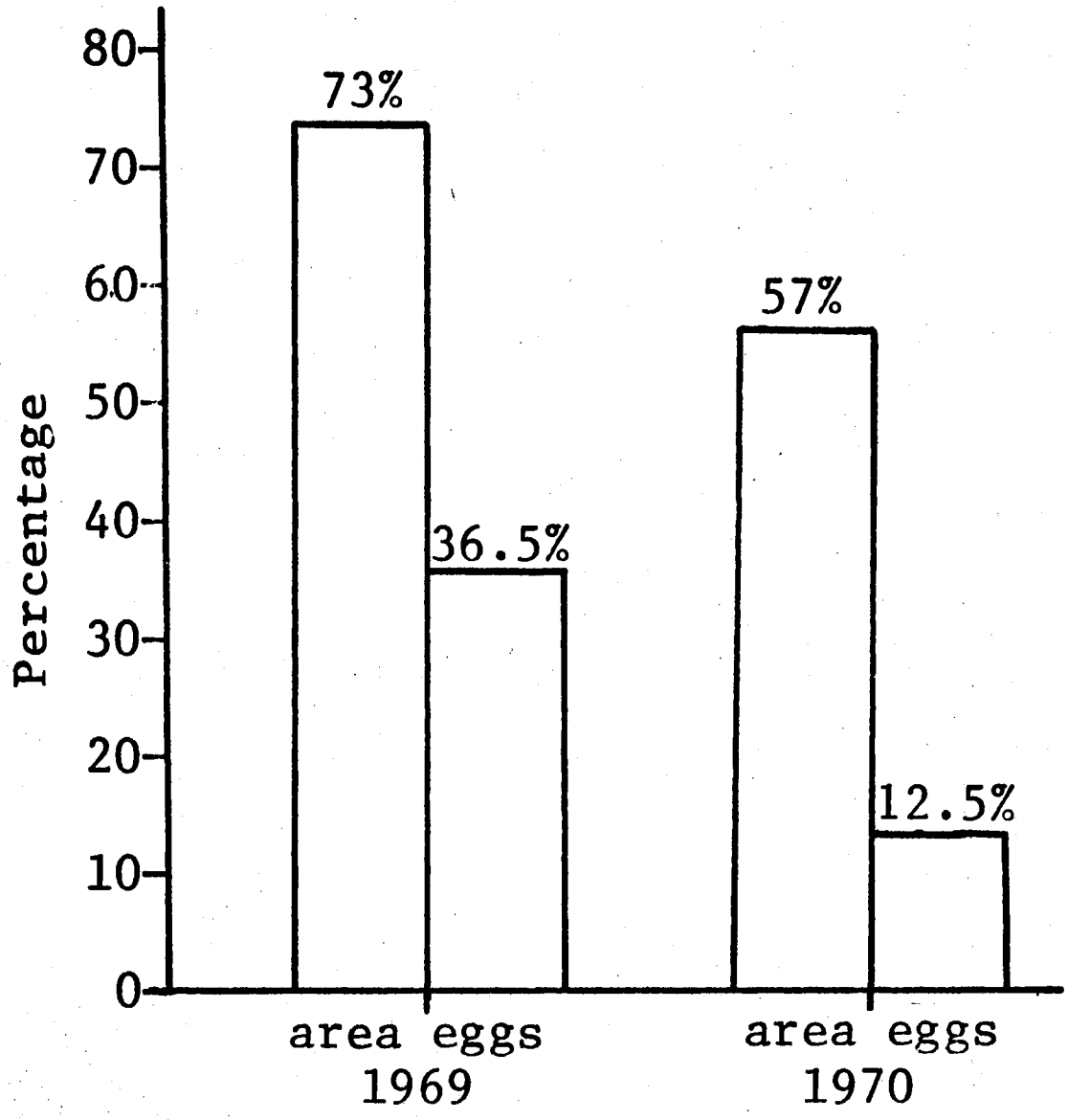

% of total area
% of total number of eggs
Stage I
II
III
IV
V
VI
VII
PERCENTAGE
MONTH F M M A A A M M J J J J J A A S S O O N N D D J F F M M A A M M
DAY 20 6 20 3 17 24 8 22 5 19 3 17 31 14 28 11 25 9 23 6 20 4 18 26 9 23 9 23 6 20 5 18
YEAR 1969 1970
COLLECTION DATES

Figure 18. Comparison of Production of Stage VI Oocytes in 1969 and 1970 (Percentage of Total Area = White; Percentage of Total Number of Oocytes of All Stages = Crosshatched)


80
70
60
50
40
30
20
10
0
Percentage
73%
36.5%
57%
12.5%
area eggs
1969
area eggs
1970

only two weeks for each stage (I to V). However, when the water temperature lowers in the fall and winter the rate is much slower. Six weeks (October 23 to December 4) were required for the peak of stage IV oocytes to shift to a peak of stage V. The oocytes "overwinter" in stage IV, then in the spring, the rate of maturation increases to two weeks or less for each egg stage.

These results can only be applied to oogenesis in golden shiners infected with P. ovariae as all fish examined were infected, but there was no indication that the parasitism altered the rate of oogenesis.

There is a definite decrease in oocyte production as the infection spreads throughout the ovary. However, the large areas of atresia (see section on atresia) following the 1969 spawning season appeared not to limit the production of stage I and II oocytes. In July, August, and September, when stage I oocytes were most numerous (Figure 17), the percentage of the ovary affected by the parasite ranged from 35 to 65%, yet stage I increased in 1970. The reduction began with stage III oocytes. This conclusion is supported by the fact that parasites are not observed in stage I and rarely in II oocytes (see section on parasite life cycle), but become prevalent in stage III oocytes.

Atresia

The process of atresia is the same fundamentally in all oviparous teleosts, but differs somewhat in appearance depending on the amount of yolk present in the resorbing eggs (Beach, 1959). Braekevelt and McMillan (1967) found that atresia was most widespread immediately after spawning when unspawned eggs were removed from the ovary. They described

two forms of atresia, nonhypertrophic and hypertrophic-phagocytic, in the brook stickleback, Eucalia inconstans.

Atresia is most widespread immediately following the spawning season in golden shiners. Both forms of atresia are observed in the golden shiner, although the non-hypertrophic type is less common (Table II).

Hypertrophic-Phagocytic Atresia

Hypertrophic atresia is the most widespread type of atresia in the golden shiner and is basically the type described in four stages (a through d) by Bretschneider and de Wit (1947) for the bitterling (Rhodeus amarus). The a-stage of atresia is transitory and characterized by hypertrophy of the follicular cells and erosion of the zona radiata. The end of the a-stage is marked by eruption of the zona radiata. During the b-stage the ruptured zona radiata persists, most of the egg material is removed, and the follicle cells show further hypertrophy. The c-stage shows further thickening of the follicular layer and massive migrations of follicular cells into the area formerly occupied by the egg cytoplasm. In the d-stage, the follicular layer collapses and fills the space previously occupied by the egg cytoplasm. All remaining yolk granules have been removed.

Lewis and McMillan (1965) give a similar description of phagocytic atresia in the sea lamprey, Petromyzon marinus. They find that the concentration or coalescence of yolk particles and the resulting intensification of the staining reaction are the first signs of atresia. Early stages of atresia in basophilic oocytes are difficult to distinguish from yolk uptake. Phagocytes derived from follicular cells

TABLE II

COMPARISON OF NUMBER AND PERCENTAGE OF EGGS UNDERGOING NONHYPERTROPHIC AND HYPERTROPHIC ATRESIA DURING SAMPLING PERIODS. A TOTAL OF 1000 EGGS (100 PER FISH FOR 10 FISH), REPRESENTING ALL STAGES WAS CHECKED EACH SAMPLING PERIOD AND THE PERCENTAGE OF THE TOTAL NUMBER OF EACH STAGE WAS CALCULATED

Collection Date	Number Uninfected Fish	Nonhypertrophic							Hypertrophic							% Total Oocyte Number
		% Oocytes Atretic					Number Unknown	% Total Number	% Oocytes Atretic					Number Unknown	% Total Number	
		III	IV	V	VI	VII			III	IV	V	VI	VII			
2/20/69	10	-	3	-	-	-	2*	.6	-	-	-	-	-	-	-	.6
3/6/69	10	.3*	.6*	-	-	-	-	.2	-	-	-	-	-	-	-	.2
3/20/69	10	-	8*	-	-	-	7*	3.3	-	-	-	-	-	-	-	3.3
4/3/69	10	7*	2*	-	-	-	-	2.8	-	.2*	-	-	-	-	.1	2.9
4/17/69	10	-	5*	1*	-	-	-	1.0	-	.5*	1*	-	-	-	.2	1.2
4/24/69	10	2*	3*	-	1*	-	4*	1.4	4*	5*	2*	2*	-	-	2.0	3.4
5/8/69	10	-	8*	1*	.6*	-	-	1.4	-	-	-	-	-	4*	.4	1.8
5/22/69	0	1	3	-	.9	-	1	1.0	-	.7	-	1	-	52	5.7	6.7
6/5/69	0	-	-	-	1	-	-	.4	-	5	3	7	-	37	6.9	7.3
6/19/69	0	-	-	-	3	-	-	.4	-	6	3	31	-	85	13.8	14.2
7/3/69	0	-	-	12	-	-	-	.1	-	-	62	55	-	204	23.6	23.7
7/17/69	1	-	-	-	-	-	-	-	5	-	-	10	-	77	9.3	9.3
7/31/69	0	-	-	-	-	-	-	-	-	-	-	33	-	24	2.5	2.5
8/14/69	0	-	-	-	-	-	-	-	-	-	-	-	-	10	1.0	1.0
8/28/69	0	-	-	-	-	-	-	-	-	-	-	-	-	37	3.7	3.7
9/11/69	0	-	-	-	100	-	-	.1	-	-	-	-	-	15	1.5	1.6
9/25/69	0	-	-	-	-	-	-	-	-	-	-	-	-	16	1.6	1.6
10/9/69	0	-	-	-	-	-	-	-	-	-	-	100	-	2	.3	.3
10/23/69	1	-	-	-	-	-	-	-	-	-	-	100	-	11	1.2	1.2
11/6/69	1	-	-	-	-	-	2	.2	-	-	-	-	100	7	.8	1.0
11/20/69	0	-	-	-	-	-	2	.2	-	-	-	-	50	34	3.5	3.7
12/4/69	0	-	-	-	-	-	1	.1	-	-	-	-	100	29	3.1	3.2
12/18/69	1	-	-	-	-	-	-	-	-	-	-	-	100	10	1.1	1.1
1/26/70	0	-	-	-	-	-	-	-	-	-	-	-	-	13	1.3	1.3
2/9/70	2	-	-	-	-	-	1	.1	-	-	-	25	67	51	5.9	6.0
2/23/70	0	-	-	-	-	-	-	-	-	-	-	-	-	13	1.3	1.3
3/9/70	0	-	-	-	-	-	1	.1	-	-	-	-	-	21	2.1	2.2
3/23/70	1	-	-	-	-	-	-	-	-	-	-	-	-	11	1.1	1.1
4/6/70	2	-	-	-	-	-	-	-	-	-	-	-	-	5	.5	.5
4/20/70	3	-	-	-	-	-	-	-	-	-	-	7	-	14**	2.3	2.3
5/5/70	0	-	-	-	-	-	-	-	-	-	-	29	-	42	5.9	5.9
5/18/70	0	-	-	-	-	-	-	-	-	-	-	9	-	25	2.9	2.9

*Numbers marked with an asterisk were uninfected, those not marked were infected.
**One uninfected and 13 infected oocytes.

Figure 19. Early Phagocytic-Hypertrophic Atresia Showing Thickened Follicular Epithelium

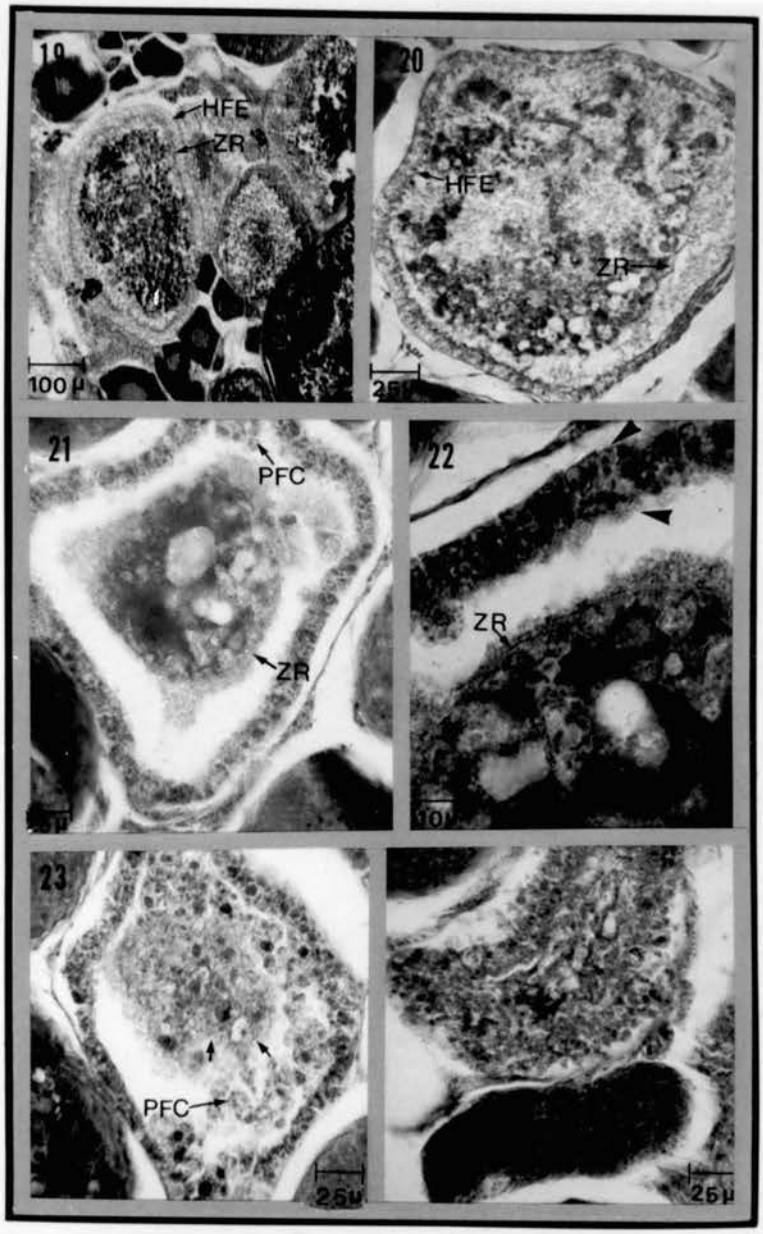
Figure 20. Early Phagocytic Atresia Showing Initial Erosion of Zona Radiata (ZR), Breakdown of Yolk, and Hypertrophy of Follicular Epithelium

Figure 21. Advanced Atresia With Phagocytic Cells Invading Ooplasm

Figure 22. Advanced Atresia Showing Hypertrophied Follicular Epithelium (Arrow Heads) and Ruptured Zona Radiata (AR)

Figure 23. Advanced Phagocytic Atresia With Numerous Phagocytic Cells and Disintegrating Zona Radiata (Double Arrows)

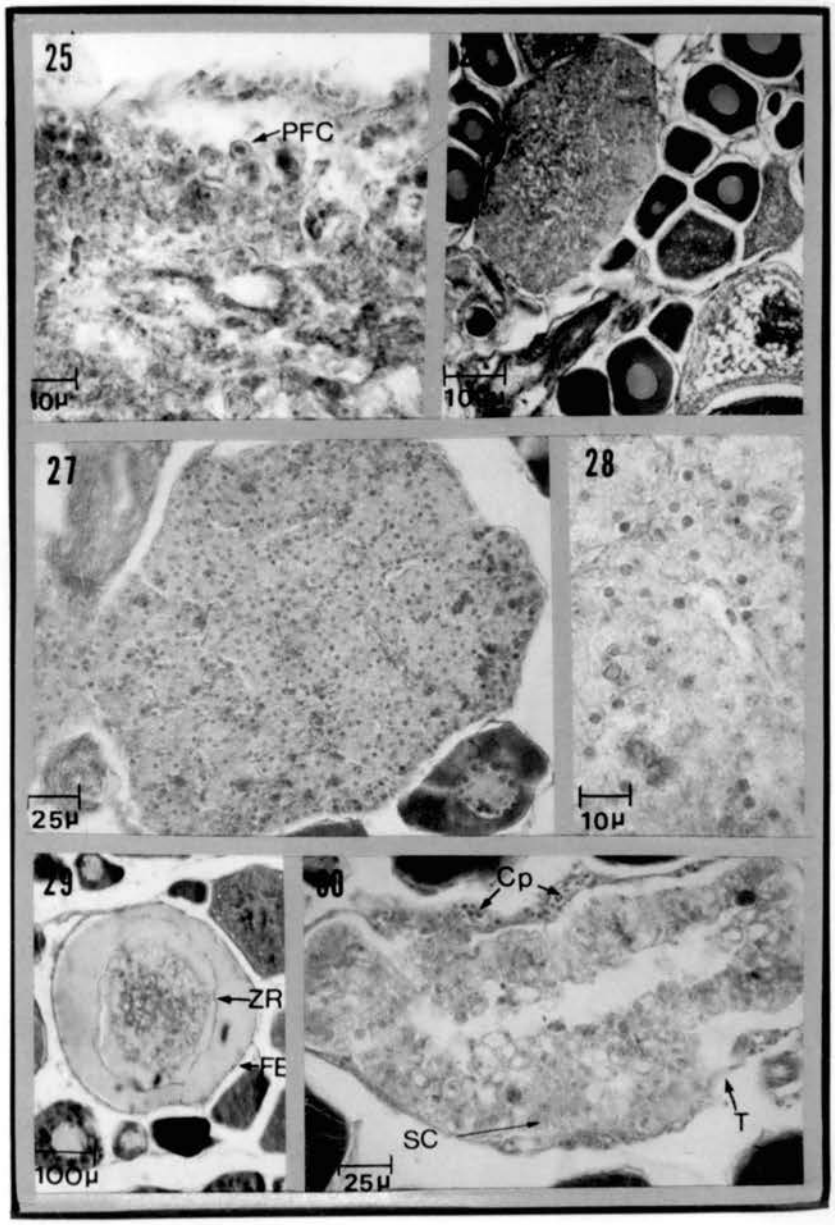
Figure 24. Advanced Atresia With Ooplasm Replaced by Phagocytic Cells


19
HFE
ZR
100 µ
20
HFE
ZR
25µ
21
PFC
ZR
22
ZR
23
PFC
25µ
25µ

congregate at the periphery of the oocyte and begin to ingest the yolk, producing an irregular mass of inward moving phagocytes surrounding a diminishing ball of yolk. The phagocytes are large amoeboid cells with a thick cytoplasmic membrane and clear pale cytoplasm which may contain yolk granules in various stages of disintegration. When the yolk has been consumed, all that remains is a ball of cuboidal or ovoidal follicular cells mixed with fibrous connective tissue.

In the first stages of phagocytic-hypertrophic atresia in uninfected golden shiners (Figures 19 and 20), erosion of the zona radiata is beginning, breakdown of yolk is apparent, and follicular epithelium is hypertrophied. The follicular epithelium continues to thicken (Figures 21 and 22), follicular cells become more numerous, and these cells begin to invade oocyte cytoplasm. In the final stages, the zona radiata is no longer evident (Figure 23) and the ooplasm completely fills with phagocytic follicular epithelial cells (Figure 24). The follicle fills solidly with phagocytic follicular cells (Figure 26), all ooplasm being removed. Atretic follicles eventually coalesce and lose their identity (Figures 27 and 28).

The process of atresia appears the same in unparasitized and parasitized oocytes. In the stage VI oocyte in Figure 31, all external membranes appear normal, but numerous sporonts are seen throughout the cytoplasm. As parasitic atresia begins, the zona radiata collapses and convolutes (Figure 32), the follicular epithelium undergoes hypertrophy and hyperplasia (Figure 33), and the oocyte continues to shrink. Variations to this pattern include the formation of a homogeneous matrix around the border of the oocyte just beneath the zona radiata (Figure 34); and, when the zona radiata and hypertrophied epithelium remain


25
PFC
10μ
27
25μ
28
10μ
29
ZR
100μ
Cp
SC
T
25μ

intact until spores constitute the major portion of the contents of the oocytes (Figure 35). Eventually, after all membranes disappear, primary oocytes begin to appear in the atretic stroma (Figure 36). Infiltration of phagocytic follicular cells (Figures 37 and 38) rapidly occurs after the zona radiata ruptures.

The phagocytic cells are large (10-15 microns) with distinct vesicular nuclei (Figure 39) and distinct cytoplasm. The nuclei are usually eccentrically located.

Following immigration of phagocytes, yolk vacuoles are quickly phagocytized (Figure 40). Spores are also phagocytized (Figure 41), but first undergo deformation and shrinkage (Figure 42). The resorption of the larger number of atretic oocytes requires more time because of the high prevalence of atresia in parasitized oocytes. In uninfected fish, the majority of mature ova are spawned and only a few remaining ova need be removed through atresia. However, most infected ova are apparently not spawned and undergo atresia before they mature.

In sections of lightly infected ovaries of fish taken in September and October, small areas of atretic stroma were found scattered throughout the entire ovary. Sections through heavily infected ovaries taken at the same time show the ovigerous lamella to be a mass of spore-filled atretic stroma with a few clumps of stage 0, I, II, and III oocytes scattered along the edges (Figure 36).

Atresia of parasitized oocytes is almost always the phagocytic-hypertrophic type. Atretic eggs eventually lose their distinct identity, the zona radiata contracts and disintegrates, and the follicular epithelium undergoes hypertrophy. Finally, the vestiges of the oocytes, some yolk, phagocytic follicular cells, and spores form a mass called

Figure 31. Stage VI Oocyte With Sporont (Sp) Filled Cytoplasm. A Double Layer of Primary Yolk (PY) Lies Just Beneath the Zona Radiata (ZR). Other Cell Structures are: Secondary Yolk (SY), Nucleus (N), and Follicular Epithelium (FE)

Figure 32. Stage VI Oocyte, Infected With Sporonts (Sp), Beginning to Undergo Hypertrophic Atresia. The Zona Radiata (ZR) is Becoming Irregular and the Follicular Epithelium (FE) is Beginning to Hypertrophy

Figure 33. Follicular Epithelial Cells (FE) are Invading Cytoplasm and Phagocytizing Yolk (YV) and Sporonts (Sp). A Stage III Oocyte is Shown Infected With Sporonts (a Rare Situation)

Figure 34. Atretic Follicle With Liquified Area (Arrow) Along One Margin. Primary Oocytes (PO) are Clumped Together as Nests

Figure 35. Outline of Two Infected Atretic Oocytes (IAF) With Intact Zona Radiata. Eventually, as a Result of Phagocytic Atresia, the Infected Atretic Follicles Will be Reduced to Stroma (St). Stage II (Primary) Oocytes (PO) are Beginning to Appear

Figure 36. Infected Ovary of Golden Shiner in Late August Showing Dense Stroma Becoming Infiltrated With Primary Oocytes (PO)

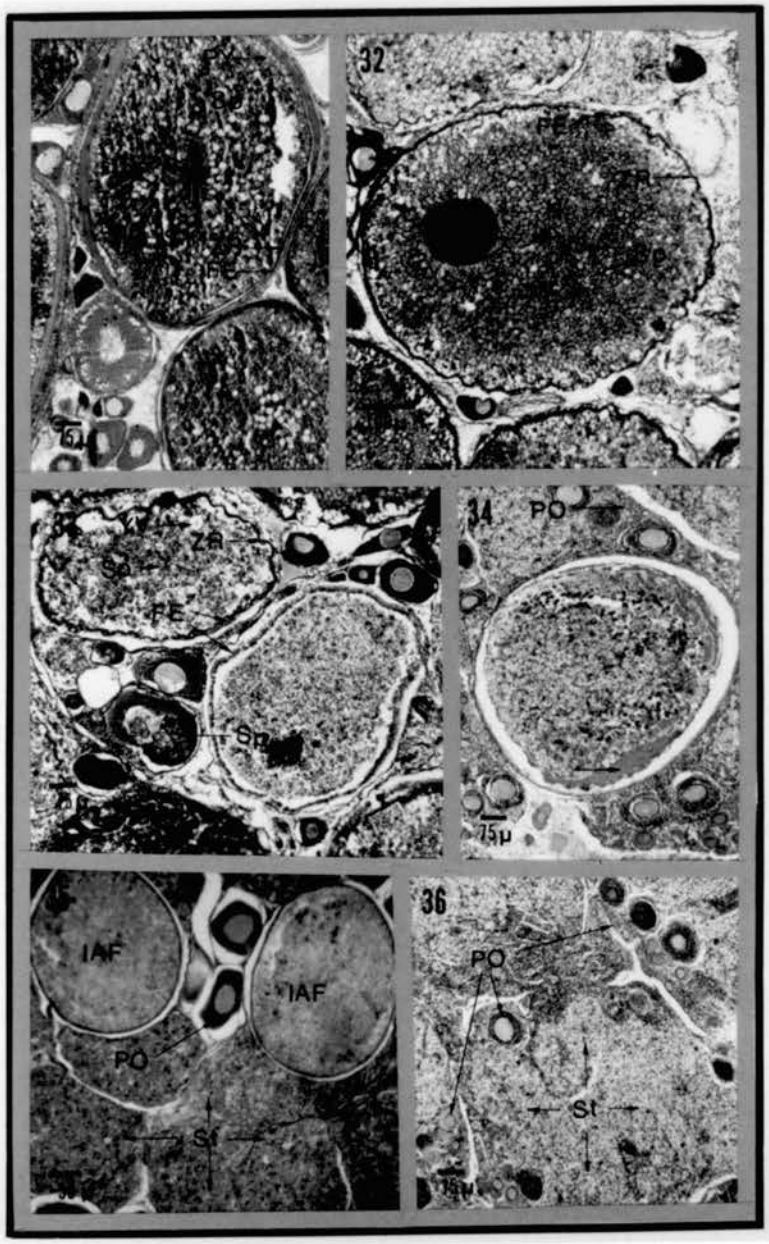

32
ZR
PE
33
ZR
Sp
PE
Sm
34
PO
75μ
IAF
IAF
PO
St
36
PO
St
75μ

Figure 37. Hypertrophied Follicular Epithelium (HPE) and Phagocytic Follicular Cells (PFC) Invading an Infected Oocyte. Spores (S) and Sporonts (Sp) are Numerous Within Oocyte. Vestige of the Zona Radiata (ZR) Still Exists

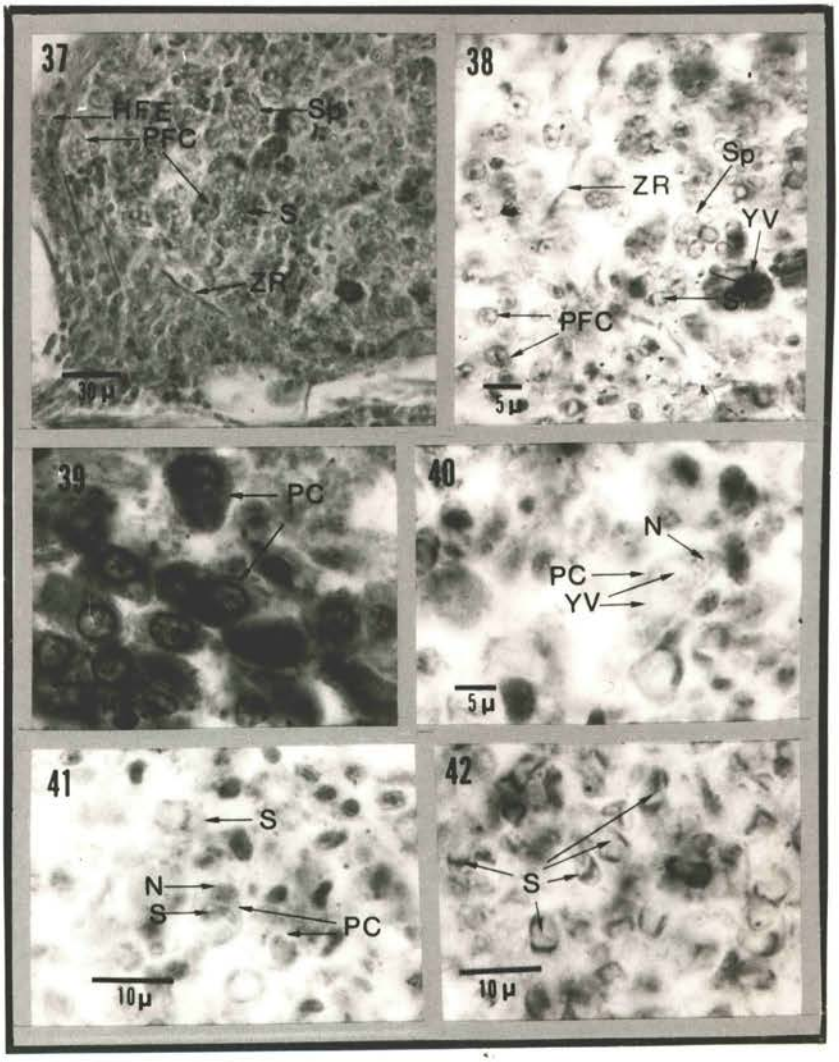
Figure 38. Phagocytic Follicular Cells (PFC) Penetrating the Ruptured Zona Radiata (ZR) to Phagocytize the Spores (S), Sporonts (Sp), and Yolk Vacuoles (YV)

Figure 39. Cellular Detail of Phagocytic Cells (PC)

Figure 40. Phagocytic Cell (PC) Which Presumably Engulfed Two Yolk Vacuoles (YV). Nucleus (N)

Figure 41. Phagocytic Cells (PC). The Remanent of Phagocytized Spores (S) are Visible in One Cell. Above is Another Spore (S) Surrounded by Phagocytes

Figure 42. Spores in Various Stages of Necrosis


37
HRE
Sp
PFC
S
ZR
30 µ
38
Sp
ZR
YV
S
PFC
5 µ
39
PC
40
N
PC
YV
5 µ
41
S
N
S
PC
10 µ
42
S
10 µ

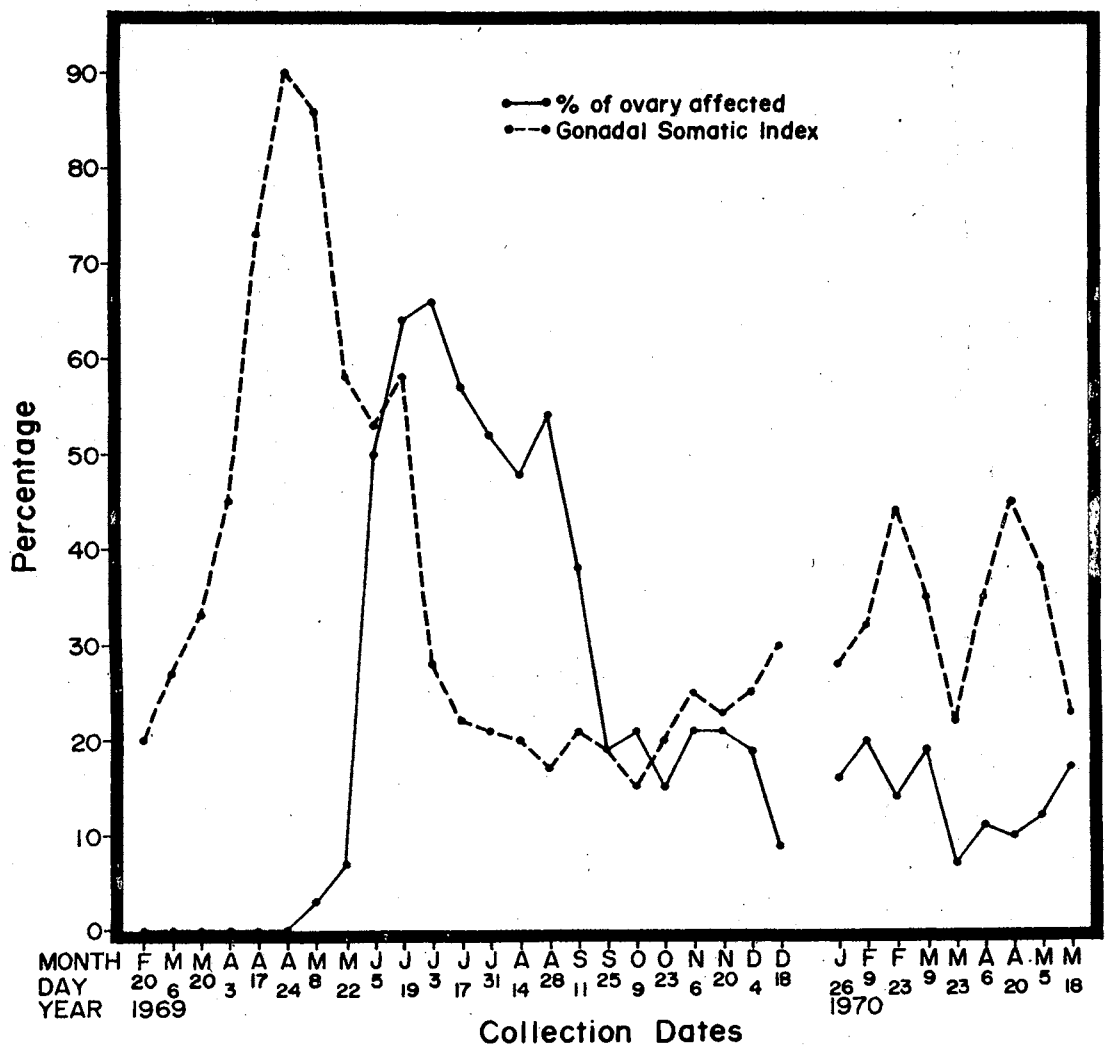
"stroma". The spores, which have been liberated from the sporonts, are mixed diffusely throughout the stroma. During the process of atresia, the spores begin to round up and their sporoplasm begins to clump and disappear.

Once a patent infection becomes established within the fish, almost every oocyte undergoing atresia shows evidence of parasitism. Only one atretic oocyte was found in the 12 uninfected fish sampled after the May 8, 1969 collection period (Table II). Thus, P. ovariae apparently increases the incidence of atresia, especially hypertrophic atresia.

The percentage of ovary affected (the percentage of the ovary occupied by infected oocytes and spore-filled stroma) serves as an indicator of the intensity of parasitism in a given fish. This percentage (Figure 43) increases rapidly as stage VI eggs become abundant. The slow decrease in percentage of ovary affected after spawning indicates the time it requires for phagocytosis, atresia, and production of new oocytes. When stage I and II eggs are predominant during the late summer and fall of 1969, the percentage of ovary affected levels off between 15-20%. This level is maintained throughout the year by the few eggs that are continually maturing and becoming infected. However, this 15-20% affected level reduces the area available for development of new oocytes. The result of this reduction in available area is a corresponding decrease in oocyte production and a drop in fecundity.

All oocytes, stage III and above (Figure 17), reflect a decrease in total egg production in 1970 compared to 1969. Both total number of oocytes and per cent area occupied by oocyte stage VI are down (Figure 18). The average number of stage III and IV eggs in 1970 is 12% less than 1969. The average number of stage V eggs is 10% less and stage VI

Figure 43. Biweekly Changes in Mean Percentage of Ovary Affected


% of ovary affected
Gonadal Somatic Index
Percentage
90
80
70
60
50
40
30
20
10
0
MONTH F M M A A A M M J J J J J A A S S O O N N D D J F F M M A A M M
DAY 20 6 20 3 17 24 8 22 5 19 3 17 31 14 28 11 25 9 23 6 20 4 18 26 9 23 9 23 6 20 5 18
YEAR 1969 1970
Collection Dates

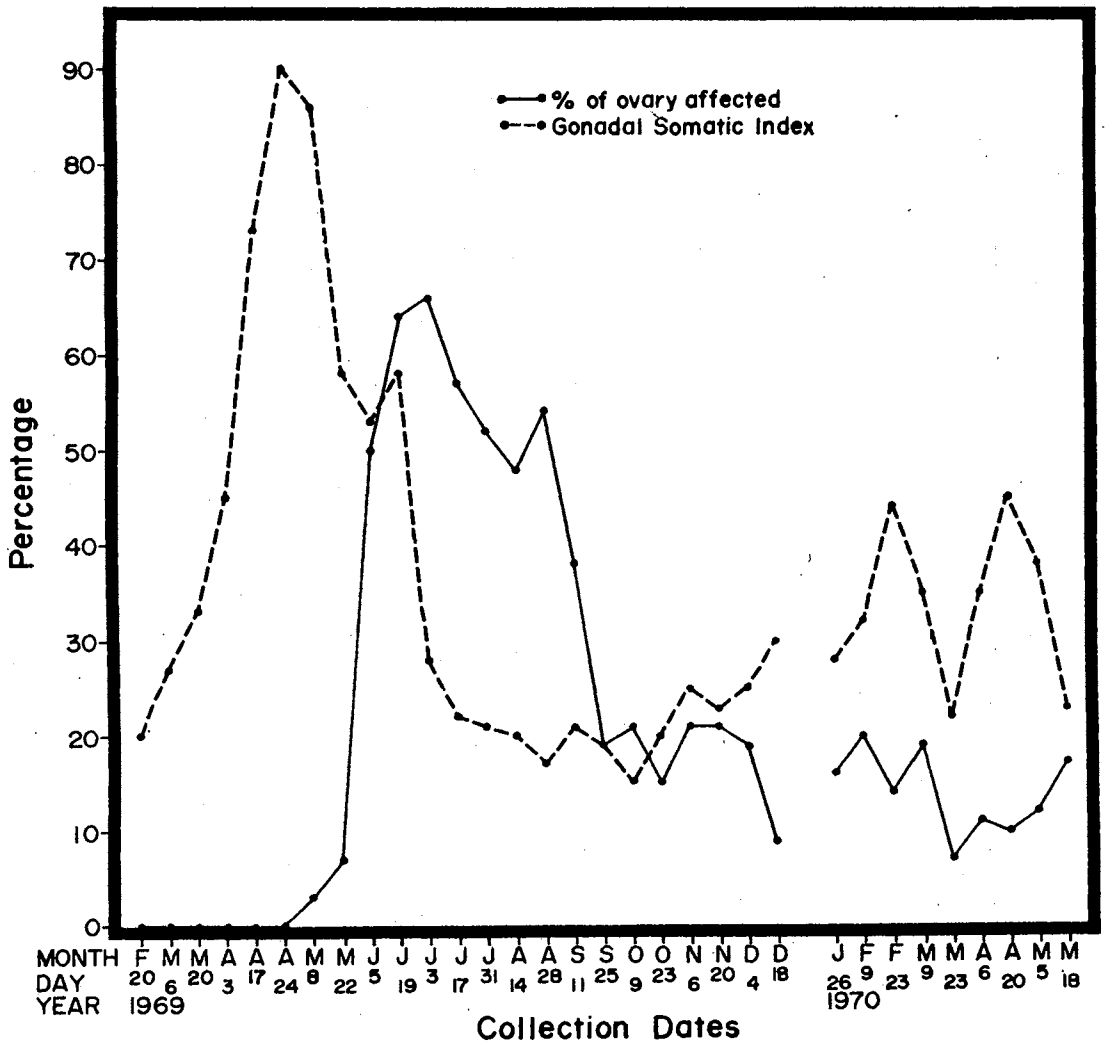
eggs 24% less in 1970 compared with 1969. The maximum gonadal-body ratio (GSI) is twice as high in May 1969 (9.0) as in May 1970 (4.5) (Figure 44). Fish ovary size increases as fish grow and, consequently, the GSI remains about the same during any given season of the year. The reduction in GSI during May 1970 also indicates a decrease in fecundity.

Nonhypertrophic Atresia

Nonhypertrophic atresia occurs without hypertrophy and phagocytosis by follicular cells (Figure 29). The usually eosinophilic yolk becomes basophilic, finely granular and sparse. The vitelline membrance erodes early or remains intact. In either case, the contents of the oocyte undergoes lysis.

Nonhypertrophic atresia is fairly common (1.5% of oocytes) prior to spawning and the first occurrence of *P. ovariae* within the oocytes. The rate of occurrence of nonhypertrophic atresia was less than 0.16% of 25,000 oocytes checked between May 22, 1969 and May 18, 1970. Nonhypertrophic atresia was not observed in parsitized oocytes. The frequency of occurrence of nonhypertrophic was considerably less in 1970 than 1969. From September 1969 on this incidence of this type atresia never exceeded 0.2%, whereas, it reached as high as 3.3% in early 1969 (Table II). The reduction in nonhypertrophic atresia in 1970 seems to coincide with an increased level of parasitism. Phagocytosis rather than proteolytic lysis is the form atresia associated with parasitism.

Figure 44. A Comparison of Biweekly Fluctuation in Mean Gonadal-Somatic Index and Percentage of Ovary Affected


% of ovary affected
Gonadal Somatic Index
Percentage
0
10
20
30
40
50
60
70
80
90
MONTH F M M A A A M M J J J J J A A S S O O N N D D J F F M M A A M M
DAY 20 6 20 3 17 24 8 22 5 19 3 17 31 14 28 11 25 9 23 6 20 4 18 26 9 23 9 23 6 20 5 18
YEAR 1969 1970
Collection Dates

Corpus Luteum

Hoar (1965) says there is no agreement concerning the locus of hormone production in the fish ovary. The ovum, follicular cells, and corpus luteum are possible sources of the estrogens. Hoar regards the pre-ovulation corpus luteum, acting under the direction of the pituitary, as the source of sex steroid hormones responsible for secondary sexual characteristics of spawning. Oviparous fish appear to have little need for an extended post-ovulatory corpus luteum function.

The structure of the corpus luteum (Figure 30) differs from those of atretic follicles. The luteal cells, apparently derived from the follicular layer, are columnar, highly vacuolated, and presumably secretory. The corpus luteum seems to be produced by luteunization without ovulation. It was highly vascularized with an abundance of small capillaries, a situation not seen elsewhere in the ovary.

Intraovarian Life Cycle

Schizogony

Schizogony, the asexual phase of the life cycle, was defined by several investigators (Chandler and Read, 1961; Hickman, 1961; Levine, 1961; Hall, 1964; Kudo, 1966) as multiple fission in which several nuclear divisions occurred before the cytoplasm divided. However, Van Duijn (1967) defined schizogony as fission (either by multiple or binary fission) without a preceding sexual process. Schizogony by binary fission in microsporidans of fish has been reported in *Glugea* sp. (Sprague and Vernick, 1967) and *Pleistophora ovariae* (Summerfelt and Warner, 1970a). Multiple fission has been reported for *P. ovariae*

(Wilhelm, 1964) and Glugea hertwigi (Delisle, 1969). Binary and multiple fission within the life cycle of a single species of microsporidan has been reported in insects (Dissanaike, 1957; Canning et al., 1964; and McLaughlin, 1969), but only by Wilhelm (1964) in fish.

Wilhelm (1964) described a sequence of stages in the development of P. ovariae including multinucleate and uninucleate schizonts and uninucleate sporonts. Wilhelm considered certain small multinucleate bodies as early schizonts. They were thought to undergo growth and additional nuclear division to produce larger multinucleate schizonts. He assumed the multinucleate form preceded the uninucleate form, although he did not account for the origin of the multinucleate form. Wilhelm, therefore, characterized the sequence of stages in the oocytes as starting with a small multinucleate form which, by growth and nucleogamy, gave rise to a larger multinucleate plasmodium. The plasmodium gave rise to numerous uninucleate schizonts which multiplied by binary fission.

Weiser (1949), in his description of P. oolytica, said the youngest forms were oval schizonts with one or two nuclei. These were seen in the vacuoles of the yolk of the eggs where they remained throughout their life. Nuclear division occurred and was followed by division of cytoplasm into multinucleate bodies. The findings of the present study agree with this type of life cycle. Wilhelm (1964), however, confused the multinucleate sporont with a schizont, but since he did not illustrate these with photomicrographs, it cannot be certain what he saw.

Pleistophora ovariae appears as a spherical schizont, about eight microns in diameter, within the cytoplasm of stage II (rarely), III, and IV oocytes (Figure 45). This regular outline suggests that the

schizont is incapable of movement. The schizont appears to be bounded by a heavy membrane (about 2 μ) and "halo". The heavy membrane around the nucleus seems to represent a basophilic cytoplasm. Chromatin granules are apparent throughout the nucleoplasm.

Schulman (1962) and Lom and Corliss (1967) assumed that this type halo was caused by saprozoic action of the schizont on the host cell cytoplasm, probably proteolytic action of enzymes. Catabolic activity of P. ovariae was indicated by a halo of lysed host's cytoplasm (Summerfelt and Warner, 1970a) (Figure 8). Lom and Corliss (1967) mentioned an envelope surrounding young unicellular schizonts of P. hypessobryconis consisting of lysed host muscle, and Kudo (1924) described a clear space surrounding schizonts of Nosema bombycis. The comment by Lom and Corliss implied that the parasite was extracellular; however, they did not illustrate this condition.

The schizont apparently divides by binary fission (Figures 46 to 53). Anaphase and telophase phases of mitosis are shown in Figures 49 and 50, respectively. However, the division of the schizont appears to differ from classical mitosis because the nuclear membrane is apparent during the entire process. The schizont divides repeatedly (Figure 54) until clumps of schizonts (Figure 55) surround the host cell nucleus. Schizogony was more prevalent during late June and early July, but significant numbers of schizonts were found in stage III and IV eggs throughout the entire year (Table III).

The process of schizogony apparently occurs very rapidly. In sampling at biweekly intervals, a sharp increase in numbers of oocytes infected with schizonts occurred between two sampling periods (May 8 to May 22, 1969) (Table III). The process of schizogony, therefore,

TABLE III

PERCENTAGE OF EACH EGG STAGE INFECTED WITH SCHIZONTS AND SPORONTS DURING EACH COLLECTION PERIOD, INCLUDING ATRETIC OOCYTES FOR WHICH THE EGG STAGE WAS STILL IDENTIFIABLE

Collection Period	Schizonts in Egg Stages				Sporonts in Egg Stages				
	II	III	IV	V	III	IV	V	VI	VII
2/20/69	-	-	-	-	-	-	-	-	-
3/6/69	-	1	-	-	-	-	-	-	-
3/20/69	-	2	1	-	-	-	-	-	-
4/3/69	-	2	-	-	-	-	-	-	-
4/17/69	-	2	1	-	-	-	-	-	-
4/24/69	-	3	-	-	-	-	3	6	-
5/8/69	1	3	3	-	-	-	2	6	-
5/22/69	-	-	-	-	-	8	12	21	-
6/5/69	2	2	4	3	-	3	53	70	-
6/19/69	1	10	6	-	-	14	50	79	-
7/3/69	3	9	24	-	1	6	100	98	100
7/17/69	1	6	-	-	1	-	9	20	44
7/31/69	-	10	-	-	-	-	-	100	33
8/14/69	2	16	-	-	-	-	-	-	-
8/28/69	1	3	-	-	-	-	-	100	-
9/11/69	-	-	-	-	-	-	-	-	-
9/25/69	-	-	-	-	-	-	-	-	-
10/9/69	-	4	9	-	-	-	-	-	-
10/23/69	-	3	6	-	-	-	-	-	-
11/6/69	-	2	4	-	-	-	-	-	-
11/20/69	-	2	2	-	-	-	-	-	-
12/4/69	-	3	1	-	-	-	39	27	-
12/18/69	-	-	3	-	-	-	-	100	-
1/26/70	-	3	6	-	-	-	-	-	-
2/9/70	-	1	-	-	-	-	25	38	100
2/23/70	-	2	3	-	-	-	-	50	-
3/9/70	1	7	3	5	-	-	-	-	-
3/23/70	-	2	2	-	-	-	-	-	-
4/6/70	-	1	5	-	-	-	1	3	-
4/20/70	-	5	7	2	-	-	2	2	-
5/5/70	-	2	-	-	-	-	-	3	-
5/18/70	-	1	4	-	-	4	26	11	-

Figure 45. Stage III Oocyte With Early Schizont: Cytoplasm (C), Nucleus (N), and Follicular Epithelium (FE)

Figure 46. Stage III Oocyte With Early Schizont Containing Chromatin Granules (CR). X1000

Figure 47. Schizont (Sc) in Cytoplasm of Stage III Oocyte. Chromatin in Schizont Heterochromatic. Nuclear Membrane (NM)

Figure 48. Schizont Beginning Binary Fission

Figure 49. Schizont in Anaphase in Stage IV Oocyte: Chromatin (Cr) and Primary Yolk (PY)

Figure 50. Schizogonic Binary Fission: Follicular Epithelium (FE)

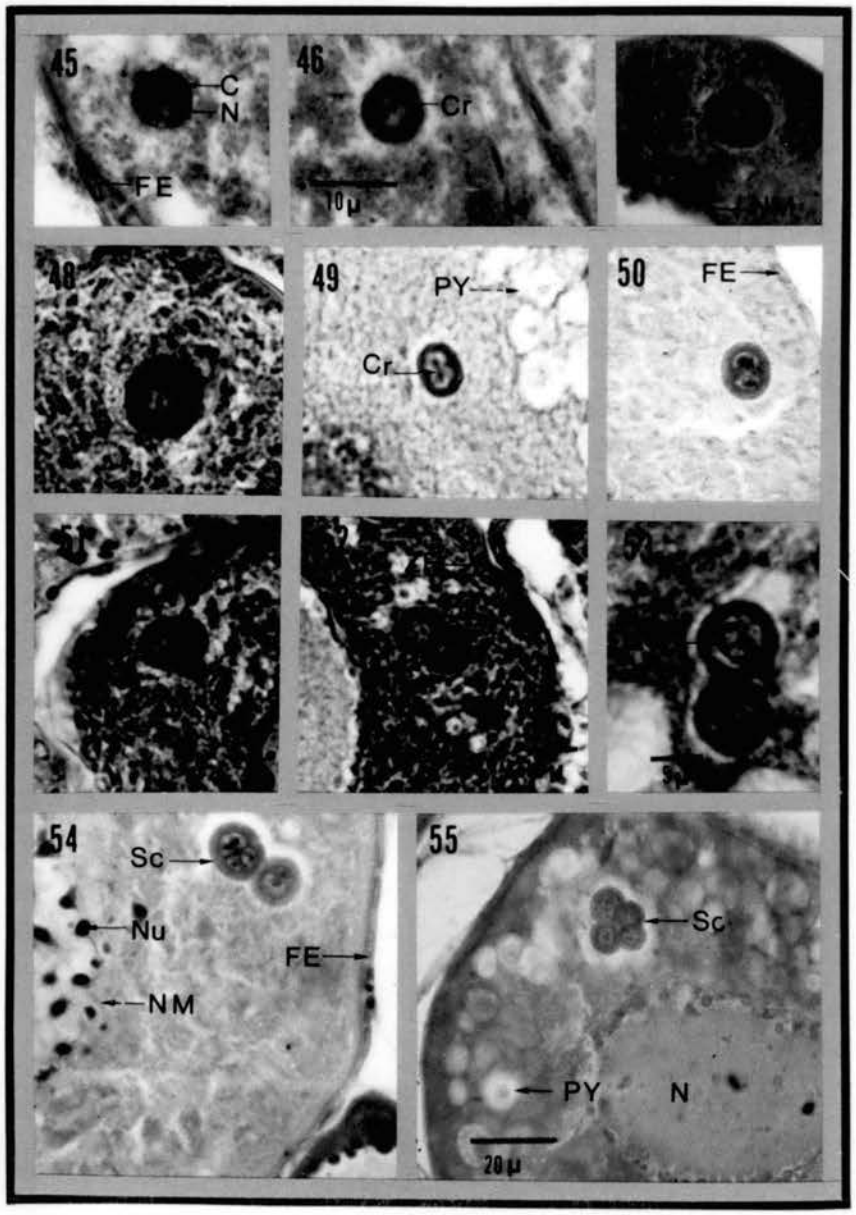
Figure 51. Schizogonic Binary Fission

Figure 52. Nuclear Division Complete: Zona Radiata (ZR) and Nuclear Membrane (NM)

Figure 53. Binary Fission Complete Showing Chromatin (Cr) in Strands

Figure 54. Repeated Binary Fission: Schizonts (Sc) With Halo of Lysed Cytoplasm, Nucleolus (Nu), Follicular Epithelium (FE), Nuclear Membrane (NM)

Figure 55. Stage IV Oocyte With Group of Schizonts (Sc) Formed by Repeated Binary Fission: Primary Yolk (PY), Nucleus (N)


45
C
N
FE
46
Cr
10 µ
48
49
PY
Cr
50
FE
54
Sc
Nu
FE
NM
55
Sc
PY
N
20 µ

probably requires less than the two-week interval. Not more than 24% of a given egg stage (stage IV) was infected with schizonts during one collection period (July 3, 1969), yet almost all eggs maturing to stages VI and VII were infected with sporonts. Maturation of the schizonts to sporonts required less than two weeks.

Wilhelm (1964) described a schizogonic sequence as going from a multinucleated to a uninucleated schizont by multiple fission. The appearance of uninucleate schizonts in stage II, III, and IV oocytes, and multinucleate forms (sporonts) in stage IV and V oocytes indicates that the uninucleate form proceeds the multinucleate forms. Therefore, the multinucleate schizonts described by Wilhelm must be early sporonts rather than Schizonts.

Sporogony

Weiser (1949) described the sporogonic sequence of *P. oolytica* in the ovary of the pike, *Esox lucius*. He described young plasmodia (schizonts) in the yolk vacuoles which divided one or more times and formed pansporoblasts (sporonts) eventually filling the entire egg follicle. Finally, after spores formed within the pansporoblasts the pansporoblast membrane ruptured and spores mixed with the egg contents. The egg membranes then ruptured and follicular cells phagocytized the follicle contents. He described follicles of the latter type as "large areas filled with spores and stroma."

Wilhelm (1964) said the sporont appeared to develop directly from the uninucleate schizont and that no evidence of a sexual process existed. The sporonts enlarged and were observed containing two, four and more sporoblasts. In later sporonts, the nucleus disappeared and

8, 16, or more than 16 spores were present. When the sporont was mature it ruptured and "scattered" its spores through the egg cytoplasm.

Differences in sporogonic phase of the life cycle have been used as a basis for separating genera among the microsporida (Kudo, 1924, 1966). Kudo used differences in the number of spores that develop within sporonts and whether or not the spores caused the host cell to hypertrophy to separate genera. However, exceptions to Kudo's criteria tend to lessen their value as taxonomic characters. Lom and Weiser (1969) suggested that Glugea should be a junior synonym of Nosema because the ripe spores of Glugea occur alone most of the time, and when paired they appeared that way because of adhesions, not because they were formed from the same sporont. Also, Nosema lophii (Bykhovskaya-Pavlovskaya, 1962) and Pleistophora salmonae (Putz et al., (1965) produced cellular hypertrophy.

In golden shiners sporogony generally begins when eggs mature to late stage IV. Sporonts first appear in clusters (the result of schizogony) near the nucleus of the oocyte. The early sporonts (Figure 56) are larger (15 μ) than schizonts, the thick cytoplasmic border surrounding the nucleus is disappearing, and chromatin more visible. The nucleoplasm undergoes sporogonic karyokinesis until three to six nuclei are visible (Figure 57). At this time, the sporont nucleoplasm appears mottled because of clumps of nuclear material. This is the stage that Wilhelm (1964) called a multinucleate schizont. Plasmotomy occurs, without disruption of the sporont membrane, forming eight or more masses, each with a single nucleus (Figure 58). These nuclei undergo another division to form eight or more dyads (Figure 59) still contained within the sporont membrane. The dyads are elongate (10-12 μ) and stain

red with Mallory's stain. They divide by binary fission, each dyad becoming two spherical sporoblasts (Figure 60 and 61) which are spore precursors measuring 3-4 μ diameter. Sprague and Vernick (1968) postulated that remanents of the nuclear isthmus, formed during division of the dyads, became the "basal" portion of the future polar filament and was the filament primordium. Sporoblasts develop into the mature spores (Figure 62). Mature spores stained with Mallory's have a yellow-red band near the center of the spore.

In stage VI eggs, the sporonts appear throughout the cytoplasm (Figure 63) and all stages of sporont development can be found in a single oocyte. Thus, the developmental rate of individual sporonts within the same oocyte is not syncronized. The time required for sporogony, like schizogony, is apparently quite short (less than two weeks).

Seasonal Variation in Life Stages of *P. ovariae* and Reproductive Cycle of the Host

The time required from penetration of a susceptible host cell to production of spores is termed the generation time. It has been determined for only a few microsporidans and ranged from 1 to 24 days.

McLaughlin (1969) reported occurrence of mature spores of *Glugea gasti* in boll weevil (*Anthonomus grandis*) within 24 hours (at 27-28° C) after exposure. *Nosema apis* spores were recovered from newly infected bees (*Apis mellifera*) after 14 days at 37° C (Burnside and Revell, 1948). The generation time of *Nosema acridophagus* in grasshoppers was six to eight days (Henry, 1967). Sikorowski and Madison (1968) reported that the generation time of *Thelohania corethrae* in the dipteran

Figure 56. Early Sporont (Arrow) With Clumped Chromatin: Cytoplasm (C), Nucleus (N), and Nucleolus (Nu)

Figure 57. Sporonts (Arrows) Undergoing Nucleogamy, Clumped Near Nucleus (N) of Stage VI Oocytes. Nuclear Membrane (NM)

Figure 58. Sporonts (SP) Undergoing Plasmotomy

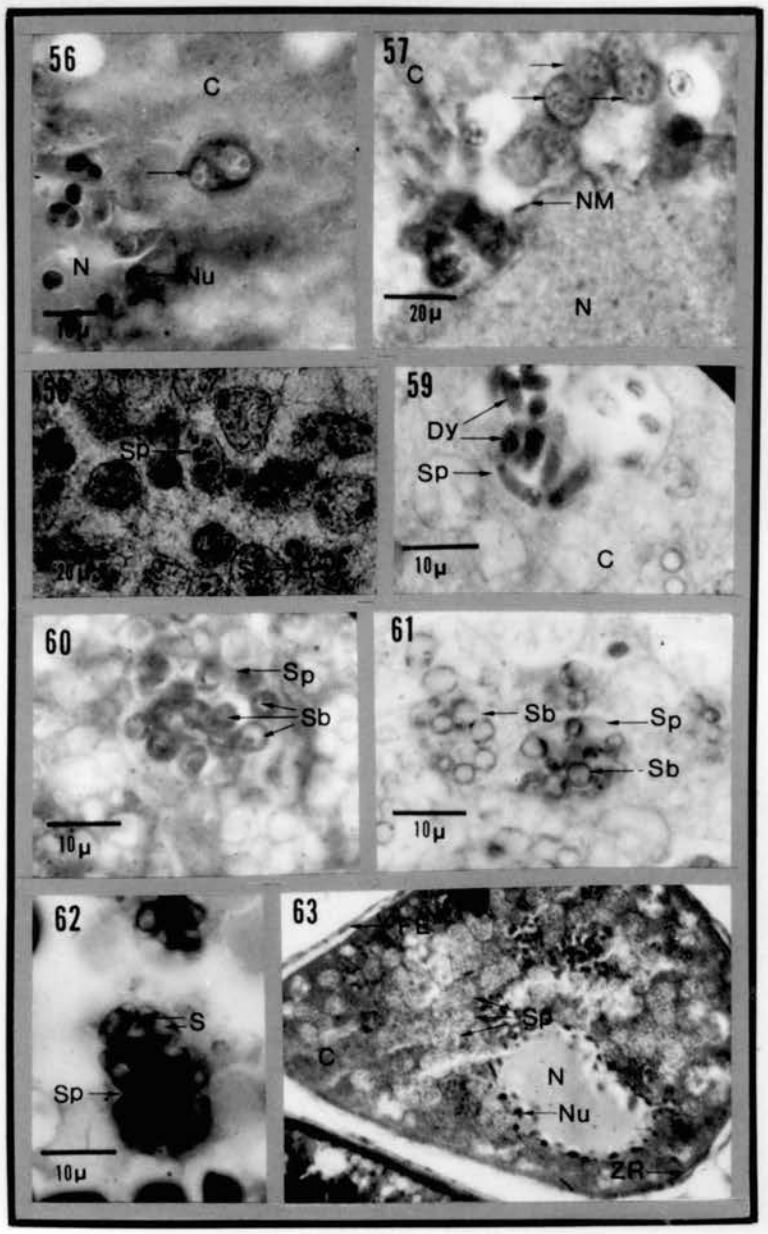
Figure 59. Dyads (Dy) Within Sporont. Each Dyad Divides to Produce Two Sporoblasts. Cytoplasm (C)

Figure 60. Sporoblasts (Sb), 12 are in Focus, Within a Sporont (Sp)

Figure 61. Cytoplasm of Stage VI Oocyte Filled With Sporonts (Sp) Containing Sporoblasts (Sb)

Figure 62. Mature Spores (S) Within Sporont (Sp) of Stage VI Oocytes

Figure 63. Stage VI Oocyte With Cytoplasm (C) Completely Filled With Sporonts (Sp) Containing Sporoblasts and Spores


56
C
N
Nu
10μ
57
C
NM
N
20μ
58
Sp
20μ
59
Dy
Sp
C
10μ
60
Sp
Sb
10μ
61
Sb
Sp
Sb
10μ
62
S
Sp
10μ
63
Sp
C
N
Nu
ZR

Chaoborus astictopus was 16 to 24 days at 20° C. The generation time of Pleistophora myotrophica, a muscle parasite of the common toad Bufo bufo, was noted as 18-23 days, measured from the time infected muscle was fed to uninfected toads until spores appeared in the toad's muscle (Canning et al., 1964). In fish, young-of-the-year smelt (Osmerus eperlanus mordax), spores of Glugea hertwigi appeared three months after hatching (Delisle, 1969).

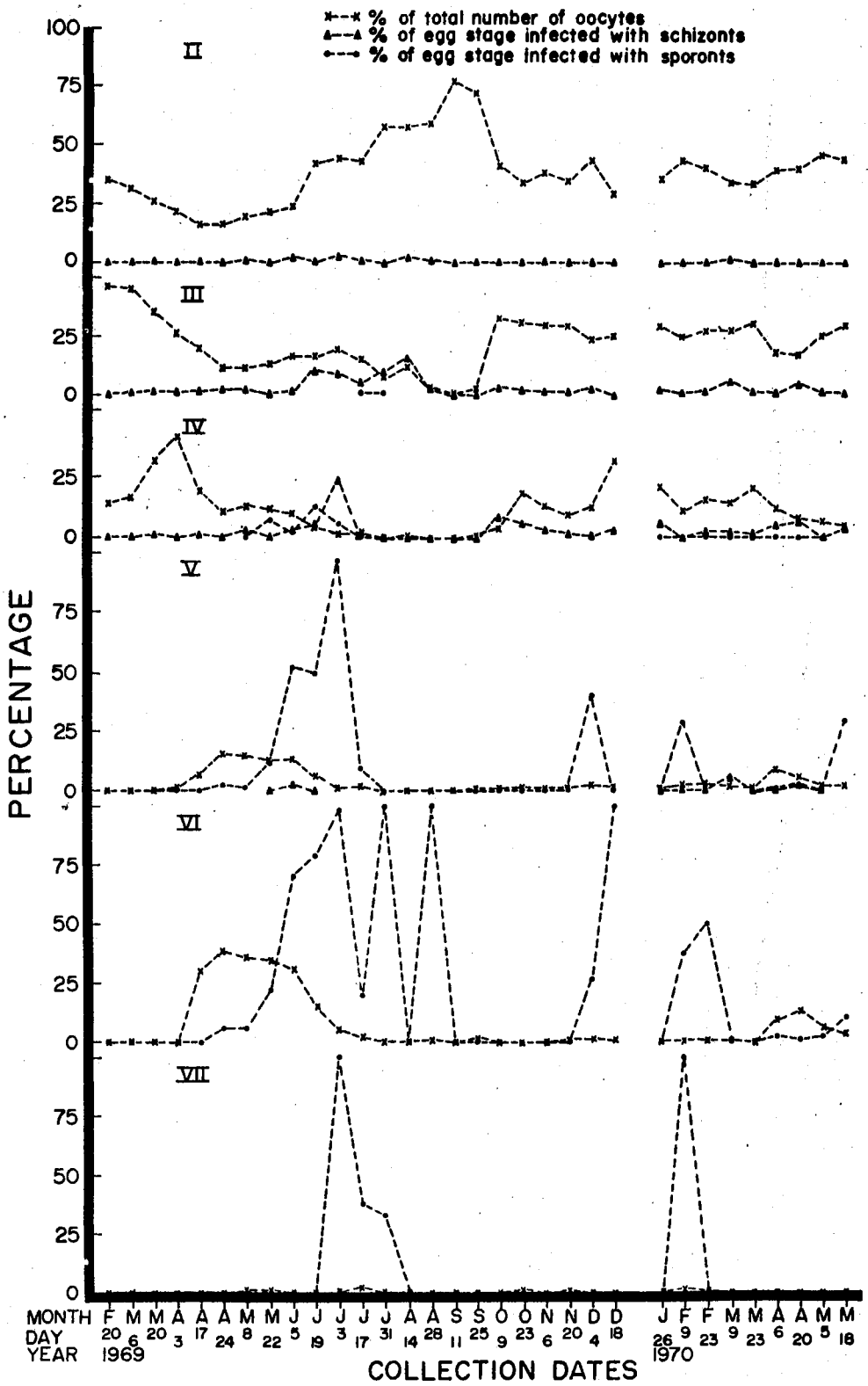
The generation time of P. ovariae apparently is highly variable. It probably depends on conditions within the ovary such as the presence of proper oocyte stages. Although the fish used in this research are progeny of parents from a population in which incidence of infection was 100%, light infections were noted in seven collections (March 6 - May 8) (Table II). However, between May 22 and January 26, the percentage of uninfected fish was generally zero. Thus, in 1969, patent infections occurred when the fish approached their first spawning. These fish had no known contact with infected fish from the stocking date (February 20, 1968). To recapitulate the facts about these fish, they were progeny of parents in which infection was 100%, showed slight infections in midwinter, they lacked apparent exposure to spores or sources of infection, but showed a progressive increase in incidence and intensity as spawning approached. Two hypotheses are offered to explain the development of parasitism in these fish. A submicroscopic stage could have existed which transformed, or developed into a microscopically visible stage because of some stimulus (hormonal or nutritive) associated with spawning. It must, therefore, be concluded that in the initial infection of golden shiners with P. ovariae, the "germ" is capable of staying latent for an extended period of time (up to 10

months in this case), alternatively, a very low frequency of occurrence of schizonts served as a foci for rapidly spreading autoinfection when stimulated by the approaching spawning season. Autoinfection would require maturation of spores and prespawning atresia to release the spores and facility germination and transmission within the ovary. Atresia was observed to obtain a high frequency coincident with development of a high frequency of occurrence and intensity of infection in prespawning fish. The former hypothesis cannot be evaluated, but the latter is tenable because the development of sporonts from schizonts and the formation of new schizonts after the development of new stage III oocytes requires less than two weeks (Table III, Figure 64) after a patent infection is established.

When examining individual ova for the presence of schizont and sporont stages of P. ovariae, Wilhelm (1964) stated that "egg stage and size could not be directly correlated with any particular stage of the microsporidan." He based this conclusion on an observation that uninucleate and multinucleate schizonts and developing sporonts could be found together. However, he did not characterize oocyte development nor did he quantify these observations. He sampled fish during "spring, summer, fall, and winter," and found "abundant" developmental stages in all seasons. From this he concluded that occurrence of "various developmental stages were not seasonal in nature."

Wilhelm's statements on the relationship of egg stage and parasite stage, and seasonal occurrence of P. ovariae are in error because P. ovariae were not observed in oocyte stages 0 and I, and only rarely are schizonts found in stage II oocytes (Figure 64). Only schizogonic, not sporogonic, stages of P. ovariae (with two exceptions) were found

Figure 64. Percentage Occurrence of Oocyte Stages II Through VII in Samples of 1000 Eggs Sampled From 10 Fish Per Collection, and Incidence of Schizonts and Sporonts in Oocyte Stages II Through VII


% of total number of oocytes
% of egg stage infected with schizonts
% of egg stage infected with sporonts
PERCENTAGE
II
III
IV
V
VI
VII
100
75
50
25
0
MONTH F M M A A A M M J J J J J A A S S O O N N D D J F F M M A A M M
DAY 20 6 20 3 17 24 8 22 5 19 3 17 31 14 28 11 25 9 23 6 20 4 18 26 9 23 9 23 6 20 5 18
YEAR 1969 1970
COLLECTION DATES

in stage III eggs. Schizogony commenced in stage II oocytes, and was very active in stages III and IV, but rare in the stage V oocyte. Schizonts were first observed in March 6, 1969 specimens (Figure 64) when they were present in about 1% of the stage III eggs. The prevalence in stage III eggs was less than 5% until July 1969 when the number of schizonts increased to 10%, then increased again on August 14 when one or more schizonts occurred in 16% of the stage III eggs.

Schizonts are the most prevalent parasitic form found in stage IV eggs (Figure 64). The maximum percentage of stage IV ova infected (24%) was recorded on July 3, 1969, the same date the maximum percentage of ovary affected (66%) was recorded (Figure 43). During late June and early July, a few sporonts were found in stage IV eggs (Table III). The latter observation demonstrates that the sporogonic cycle can be completed in oocytes containing only primary yolk. However, the absence of sporonts in stages younger than III, their rarity in stage III, and common occurrence in stage IV or older oocytes indicates that sporogony may have a nutritional dependence on primary yolk.

Often the stage IV eggs, which contain sporonts, have a homogeneous, basophilic cytoplasm apparently undergoing the first stages of atresia. Schizonts are rarely found in stage V and sporonts and spores were the only recognizable parasitic forms in stage VI and VII eggs (Table III, Figure 64).

The structure of the stage III egg lends itself well as a site for schizogony. The zona radiata is monolaminar and thin (Table I). Thus, it would be easier for the amoeboid stage of the parasite to enter, or the polar filaments to penetrate, the oocyte. Furthermore, the microsporida, which have no mitochondria, can use those which are becoming

functional in the host cell for their own needs (Sprague and Vernick, 1968). The follicular epithelium in stage IV oocytes is thicker and becomes bilaminar by the end of this stage.

Schizogony occurs primarily in stage III and IV oocytes while sporogony is most prevalent in stages V, VI, and VII. Since the ovary undergoes an annual cycle in regards to oocyte development, the preference of certain parasite stages for certain oocyte stages will make the abundance of various parasite stages seasonal in nature. Nevertheless, as Wilhelm (1964) observed, since some oocytes ripen during all seasons of the year, it is possible to find any given parasite life stage at any time during the year.

Autoinfection

Spores of certain microsporidians (*Nosema apis*, *N. bombycis*, *Perezia legeri*) germinate in the same host in which they were formed (Kudo, 1924, p. 34). Such autoinfection perpetuated these parasites in the hosts and was alluded to by many authors in reference to progressive infections (Kramer, 1964; Kellen et al., 1965). The constant occurrence of *P. ovariae* in the shiner ovary strongly suggested perpetuation by autoinfection (Summerfelt and Warner, 1970a, 1970b).

Another mode of autoinfection involved a "secondary infective form." Ishihara (1969) described "a secondary infective form" of *Nosema bombycis* in tissue culture cells of *Bombyx mori*. This form differed from the "germ" in its larger size, variable shape, and stronger affinity for stain (Giemsa). It presumably was responsible for spreading the protozoan from one cell to another within the host.

Atresia of ova infected with P. ovariae is commonplace resulting in liberation of spores, and perhaps schizonts, "infective forms," or all three. Spores of P. ovariae liberated from an atretic oocyte may germinate and produce autoinfection through the usual life cycle. Alternately, transmission may be by means of schizonts or "secondary infective forms" which might migrate from an oocyte prior to development of a thickened zona radiata. Several lines of evidence indicate autoinfection in P. ovariae occurs by germination of the spores; however, this must be regarded as inconclusive because of the possibility for continuous per os transmission.

Spores escape from the body of the fish during spawning; some adhere to the surface of eggs being spawned (Summerfelt and Warner, 1970a); and other spores released by atresia are present in prodigious numbers in the spawning roe. The spawning season limits the time during which fish are exposed to fresh spores in the external environment. Yet, as recorded in Table III and Figure 64, newly maturing stages II, III, and IV eggs become infected with schizonts throughout the entire year. It is possible that these new infections are the result of spores that exist in the pond bottom for extended periods of time. However, in light of the information on the relationship between percentage of ovary affected and numbers of stage III oocytes becoming infected, and similar findings with stage IV oocytes, it is more likely that these infections are the result of autoinfection.

When a large portion of the ovary is filled with mature spores and atretic follicles (more than 50%) on August 14, 1969, the number of newly matured stage III eggs infected with schizonts reaches a maximum (16%). However, a rapid increase in numbers and area of stage III eggs

occurred between September 25 and October 9, 1969 (stage III oocytes increase to 32% of total eggs and 60% of area), when a relatively small portion of the ovary was affected (less than 25%), and the percentage of eggs with schizonts was only 4%. This evidence suggests that most schizonts observed within these eggs are the result of autoinfection.

Transmission

The beginning of a new generation of Microsporida starts with the escape of the "germ" from the spore. Most investigators assume that the "germ" is extruded with the eversion of the polar filament. This may occur prior to or after the spore enters the host.

Several workers reported that the "germ" penetrates the new host cell directly (Hall, 1952; Dissanaike, 1957; Burnett and King, 1962; Canning, 1962; and Ishihara, 1969). Others reported that the polar filament penetrated the cell before the release of the "germ" (Gibbs, 1953, Bailey, 1954; and Ishihara, 1968). In 1968, Sprague and Vernick stated, "It now appears that we have all but conclusive proof that the polar filament plays the role of an inoculating needle."

Regardless of the method of entrance, once inside the cell, the parasite undergoes schizogony and sporogony, often filling the infected cell with spores, thus completing the life cycle of the parasite.

Microsporidan parasites are transmitted when a susceptible host either ingests free spores (*per os*), or schizonts and spores are transmitted with the eggs from infected females (transovarian), or both.

Hunter (1968) transmitted a *Gurleya* sp. to *Chironomus californicus* by placing a bottom sample from a pond, in which an epizootic had occurred three weeks previously, in an aquarium. When spores of *Nosema*

destructor, that had been held in water at 4° C for 184 days, were fed to potato tuberworm larvae, 50% became infected (Steinhaus and Hughes, 1948). Splittstoesser and McEwen (1968) obtained 100% mortality by feeding cecropia larvae, *Trichoplusia ni*, spores obtained from a *Thelohania* sp. and stored at 10° C for three years. Arthropods were infected *per os* by many investigators (Steinhaus and Hughes, 1948; Hall, 1952; Thompson, 1960; West, 1960; and Weiser, 1961; also Canning, 1962; Gingrich, 1965; Cali and Briggs, 1967; Hunter, 1968; Sikorowski and Madison, 1968; and McLaughlin, 1969). Two species of *Pleistophora*, *P. mytrophica* (Canning et al., 1964) and *P. husseyi* (Michelson, 1963), from the common toad (*Bufo bufo*) and aquatic pulmonate snails, respectively, were transmitted *per os*. Dissanaike (1957) transmitted *Nosema helminthorum* to tapeworms by letting the host of the cestodes ingest the spores. Wissenberg (1921) successfully transmitted *Glugea anomala* by feeding spores to young stickleback (*Gasterosteus aculeatus*).

Lom (1969) maintained *P. hyphessobryconis* (normal host is neon tetra) infection in goldfish by mixing spores with their feed. In golden shiners the incidence of infection increased with age through the third year (Summerfelt and Warner, 1970b) indicating either continued *per os* transmission, the appearance of latent infections, or that the higher incidence in older fish was due to a higher intensity of infection which made it easier to recognize.

Transovarian transmission of Microsporida was reported by Kudo (1924), Spangenberg and Claybrook (1961), Kellen and Wills (1962), Chapman and Kellen (1967), Anderson (1968), and Drea et al. (1969) in invertebrates, but was never described in vertebrate hosts.

Several authors cited evidence of transovarian and per os transmission. Lipa and Martignoni (1960) transmitted Nosema phryganidiae to Phryganidia californica per os and transovarially. Perezia pyraustae was transmitted both ways to European corn borers, Phrausta nubilalis (Thompson, 1960). Raun (1961) and Henry (1967) described the same routes for Nosema acridophagus in grasshoppers, Melanoplus sanguinipas. Nosema heliothidis, in the corn earworm Heliothis zea, was reported to be transmitted both transovarially and during copulation (Brooks, 1968).

Attempts by Summerfelt (1964) and Wilhelm (1964) to effect oral transmission of P. ovariae failed, but their cursory efforts did not disallow its possibility. Successful per os transmission of P. ovariae was obtained later in young and adult golden shiners using fresh spores mixed with dry feed (Summerfelt, personal communication). In the latter case, a long incubation phase was necessary. Incubation, or latency, was the length of time required for oocytes to mature to stages suitable for development of the parasite. Pleistophora hyphessobryconis had been the only other microsporidan experimentally transmitted in fish hosts.

Shiners are intermittent spawners and their eggs do not all mature at one time. Infected shiners apparently produce some viable offspring early in the spawning season. Spawning occurred in early May 1969, yet it is the first sample in June when the percentage of stage VI oocytes infected exceeded 50% (Figure 43). After this time, production of viable eggs was greatly curtailed because all maturing eggs underwent atresia.

Eggs may mature, be fertilized, and produce viable young while containing an infective form of the parasite. This would make

transovarian transmission a part of the life cycle. Transovarian transmission was also implicated by Summerfelt and Warner (1970b) when they found the incidence of parasitized shiners varied among producers, but, was constant among the various year classes of fish at a given fish farm. Fish coming from heavily infected fish were heavily infected, and fish from parent populations with a light incidence of infection had a light incidence of infection. However, transovarian transmission has not been demonstrated in golden shiners.

Effect of Oocyte Maturation on Number of Sporonts Per Unit Volume of Oocyte

Before determining the mean number of sporonts per unit volume oocyte, it was necessary to determine if certain egg stages would shrink more drastically than others during fixation and dehydration. If indeed this did occur, a corrective factor would need to be calculated for each egg stage in order to use the equation for determining the number of particles per unit volume (Haug, 1967).

The effects of two separate fixatives and of freezing on mean diameter are presented in Table IV. Only stages IV, V, and VI are used for calculating the sporonts per unit volume. Counts for stages III and VII are not used because of insufficient data. The relative size difference in the eggs is quite constant between fixatives for IV, V, and VI stage eggs (Table IV). These stages fixed in Bouin's fluid (Humason, 1967) average 37, 33, and 30 microns smaller in diameter, respectively, than corresponding eggs fixed with Kaformacet. Stage V and VI eggs average 20 and 33 microns larger in frozen sections than corresponding Kaformacet fixed eggs. From these data, it is concluded

that the fixative used for this study, Kaformacet, has approximately an equal shrinkage effect on stage IV, V, and VI oocytes.

TABLE IV

EFFECTS OF FIXATION AND FREEZING ON THE MEAN DIAMETER OF VARIOUS EGG STAGES

Egg Stage (avg. diameter μ)	Fresh Frozen	Fixative	
		Bouin's Fluid	Kaformacet
III	134	169	175
IV	---	236	273
V	375	322	355
VI	523	460	490
VIII	674	617	---

The mean volume for each egg stage (Table V) after fixation in Kaformacet was calculated by the equation $V = 4/3 \pi R^3$.

The mean number of sporonts, in stage IV eggs was 2580; there were 34,507 in stage V, and 57,626 in stage VI. The number of sporonts increased 22 fold between stage IV and VI, but evidence indicates that sporonts do not multiply and arise only from schizonts. Hence, lacking schizonts in stage V oocytes makes it difficult to account for the increase in stage VI oocytes, except that the length of time required

to pass through these stages is so short that, in fact, the increase in sporonts in stage VI results from the schizogonic activity in stage IV.

TABLE V

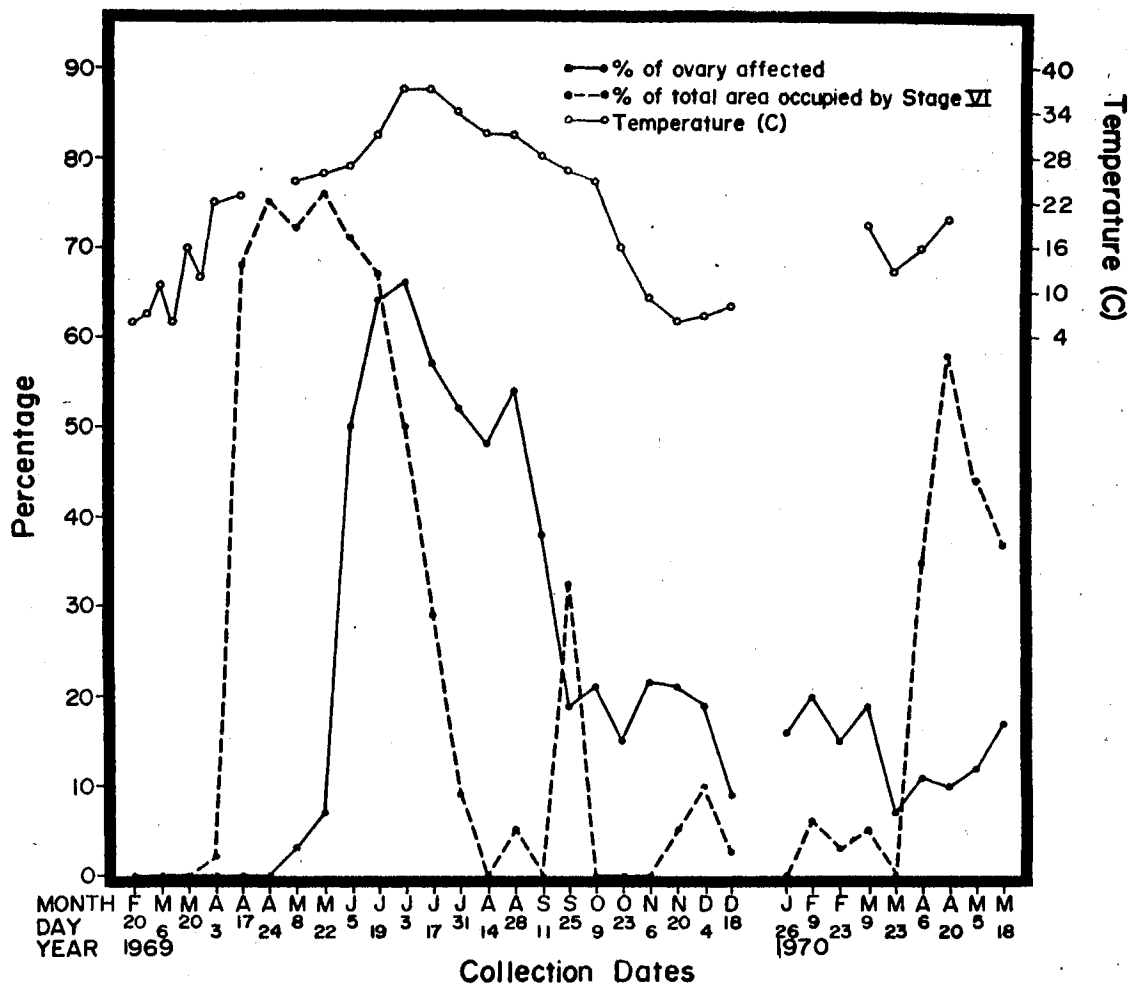
MEAN VOLUME OF EGG STAGES IN KAFORMACET FIXED OVARIES

	Egg Stage						
	I	II	III	IV	V	VI	VII
Volume $\times 10^4$ (cubic microns)	.8	4.3	24	58.5	98.9	188.5	295

Relationship of Temperature to Parasitism

Water temperature was taken on all 1969 collections, but only during March and April of 1970. The percentage of the ovary affected coincides with the temperature throughout the year (Figure 65). This is because oogenesis is closely related to water temperature, and parasitism is closely related to oogenesis. Oogenesis reached its peak (mature oocytes) at the beginning of spawning (May) when water temperatures range from 25-26° C. The maximum percentage of ovary affected occurred at the end of the spawning season when the ovary had large areas of spore filled stroma. High summer temperatures (38° C on July 3) terminate spawning.

Figure 65. Comparison of Temperature, Percentage of Ovary Affected, and Percentage of Ovary Occupied by Stage VI Oocytes


% of ovary affected
% of total area occupied by Stage VI
Temperature (C)
Percentage
Temperature (C)
90
80
70
60
50
40
30
20
10
0
40
34
28
22
16
10
4
MONTH F M M A A A M M J J J J J A A S S O O N N D D J F F M M A A M M
DAY 20 6 20 3 17 24 8 22 5 19 3 17 31 14 28 11 25 9 23 6 20 4 18 26 9 23 9 23 6 20 5 18
YEAR 1969 1970
Collection Dates

CHAPTER V

SUMMARY

The seasonal cycles of oogenesis and parasitism in golden shiners was studied by sampling, at biweekly intervals, a population of shiners infected with *Pleistophora ovariae*. Oogenesis of the host was described in seven stages recognized on the basis of morphological characteristics of oogonia, and primary and secondary oocytes. The morphology of Mallory's trichrome stained oocytes was described. The seasonal cycle of oogenesis was evaluated by comparing the incidence of each oocyte stage within the ovary at each sampling period. A description of post-ovulatory atresia was presented and a comparison made between the occurrence of hypertrophic and nonhypertrophic atresia.

The processes of schizogony and sporogony within the developing oocytes were described. The infective form of *P. ovariae* was not demonstrated, but evidence supporting autoinfection was presented. The relationship between oogenesis and parasitism was discussed in terms of the seasonal cycle.

Morphology and Dynamics of Oogenesis

Stage 0 oocytes (oogonia) were small (6-7 μ), somewhat spherical to cuboidal cells, with a slight, thin rim of lightly basophilic cytoplasm, and a large vesicular nucleus, containing a broad ring of peripheral chromatin and a single, large nucleolus. They often appeared in nests

along the ovigerous lamellae.

Stage I oocytes had a homogeneous, basophilic cytoplasm with a large (16-19 μ) basophilic and centrally located nucleus. One or two prominent nucleoli were present lying adjacent to the nuclear membrane. Mean oocyte diameter was 32 microns. This stage was most prevalent in August, immediately following the spawning season. Greater percentage of stage I oocytes were found during the early spring of 1970 compared to 1969 even though parasitism in other oocytes was much higher in 1970. No stages of the parasite were found in stage I oocytes.

Mean cell diameter was 74 microns, three to five visible nucleoli were present, and cytoplasmic basophilia was maximum in stage II oocytes. The zona radiata was not visible, but several thin flattened follicular cell nuclei comprised a thin monolayered follicular epithelium. This oocyte stage, which developed directly from stage I oocytes, was most prevalent September 11, 1969, the next sampling period after stage I had reached its maximum. The percentage of total eggs represented by stage II was 15% higher in 1970 compared to the same period in 1969. From this, it was concluded that parasitism had little or no effect on production of stage II oocytes, but schizonts occurred in a few of these oocytes.

Stage III oocytes (secondary) often had polar bodies in the periphery of the cytoplasm, had a mean diameter of 175 microns, 20-35 nucleoli visible per section, a frothy basophilic cytoplasm, and a single layer of follicular cells bordering the cytoplasm. The nucleus was pale, acidophilic, and had an irregular membrane. A thin, unstriated zona radiata was present. Stage III oocytes were most prevalent during late September and early October, coinciding to the decline

in stage II eggs. The maximum percentage of area occupied by stage III oocytes was 50% in 1969 and 34% in 1970. The reduction in stage III, which occurred following increases in both stages I and II in 1970, lead to the conclusion that P. ovariae had its first impact on fecundity with this stage. Schizonts were common but sporonts were not observed in this stage.

Stage IV oocytes had a mean diameter of 273 microns, 50-65 nucleoli vesicles of basophilic primary yolk forming around the periphery of the cytoplasm, and thin vitelline membrane. The nucleus was acidophilic and the nuclear membrane irregular in shape. The stage was most prevalent from October until March and was the stage in which the oocytes "overwinter." Fewer stage IV oocytes were produced in 1970 than in 1969. Since no uninfected control was used during the sampling, it was not possible to demonstrate that the reduction in this stage and stage III was not an age induced change rather than a type of partial parasitic castration. However, because of the observed changes in intensity of parasitism, it was concluded that the reduction was parasite induced. More work including uninfected controls is needed to answer this question.

Stage V oocytes had a mean diameter of 355 microns, cytoplasm essentially filled with primary yolk, and a distinct zona radiata staining blue to red with Mallory's. The nucleus was acidophilic and the follicular epithelium bilaminar. Stage V oocytes occupied a variable percentage of the ovary, representing 15% on February 9, 1970, 5% on March 23, 1970, and 24% on April 6, 1970. Possibly the time required for maturation from IV to VI was so rapid that at any given time only a few oocytes were observed in this stage. Furthermore, late stage IV and

some early stage VI oocytes were difficult to distinguish from stage V oocytes. Therefore, many stage V oocytes could have been classified as either of these stages. In future work, it would be much easier to classify stage IV and V oocytes as the same stage. Thus, stage IV oocytes would be any oocyte with only primary yolk present.

Stage VI oocytes had a mean diameter of 523 microns, strongly acidophilic cytoplasm with secondary yolk (acidophilic) forming in the perinuclear region and migrating outward. Zona radiata was striated and vitelline membrane well developed. The presence of large numbers of stage VI oocytes occurred during the spawning season. The maximum percentage of total area occupied by stage VI was down 16% in 1970 from 1969 and the maximum percentage of total eggs decreased 24% in 1970. These reductions were believed to be the result of infection with *P. ovariae*.

Stage VII oocytes had a mean diameter of 617 microns, peripheral nucleus, and showed coalescence of secondary yolk. Only a few stage VII oocytes were found which probably resulted from the relatively short time period this stage existed within the ovary before being spawned; the destruction of early egg stages before they fully matured; and the difficulty of preparing stage VII oocytes for histological sectioning (they were difficult to keep from disintegrating).

The rate of oocyte maturation varied with the season of the year. In warm weather, the rate was rapid, requiring only about two weeks for each stage (I to V), while in cooler weather as much as six weeks was required for development of stage V oocytes from stage IV.

Atresia

The two forms of atresia (hypertrophic-phagocytic and nonhypertrophic) described by Braekevelt and McMillan (1967) for the brook stickleback, Eucalia inconstans, were found in golden shiner ovaries. Hypertrophic-phagocytic atresia was characterized by hypertrophy of the follicular epithelial cells. The zona radiata showed signs of erosion and finally ruptured, and the follicular cells migrated into the oocyte and phagocytized its contents. In the final stages, the cytoplasm was completely filled with follicular cells, and borders between adjacent atretic cells were disappearing, thus forming stroma. Atresia was similar in parasitized and unparasitized ova; however, the process required a longer time in parasitized ovaries. In uninfected fish, the majority of mature ova were spawned and only a few remaining ova needed to be removed through atresia. However, in the infected ovary many ova underwent atresia prior to maturing. The larger number of atretic oocytes plus the presence of spores and sporonts increased the time required for atresia. Atresia of parasitized oocytes was, with only four exceptions, the hypertrophic-phagocytic type.

Nonhypertrophic atresia did not involve hypertrophy and phagocytosis by follicular cells. The yolk became basophilic, finely granular, and sparse, the vitelline membrane often eroded, and the oocyte contents underwent lysis. This type of atresia was fairly common prior to the first spawning season and the first occurrence of P. ovariae within the oocytes.

Intraovarian Life Cycle

Pleistophora ovariae appeared first as a spherical schizont, about

eight microns in diameter, within the cytoplasm of stage II oocytes. Schizonts were most common in stage III and IV oocytes, although a few were found in stage II oocytes. Schizonts were not found in stage V, VI, and VII oocytes. Schizonts divide by binary fission. Schizogony was more prevalent in postspawning fish in June and July when spore-filled stroma was abundant. As inferred from changes in relative abundance of schizonts and sporonts, schizogony required less than two weeks.

Schizonts occurred together with sporonts in a few stage IV oocytes, but only sporonts were found in stage V, VI, and VII oocytes. Sporonts were not found in eggs lacking primary yolk, hence, they were not found in oocytes less mature than stage IV.

Sporonts first appeared in clusters near the nucleus of the oocyte. Nucleogamy of the sporont first produced a multinucleate body which underwent plasmotomy to form a pansporoblast containing 8 to 12 dyads. Each dyad gave rise to two spherical, red stained, sporoblasts. Each sporoblast matured into one spore which was retained within the original sporont membrane. The time required for sporogony, like schizogony, was two weeks or less.

Seasonal Variation in Life Stages of *P. ovariae* and Reproductive Cycle of the Host

The generation time of *P. ovariae* apparently was highly variable. It probably depended on conditions within the ovary such as the presence of proper oocyte stages. Stage III oocytes lacked a well-developed zona radiata which would facilitate penetration. Furthermore, the microsporida, which lack mitochondria, could use those which had just become

functional in the host cell for their own needs.

Since the ovary underwent an annual cycle in regards to oocyte development, the preference of certain parasite stages for certain oocyte stages made the occurrence of various parasite stages seasonal in nature. Nevertheless, since some oocytes ripened during all seasons of the year, it was possible to find any given parasite life stage at any time during the year.

Autoinfection

Although autoinfection was not demonstrated in the golden shiner ovary, there was much evidence to support this phenomenon. The spawning season was essentially the only time that fish were exposed to spores in the external environment, yet, newly maturing stage II, III, and IV oocytes continually became infected with schizonts throughout the year. When a large portion of the ovary was filled with mature spores, the number of newly matured stage III eggs infected with schizont was 16%. However, when an increase in numbers and area of stage III eggs occurred when a relatively small portion of the ovary contained mature spores, only four percent of the oocytes became infected. These evidences suggested that most schizonts observed within these eggs were the result of autoinfection.

Effect of Egg Maturation on Number of Sporonts Per Unit Volume of Oocytes

The mean number of sporonts in stage IV oocytes was 2580, there were 34,507 in stage V oocytes and 57,626 in stage VI. The number of sporonts increased twenty-two fold between stage IV and VI.

Relationship of Temperature to Parasitism

The percentage of the ovary affected with *P. ovariae* coincided with the temperature throughout the year. This is because oogenesis was closely related to water temperature, and parasitism is closely related to oogenesis.

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