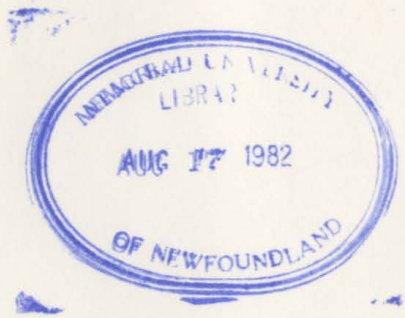
A SURVEY OF THE PREVALENCE AND STUDY OF THE EFFECTS OF AN OVARIAN PHYCOMYCETE IN SOME NEWFOUNDLAND BLACKFLIES

DANIEL OWUSU YEBOAH



A SURVEY OF THE PREVALENCE AND STUDY OF THE EFFECTS OF AN OVARIAN PHYCOMYCETE IN SOME NEWFOUNDLAND BLACKFLIES

by

© Daniel Owusu Yeboah, Dip. N.Ed., B.Sc.

A Thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

Department of Biology
Memorial University of Newfoundland
September 1980

St. John's Newfoundland

ABSTRACT

The ovarian phycomycete parasitizes *Stegopterna mutata* and *Prosimulium mixtum*. The rate of infection in *St. mutata* was found to be 40%, but the rate in *P. mixtum* could not be accurately determined because of insufficient data. *Simulium verecundum* is a suspected host because the fungus is found on its egg masses.

The spore released by the fly during oviposition did not withstand desiccation and did not develop on the artificial media tested; development occured only in water. At 4°C development was slow, and sporangia took between 5 and 7 days to develop. Above 10°C, the sporangia developed faster, within 2-3 days. The developmental process included the formation of sporangia from which discharge tubes formed, from these structures zoospores were presumed to be released. Evidence suggested that discharge tube development coincided with egg development, and that the life cycle of the fungus coincided with that of the host. A dormancy period equivalent to egg diapause of the host, or the involvement of an intermediate host in the life cycle of the fungus is discussed. Evidence collected suggests there were two seasonal forms of the fungus — winter and summer — each form parasitizing the simuliid species present in the appropriate season. Based on the evidence

collected to date, it is thought that infection occurs during the host's early instars. Parasitism does not produce any detectable external symptoms in the host but results in a complete destruction of the ovarian tissues and eggs. Infected flies did not bite after the first gonotropic cycle and did not oviposit again. The infection rate in an ovipositing fly population was found to decrease with time. Longevity of the host did not appear to be affected by the presence of the fungus.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Memorial University of Newfoundland (M.U.N.) for the Fellowship awarded me for the duration of my studies. I am greatly indebted to my thesis committee, Dr. Marshall Laird, supervisor, and Drs. Albert Undeen, Richard Nolan, and later Dr. Murray Colbo, who stood in for Dr. Undeen, for their suggestions during the course of the research and for reading the manuscript. I also wish to express my appreciation to Dr. Jean Finney for reading the initial draft and making useful suggestions. Dr. Colbo, in addition to providing me with some samples, gave an invaluable help in the taxonomy of the flies and rearing techniques to which I am grateful. I am also grateful to Mr. Joseph Mokry of the Research Unit on Vector Pathology (R.U.V.P.) for his advice in the longevity experiment and also for information on egg development. The additional financial assistance in the form of research assistantship and facilities provided by R.U.V.P. through research grant No. HEW/NIH US to Dr. Laird, are gratefully acknowledged. I am grateful to Mr. Roy Ficken, photographer, and Mr. William Gin, both of the Department of Biology, M.U.N. for the photographic assistance. I am also thankful to Mr. Phil Hyam and Ms Lisa Lee of the Electron Microscopy (E.M.) Unit of the Faculty of Medicine, M.U.N. for their assistance in the E.M. work.

I am grateful to Ms Darlene Pinsent for finding extra time to type the thesis for me. Lastly, but by no means the least, I wish to thank my dear wife, Sophia, for her love and understanding during the course of studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Fungal infections of insects are well known and widely reported in the literature (Debaisieux, 1920; Keilin, 1921; Couch and Umphlett, 1963; Macleod, 1963; Madelin, 1963; and McEwen, 1963). Entomogenous fungi attack a variety of terrestrial and aquatic insects; fungi known to cause infection and disease in insects are classified in each of four classes: Phycomycetes,* Ascomycetes, Basidiomycetes and Deuteromycetes (Steinhaus, 1967). The Phycomycetes will be considered here.

The "Phycomycetes" are generally considered the most primitive of the true fungi and include a wide diversity of forms, some showing definite relationship to the flagellates, others closely resemble colourless algae, and still others being true moulds (Sparrow, 1960). The primitive members of the class are chiefly aquatic (Fitzpatrick, 1966). The aquatic "Phycomycetes"

---

*Phycomycetes is a term which means "algal fungi" and reflects an ancient belief that all fungi were merely degenerate algae (Ross, 1979). In many books and papers, including many recent texts, Oomycetes, Hyphochytridiomycetes, Chytridiomycetes and Zygomycetes are often placed as an order in the class "Phycomycetes". Modern intepretations no longer allow the validity of such a grouping, and the term Phycomycetes has no taxonomic status (Ross, 1979; Whittaker and Marguilis, 1978). The fungus involved in this study has not been diagnosed yet and the term, Phycomycetes, is used here for convenience.

occur on a variety of substrata, principally in fresh, but also, to a lesser extent, in marine waters (Johnson and Sparrow, 1961). Generalizations concerning the morphology of the "Phycomycetes" are difficult because of the great diversity of the group (Fitzpatrick, 1966). The thallus may be simple or may bear rhizoids, or may develop an extensive mycelium(a). The thalli are often characterized by a nonseptate and multinucleate (coenocytic) mycelium and endogenous asexual spores (Alexopoulos and Mims, 1979). The thallus is usually provided with a membrane or wall, but in some of the lower groups it is naked throughout. Asexual reproduction in the "Phycomycetes" occurs in most cases by means of spores borne in sporangia. In the lower forms the sporangiospores are usually motile and are called zoospores. The zoospores may be uniflagellate or biflagellate. The structure of the zoospore is considered significant in the identification of most fungi (Sparrow, 1960; Fuller, 1977; Alexopoulos and Mims, 1979). The zoospores in some forms escape from the sporangium through a pore or by an exit tube of definite form and position. In others, the sporangial wall disintegrates or bursts in an indefinite fashion (Fitzpatrick, 1966). In parasitic species, infection is accomplished by either zoospores or zygotes (Fitzpatrick, 1966; Alexopoulos and Mims, 1979). Usually when a zygote infects a host cell it develops into

a thick-walled resting sporangium that does not immediately discharge zoospores and is capable of overwintering (Alexopoulos and Mims, 1979). Some of the parasitic forms may require an intermediate host to complete their life cycle, e.g. *Coelomomyces* (Whisler and Zebold, 1974; Whisler *et al.*, 1975; and Pillai *et al.*, 1976). In numerous genera of the "Phycomycetes", fungi have been described in which a sexual stage is not yet known to occur.

The entomogenous members of the "Phycomycetes" are found in the Entomophthorales, Mucorales, Blastocladiales, and Chytridiales (Steinhaus, 1967). Fungi infecting insects are usually parasitic. The ovarian "phycomycete" which is the subject of investigation in this study is a parasite found in blackflies.

Only a few reports exist in the literature of blackfly ovarian infections by "Phycomycetes" or "Phycomycete"-like fungi. Garms (1975), observed a fungus developing in the ovaries of *Simulium metallicum, S. ochraceum, S. gonzalezi* and *S. callidum* in Guatemala. Two forms (or stages) were observed; rod-like bodies in nulliparous flies and oval bodies in parous flies. He identified these to be morphologically identical to those frequently observed in the ovaries of *S. damnosum* in Liberia (reference not cited),

identified by Dr. Müller-Kögler (Darmstadt) as "Phycomycetes". It appears that the rod-like and oval bodies could be two different forms of the same organism; the rod-like form being an earlier stage than the oval form. Rod-like bodies were also noted in *S. damnosum* complex from the Cameroons and Liberia (Lewis, 1960a) and from the *S. neavei* complex in Tanzania (Lewis, 1960b). He referred to those from *S. damnosum* as a species of *Serumsporidium* of the protozoan order, Haplosporidia. Although no morphological descriptions of the pathogens were given, Lewis was, perhaps, looking at a "Phycomycete" because it appears that only larval infections normally occur with haplosporidian infections (Beaudoin and Wills, 1968). Until Sprague's revision of the genus *Haplosporidium* in 1963, there was a great deal of confusion regarding the identity of Haplosporidia (Sprague, 1963). Lewis may have overlooked the fact that Serumsporidium is synonymous with *Coelomomycidium*, a "Phycomycete" of the order Chytridiales (Weiser, 1963; and Karling, 1977). Lewis (1965) also noted a more amorphous body in the abdomen of nullipars of the blackflies which he believed to be a fungus. These bodies were seen previously by him in a Sudan *S. damnosum* complex population. He believed these were also a fungus. In addition he reported an *Entomophthora*-like fungus.

Lewis in the same study reported the observation of rectangular hyphal bodies of a fungus in the ovaries of 0.3% nulliparous *S. damnosum* from the Kumba area in Western Cameroons, and a protozoan parasite resembling Haplosporidia in 0.3% of nullipars. Garnham and Lewis (1959) observed an infection of the ovaries of *S. metallicum* by a species of *Coelomomyces* — a genus which includes some common parasites of mosquitoes. Thus, representatives of several fungal groups have been observed in simuliids. The fungal infection under consideration was first observed by Undeen and Nolan (1977) in 10.8% of ovipositing *Prosimulium mixtum* in St. John's, Newfoundland. This was identified as a "Phycomycete". Later Undeen (1978) found similar fungal pathogens in over 50% of *Stegopterna mutata* and fungal spores on masses of *S. verecundum* eggs on trailing vegetation in streams. His observation on the spores held in the laboratory revealed that sporangia grew out from the spores while they were kept in water. Undeen (*op. cit.*) speculated that early instar larvae are the target hosts after formation of these sporangia.

The present study was aimed at determining the rate of infection, the effect on the host and the life cycle of the fungus.

MATERIALS AND METHODS

Larvae and pupae of *Stegopterna mutata* were collected in April, 1979, and larvae only in February and April, 1980 from the stream draining Little Power Pond near St. John's, Newfoundland, at a site about 100-150 metres downstream from the outlet of the pond (a site where the fungus had previously been reported by Undeen and Nolan, 1977). *Stegopterna mutata* larvae and pupae were selected for histological study for two reasons: (1) The local *St. mutata* population was triploid, therefore always female; and this eliminated the difficult task of sexing larvae before sectioning; and (2) The high incidence of infection in the fly population reported by Undeen (1978) at the same site, increased the probability of finding infected larvae in the sectioned material. Larvae in the 3-4th, 5-6th and last instars, and pupae were selected for study for the purpose of determining the stage of infection. Early instar larvae were found to have too small an ovary making it very difficult to examine them in paraffin sections. Part of the collection was fixed in Bouin's fixative and part in Kahle's fluid (Strickland, 1911). After fixation specimens were dehydrated in graded ethanol series, embedded in paraffin wax, sectioned on a rotary microtome (5 µm sections) and stained with

Heidenhain's iron haematoxylin. Both fixatives gave good results. Some pupae of *St. mutata* and *Prosimulium mixtum* were also collected from Little Power Pond and reared in the laboratory to adults. Some of the *St. mutata* adults were fixed in Bouin's solution for histological study on the first and third day after emergence to determine the development of the fungus in the adult fly. The remainder of *St. mutata* and *P. mixtum* adults were kept in captivity and examined on the fifth day after emergence for the presence of infection. Gravid flies were collected from the outlet of Little Power Pond during May and June, 1979 and in June, 1980 using a sweep net as they came to oviposit along the stream. Collecting in 1980 could not start until June because of unfavourable weather conditions. Attacking *P. mixtum* (those seeking blood), were collected around the investigator with a net, and by aspirator. The flies were taken to the laboratory and in almost all cases, dissected the same day. Dissection was either in saline, saline and detergent (Lewis, 1958) or in Lum's solution (Undeen; personal communication). All solutions were found to be satisfactory; however, the solution containing detergent was not used when material was required for culturing. Dissections were done using a dissecting microscope. Some of the 1980 ovipositing adults were kept in captivity to determine the longevity

of infected flies. The flies were held individually in 3 x 5 cm plastic vials (Mokry, 1979) containing a few millilitres of water to allow oviposition. A small strip of paper folded into "V" was placed base-downward in the bottle to provide a resting place. The bottle was covered with gauze held in place by a rubber band. A cotton-wool wick was placed in a hole made in the gauze and wetted daily with 10% sucrose solution as a source of food. The flies were examined after death to determine the condition of the ovaries. Dr. Murray Colbo (R.U.V.P., M.U.N.) also provided a sample of *P. mixtum* which he had reared from pupae collected from Beachy Cove Brook, near St. John's. In addition *S. verecundum* egg masses were collected from trailing vegetation in Voisey's Brook near St. John's and examined for the presence of the fungus. Dr. Colbo in July, 1980, called my attention to extensive infected egg masses from Beaver Brook near Bay Bulls, as well as occasional infection from a few other streams in the St. John's area. Some infected eggs from the field were reared to larvae at room temperature (20°C) whilst some were also kept with thin layer of water at 4°C. Some of the field material was placed on Brain Heart Infusion (BHI) and Emerson Yp Ss agar media with Penicillin 100 I.U./ml

and Streptomycin 100 µg/ml (R.A. Nolan; personal communication). The cultures were incubated at 10°C, 12°C, 15°C and room temperature (20°C). The fungus incubated with eggs at room temperature and that kept at 4°C was examined daily after the eggs hatched to determine any relationship between egg-hatching and development of the fungus. Pupae collected from Voisey's Brook in June, 1980, were taken to the laboratory and reared to adults. These were fed on glucose, and the ovaries examined on the 7th day after emergence. On emergence the flies were found to be *Simulium vernum* and a mixture of the *S. verecundum/venustum* complex. The latter two species which could not be separated very easily will be referred to as *S. verecundum/venustum*.

Fungal spores dissected from ovipositing flies were incubated in tap water, water from the stream, in normal saline and on Sabouraud's agar (Collins and Lyne, 1970). These non-sterile cultures were incubated at 4°C, 10°C, 15°C and room temperature (20°C). Some of the spores were fixed and stained by Bartch's method for the preservation of *Blythiomyces spinulosus* (Sparrow, 1960). Spores were also stored dry in 5 x 1½ cm bottles, on ice, at 4°C and 10°C for two months and then placed in water at weekly interval to determine the resistance capabilities of the spore. Infected and non-infected ovaries from adult flies were fixed in

Bouin's fluid and prepared for histological study as described for the handling of the larvae and pupae. Part of the infected and non-infected material was also fixed in Karnovsky's fixative (Karnovsky, 1965) dehydrated through graded alcohol series to acetone and embedded in Epon 812. Sectioning was done on a Reichert OM U3 microtome (0.5 µm and 600-900 Å sections). The "thick" (0.5 µm) sections were stained in 1% Toluidine Blue and 1% Sodium Borate. The thin (600-900 Å) sections were stained in lead citrate. Photomicrographs of the thin sections were taken with Phillip's 300 Electron Microscope. The E.M. procedure (fixation through Electron Photomicrography) was performed by Ms. Lisa Lee of the E.M. Unit of the Faculty of Medicine. All other photomicrographs were taken by the author with a Zeiss photoscope 1, using Kodak Panatomic X film. Kodak No. 58 Wratten green filter was used where necessary. Statistical methods used for describing measurements are presented in Mendenhall (1975).

RESULTS

Infections in larvae and pupae

The infection rates in both larvae and pupae of *St. mutata* were about 10% (4 out of 45 and 4 out of 42 respectively). There were no detectable external signs of the infection in these pre-imaginal stages, and the infection was only recognized by histological techniques. Infection was found in the fat body and ovaries.

The structure of a normal (uninfected) larval fat body is shown in Fig. 1. The cells of the fat body are small and appear consistent. In contrast, the fat body of an infected larva appears disintegrated, and abnormal bodies were found within the cellular matrix (Fig. 2). The abnormal bodies are granular and lack an enclosing membrane. However, these structures may not be necessarily the ovarian "phycomycete" because of the larva's vulnerability to other parasitic organisms in this area.

The ovaries of a normal larva consist of a tubular ovary along which develop the ovarioles. In 3-4th instar larva the ovaries are grouped together and possess prominent oocyte nuclei (Fig. 3a). Each ovariole is contained in a follicular sac, and as they mature the follicular epithelia appear as separations between the ovarioles (Fig. 3, b-e). In the centre of each

Figure 1. Photomicrograph of section of *St. mutata* larva showing normal appearance of uninfected fat body.

Magnification: 300X

Stain: Heidenhain's iron haematoxylin and eosin.

Figure 2. Photomicrograph of section of *St. mutata* larva showing abnormality of the fat body (indicated by lines). Note the absence of enclosing cell membrane.

Magnification: 500X

Stain: Heidenhain's iron haematoxylin and eosin.

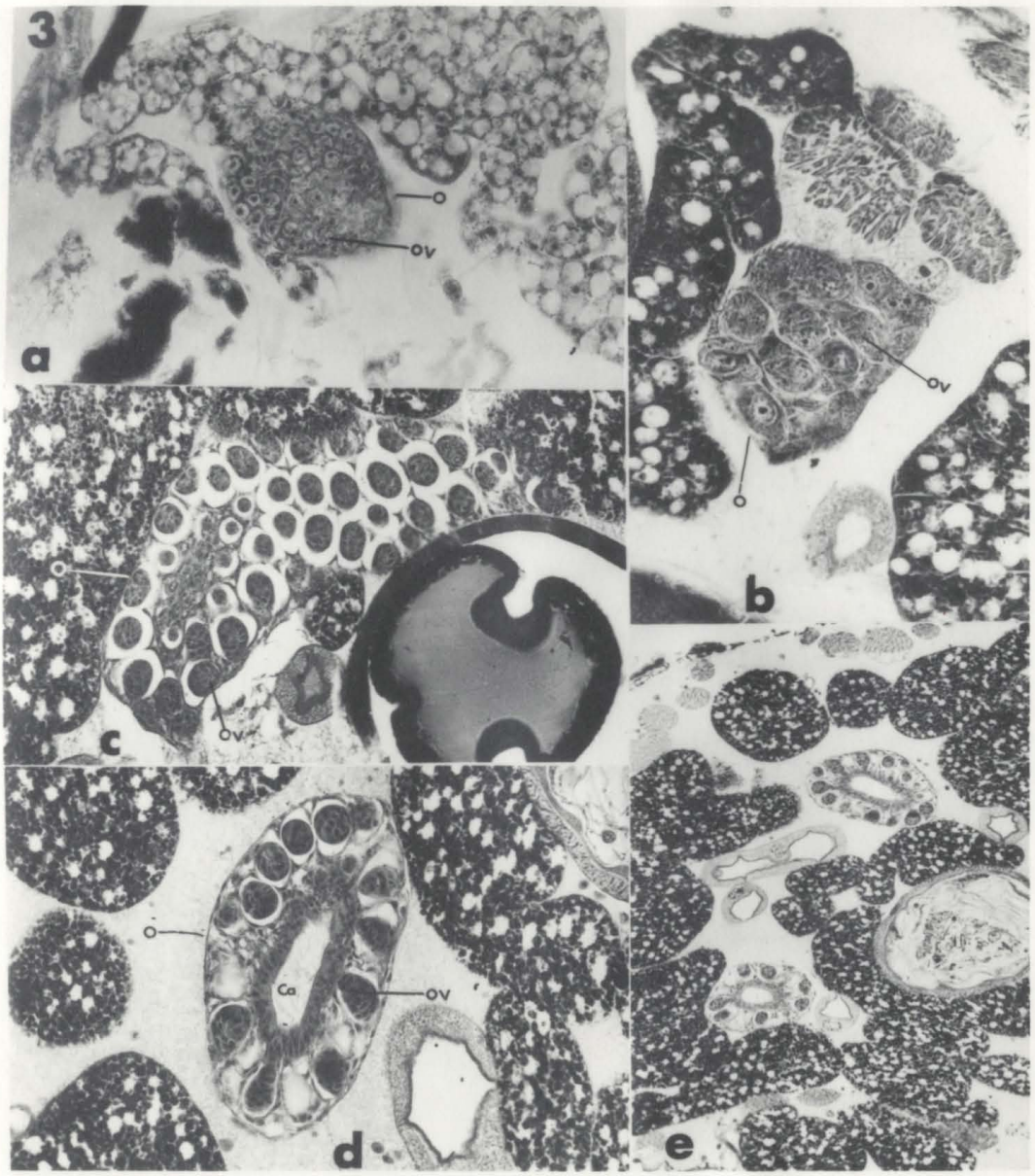
1
2
abnormality in fat body

Figure 3. Photomicrograph of section of *St. mutata* larva showing various stages of normal development of the ovaries.

a) 3-4th instar; Magnification: 416X
b) 5-6th instar; Magnification: 666X
c-e) late instar; Magnification: c & d: 260X
e) the two ovaries in situ; Magnification: 104X

Stain: Heidenhain's iron haematoxylin and eosin.

ca = calyx
o = ovary
ov = developing ovarioles (follicles)


3
a
ov
b
ov
c
ov
d
Ca
ov
e

ovary is a hollow area, the calyx (Fig. 3, d,e), which becomes the future oviduct through which the eggs pass to the exterior. Near the apex of the ovary the ovarioles appear grouped together and the calyx may not be seen (Fig. 3c). The most striking change during maturation of the ovary is the accumulation of ooplasm (Smith, 1968)— Fig. 3, b-e. Normally the two ovaries are symmetrical (Fig. 3e). The sectioned ovaries in Fig. 4 differed from the uninfected one shown in Fig. 3, in that ooplasm did not accumulate. Neither were the two ovaries symmetrical in shape and structure. There appears to be a proliferation of the cells around the calyx. The ovarioles above the calices appear to be poorly differentiated resembling, to some extent, the rudimentary structure of 3-4 instar ovary (Fig. 3a) even though these were 5-6th instar larvae. The ovaries show the presence of large abnormal cells (f) in Fig. 4. It was presumed that these abnormalities were attributable to the fungus.

The normal pupal ovaries (Fig. 5) are basically of the same structure as the larval ovaries but show signs of maturation. The ovarioles change from the circular shape (Fig. 5a) seen in the larva (Fig. 3 c-e) to an oval shape (Fig. 5 b-d). The symmetry of the ovaries are still retained (Fig. 5d). The ovaries of an infected pupa (Fig. 6) are different in structure from those of the normal ovary (Fig. 5), and the ovaries

Figure 4. Photomicrograph of section of *St. mutata* larva showing ovaries considered infected (5-6th instar) compare with Fig. 3b, the normal ovary of same age.

a) the right ovary as it differed from the left ovary, b; in the same larva
b-c) the left ovary as it changed in the same larva. Notice proliferation of cells around the calyx.

Magnification: 720X

Stain: Heidenhain's iron haematoxylin and eosin.

ca = calyx
f = ? fungal infected ovarioles
o = ovary
ov = ? developing ovarioles (follicles)

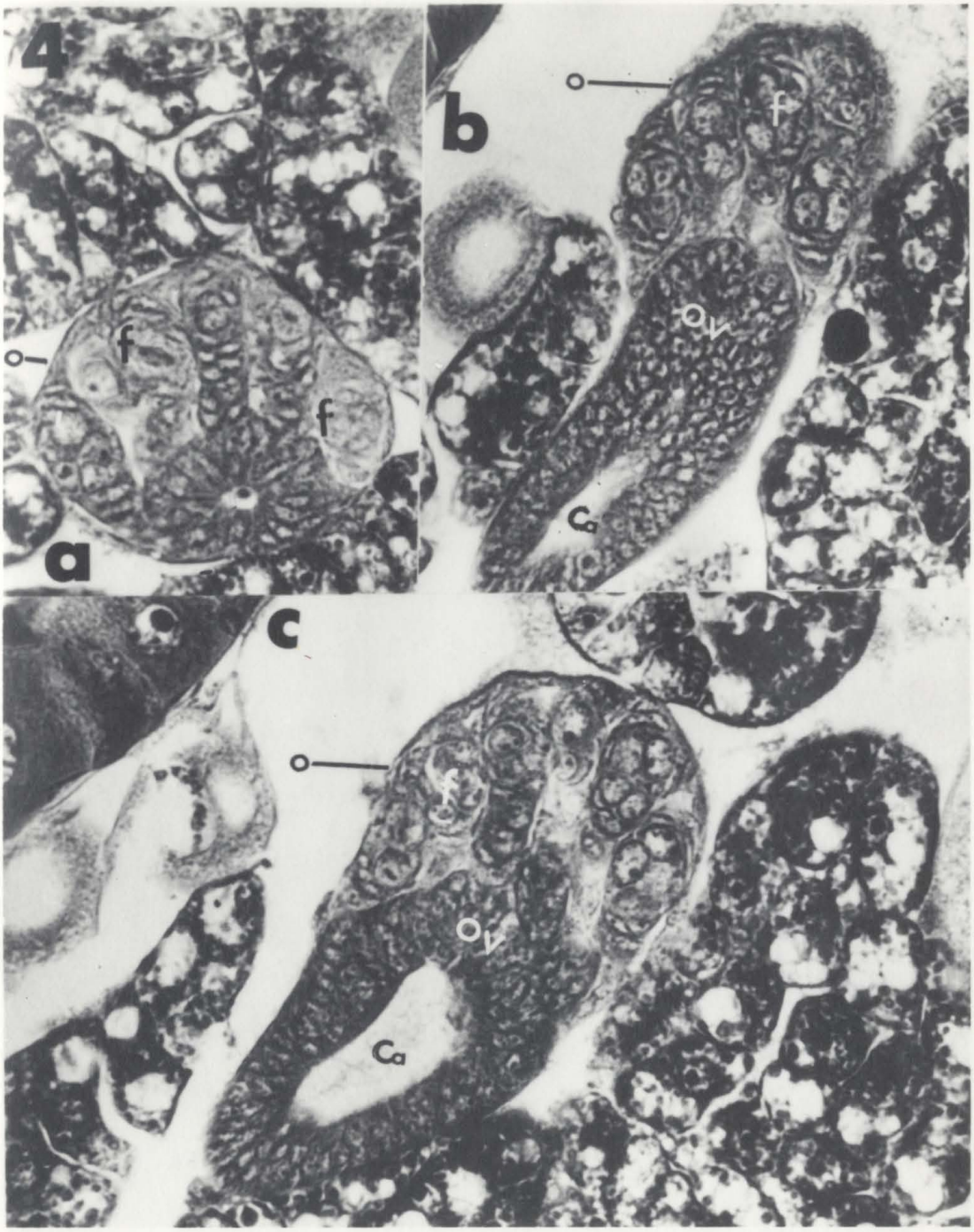

4
a
f
f
o
b
o
f
ov
Ca
c
o
ov
Ca

Figure 5. Photomicrograph of section of *St. mutata* pupa showing various stages of normal development of the ovaries.

a) late larval - early pupal ovaries
Magnification: 328X
b-c) maturing pupal ovaries
Magnification: 328X
d) the two ovaries in situ
Magnification: 132X

Stain: Heidenhain's iron haematoxylin and eosin.

c = calyx

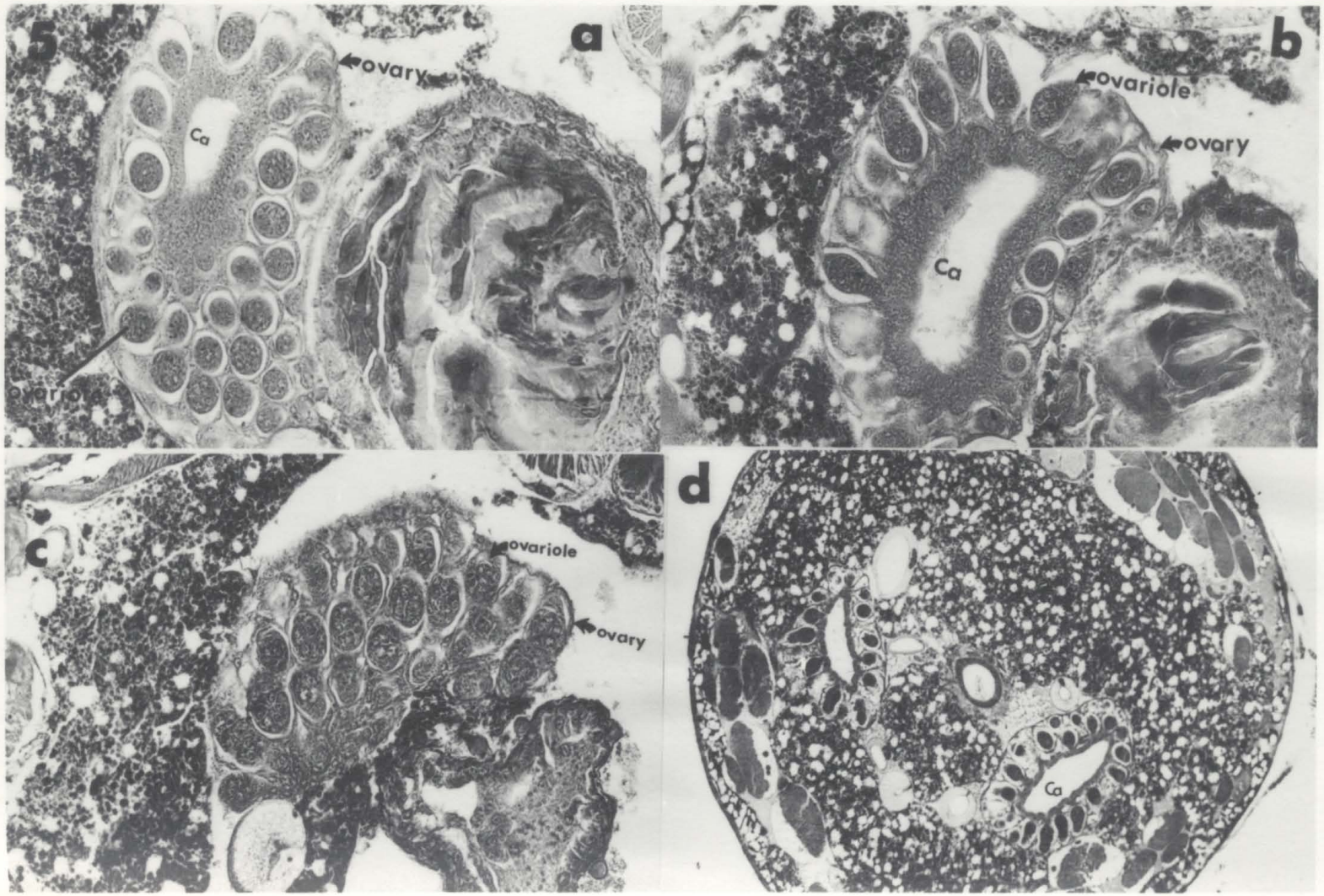
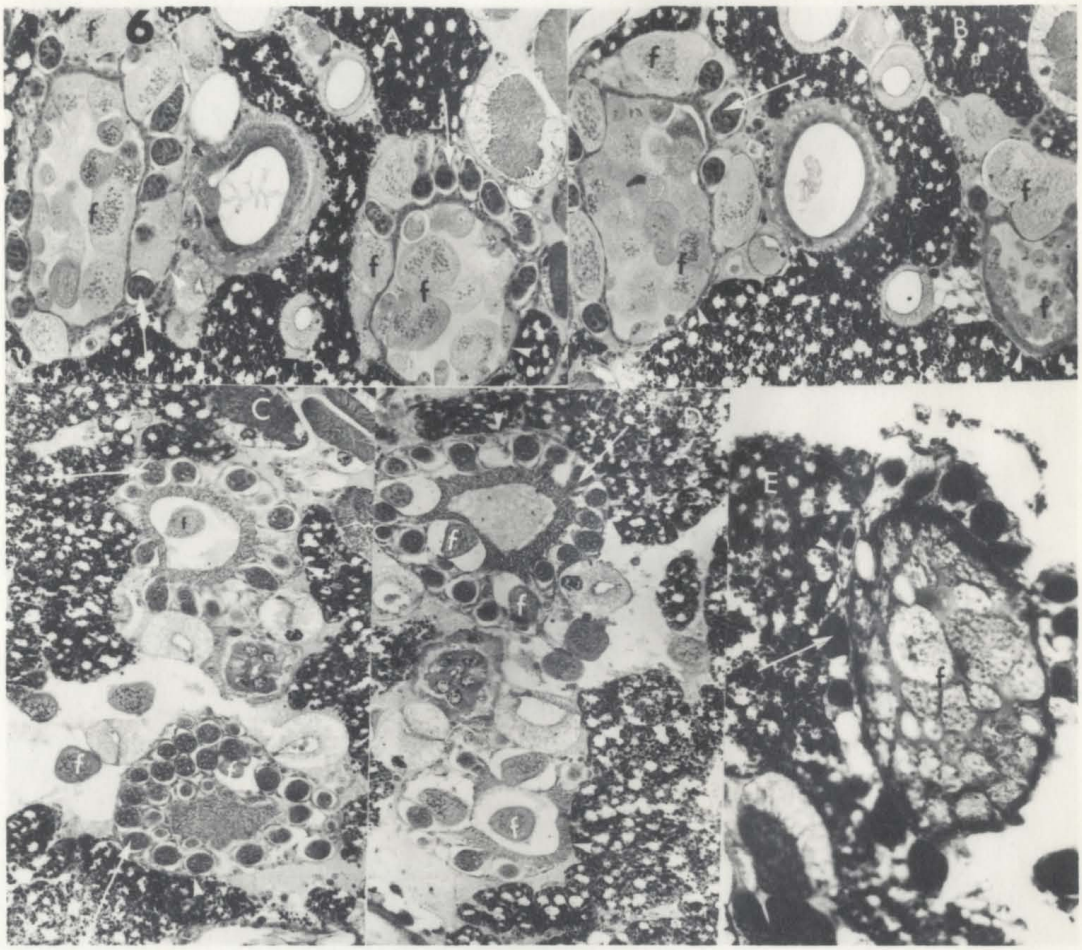

5
a
ovary
Ca
ovariole
b
ovariole
ovary
Ca
c
ovariole
ovary
d
Ca

Figure 6. (A-E) Photomicrograph of section of a *St. mutata* infected pupa showing various stages of development of the fungus.

Magnification: A-D; 154X, E; 240X

Stain: Heidenhain's iron haematoxylin and eosin.

arrows = developing ovarioles (follicles)
arrow heads = ovaries
f = fungus


6
A
B
C
D
E
f

of the infected pupa, as was in the case of the larva, were not symmetrical in shape (Fig. 6 A-D). The ovarioles at this stage are of the same shape in the infected pupa as in the uninfected pupa. However, the number and size of the ovarioles are remarkably reduced and their place occupied by the fungus. The infected ovary appears as one large cavity filled with the somatic cells of the fungus. The somatic cells are large, coenocytic, irregularly-shaped and measure $60.4 \pm 8.4 \times 99.9 \pm 16.7$ µm.** Some somatic cells were also present in the fat body at the same time as they occured in the ovaries (Fig. 6, C,D). Division of the somatic cells to produce spores appears to occur in late pupa, and the fly emerges with immature spores. By the time the parasites have completed spore development, the adult fly would be ready to oviposit.

**small sample confidence interval for µ is given by $\bar{y} \pm \frac{t\alpha/2s}{\sqrt{n}}$, where $s/\sqrt{n}$ is the estimated standard deviation of $\bar{y}$. [n in above measurements = 20, $\alpha = 0.05$.]

Infection in adults

Table 1 shows the change in infection rate in a population of *St. mutata* over a two month period in 1979. From May 22-29 the infection rate was 40.5%; however, after this date the infection level drops remarkably and this corresponds with the appearance of parous flies. It could also be the result of fungal-infected flies being ready to oviposit earlier than normal flies.

TABLE 1

Field-collected adults — *St. mutata* from Little Power Pond, 1979.

Date Collected	No. Dissected	No. Infected	No. Uninfected	No. already Oviposited	% Infection
May 22	113	42	71	0	37.2
May 23	61	23	38	0	37.7
May 24	69	26	43	0	37.7
May 29	73	36	37	2	49.3
May 31	204	34	170	57	16.7
June 5	68	13	55	19	19.2
June 7	200	14	186	114	7.0
June 11	14	1	13	2	7.1
June 12	192	8	124	91	4.2

Only one collection of females was made on June 10, 1980; out of 957 flies dissected only 98 were found to be infected — an infection rate of 10.2%. This rate agrees with that of 1979 at that time of the year. The collections in 1980 were hampered by highly variable weather conditions which meant periods of oviposition were unpredictable and probably concentrated into short

times of favourable weather and easily missed. Because the weather was cold the rate of infection in 1980 may not be comparable to that of 1979, making statistical analysis of little value. Out of 31 flies reared in the laboratory in 1979, 6 were found to be infected — an infection rate of 19.4; and out of 220 flies reared in the laboratory in 1980, 21 were infected — an infection rate of 9.5%. The discrepancy between the two rates of infection could be due to an uneven distribution of infection along the stream. The pupae which were reared to adults were collected from different areas along the stream. The lower infection rate between laboratory-reared flies and field-collected flies indicate three possibilities: (1) either infection was high at the pond outlet and not evenly dispersed downstream (2) that the fungal-infected flies emerged earlier than uninfected ones or (3) the fungal spores matured faster than eggs so parasitized flies oviposited earlier. In 1980, the weather was cold and emergence delayed. The effect of this delay on the infected larval and pupal *St. mutata* is not known.

Table 2 shows the infection rate in *P. mixtum* for the same two month period in 1979.

TABLE 2

Field-collected adults — *P. mixtum* from Little Power Pond, 1979

Date Collected	No. Dissected	No. Infected	No. Uninfected	No. already Oviposited	% Infection
May 24	134	0	134	125	0
May 29	171	2	169	154	1.2
May 31	603	17	586	211	2.8
June 5	299	2	297	37	0.7
June 7	573	20	553	70	3.5
June 11	38	1	37	8	2.6
June 12	102	1	101	18	1.0

The data show a lower average infection rate, 1.7%; however, it appears collection started when the number of parous flies had started to increase and the infection rate was also decreasing in the fly population. Indeed the infection rate in laboratory reared *P. mixtum* from the same site was 10.5% (7 infected out of 67 dissected). An infection rate of 6.8% was found in the same species collected from Beachy Cove River and reared in the laboratory. Although this may not be a true reflection of the rate of infection in the field, it gives an indication that the rate of infection is likely to be higher than 1.7% in *P. mixtum*. A population of attacking *P. mixtum* was examined in 1979. These flies had completed the first cycle of eggs and were seeking blood meal to develop the eggs. None of the 246 attacking flies was infected. Therefore infected flies must either die shortly after first oviposition

or, at least, do not seek a blood meal. No infection was found in the 318 laboratory-emerged *S. verecundum/ venustum*. These species are anautogenous, and the eggs would not develop without a blood meal. Because the laboratory-reared flies were fed only on glucose, the ovaries themselves were poorly developed at the time of examination. Therefore, it was not easy to determine, under dissecting microscope, whether a poorly developed ovary was normal or infected. No ovipositing adults were obtained from the field; only those seeking a blood meal. Again none of these parous flies was infected. No infection was found in the few (31) *S. vernum* flies that were examined.

Recognition of infection in adult flies

Parasitized flies have no visible external characteristics that indicate infection except when wet and the whitish spore mass is visible through the poorly pigmented junctions between the abdominal segments, under the dissecting microscope. The only positive method for confirming infection was by dissection. The spores as seen in the dissected fly appear white as does the infected ovary. When the ovary is punctured the spores appear as tiny granules under the dissecting microscope. At higher magnification mature spores are round to oval in shape, coenocytic, and have a definite cell wall (Fig. 7).

The mature rounded spores measure 13.3 ± 1.64 µm in diameter (ranges 10.5 - 17.5; n = 10, α = 0.05), and the more oval measure 22.8 ± 3.9 x 29.9 ± 3.1 µm (n = 10, α = 0.05). In the early stages of spore development the somatic cells become elongated and measure 22.75 ± 4.2 x 88.9 ± 12.4 µm (n = 10, α = 0.05) — Fig. 8. Occasionally eggs were found together with the fungus (Fig. 9); but it is likely that such eggs were degenerate and nonviable as they contrasted sharply with normal eggs (Fig. 10). Apart from destruction of ovarian tissues and eggs, there were no obvious pathological changes that could suggest that the parasite interfered with the functioning of other tissues of the host. Neither were external physical symptoms of the infection observed.

Sectioned and stained adult ovaries

The ovaries of the newly emerged flies Fig. (11A) were found to be in stage II (Mokry, 1979). By the third day the nurse cells were contracted and the follicles almost filled with yolk (Fig. 11B). The oocyte nucleus is still seen at this stage. The infected fly emerges with only a few poorly developed follicles (Fig. 12A). The nurse cells could hardly be seen; and the follicular epithelia appeared degenerated. No yolk was seen in the follicles of the

Figure 7. Photomicrograph of mature spores of *St. mutata* released at oviposition.

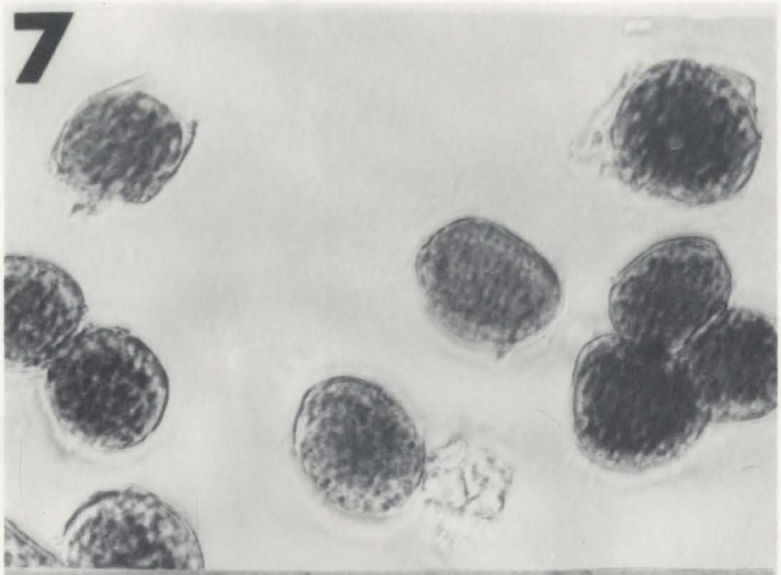
Fixed and stained: Bartch's method

Magnification: 768X

Figure 8. Photomicrograph of early stages of *St. mutata* spore formation.

Fixed and stained: Bartch's method

Magnification: 768X


7

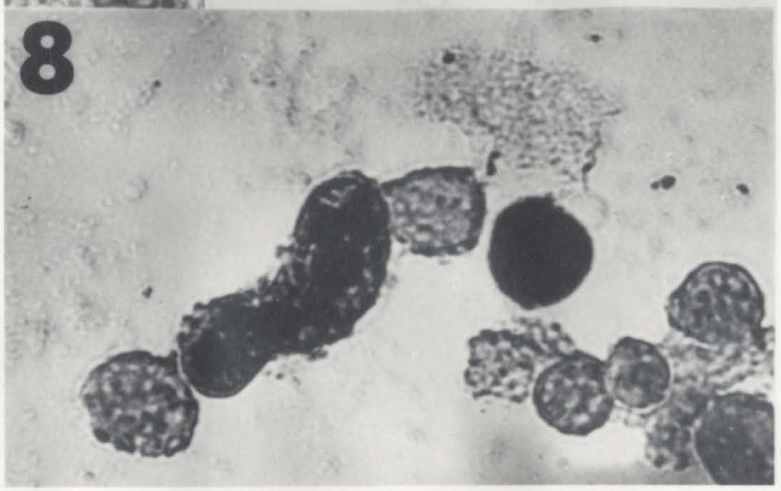

8

Figure 9. Photomicrograph of infected ovary showing spores and degenerated eggs of *St. mutata* (labelled in picture).

Magnification: 192X (wet mount)

Figure 10. Normal ovary showing healthy viable eggs of *St. mutata*.

Magnification: 192X (wet mount)

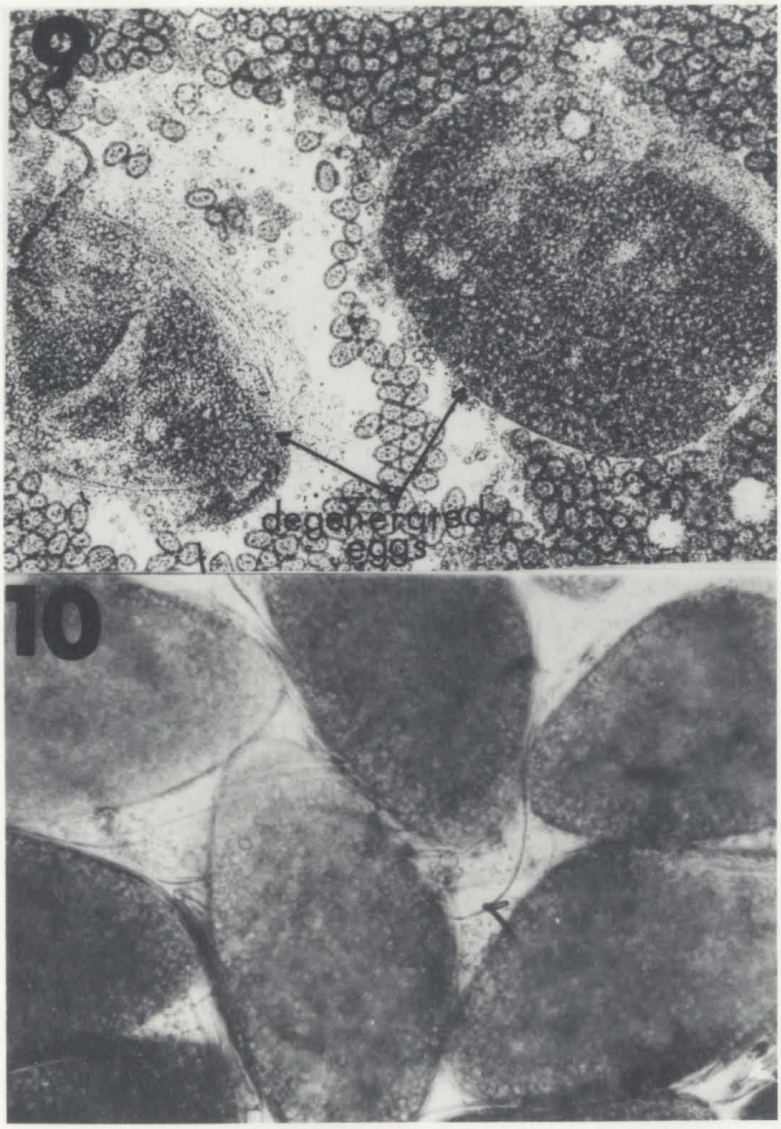

9
degenerated eggs
10

Figure 11. Photomicrograph of section through a normal *St. mutata* showing the development of ovaries after emergence.

A) 1st day after emergence
   Magnification: 208X
B) 3rd day after emergence
   Magnification: 170X

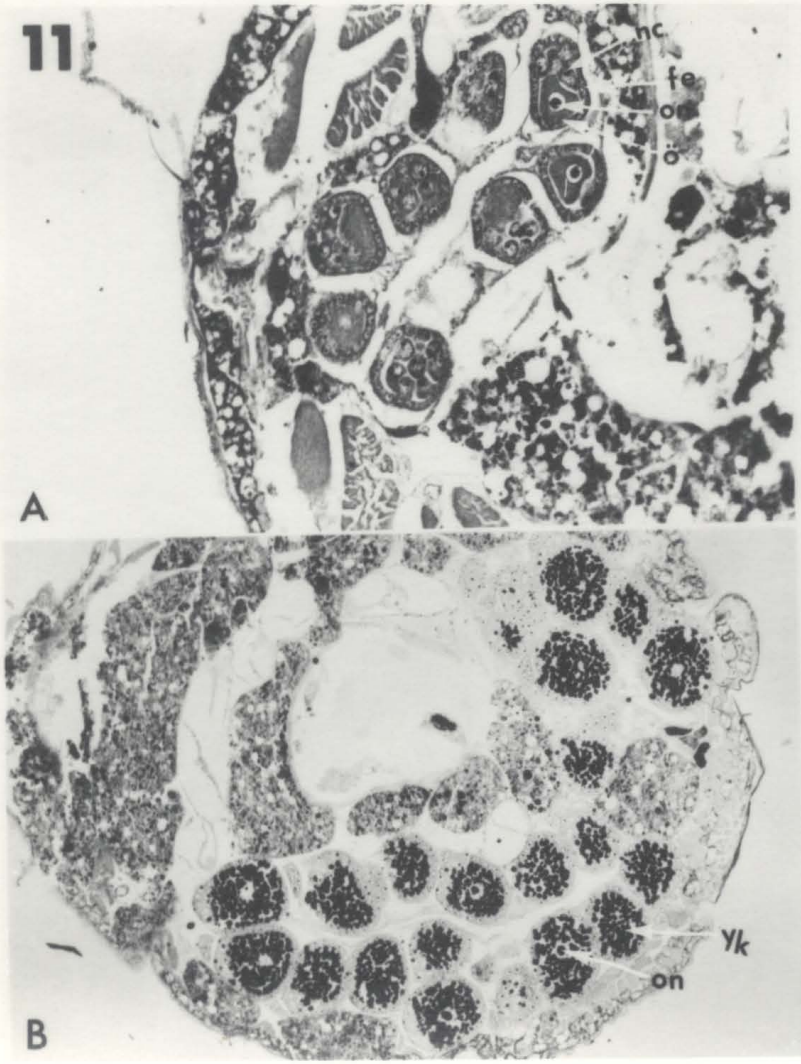
Stain: Heidenhain's iron haematoxylin and eosin

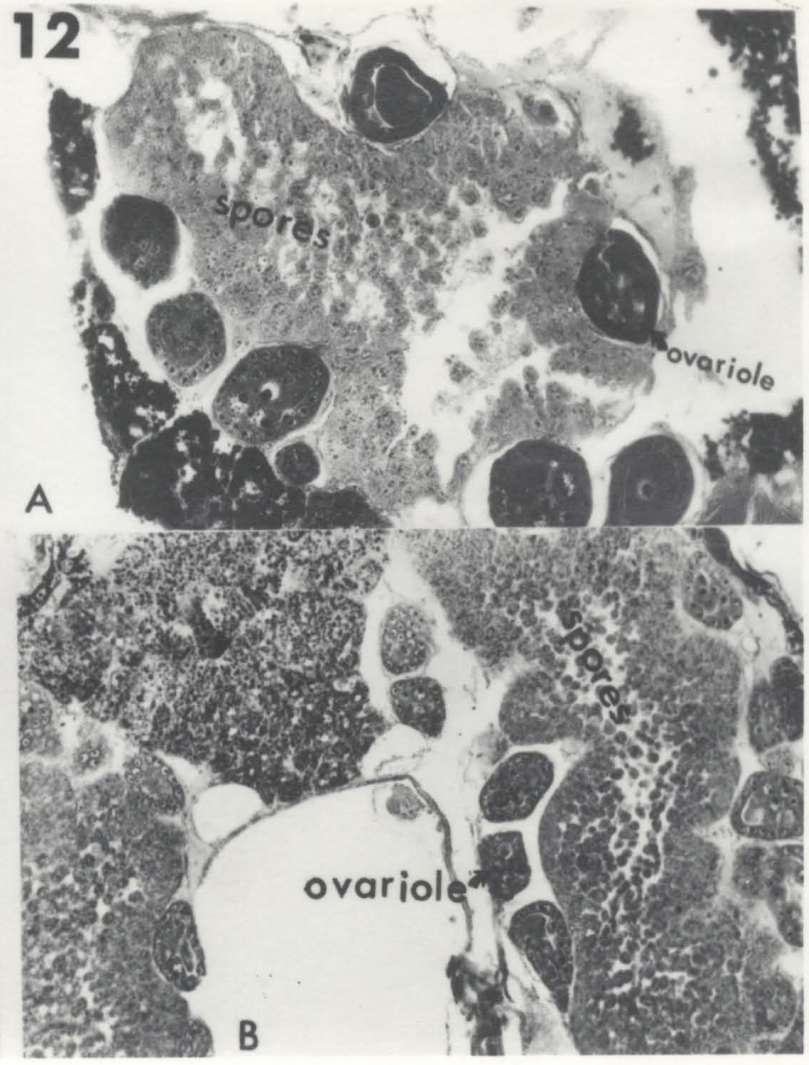
fe = follicular epithelium
nc = nurse cells
ö = oocyte
on = oocyte nucleus
yk = yolk granules

Figure 12. Photomicrograph of section through an infected *St. mutata* showing development of the fungus after emergence.

A) 1st day after emergence
   Magnification: 325X
B) 3rd day after emergence
   Magnification: 208X

Stain: Heidenhain's iron haematoxylin and eosin


11
nc
fe
o
ö
A
Yk
on
B


12
spores
ovariole
A
spores
ovariole
B

infected fly on the third day (Fig. 12B). A comparison of Fig. 12A and B shows that the division of cells to produce spores is continued in the newly emerged fly (Fig. 12A) and by the third day after emergence the spores were nearing maturity. An infected fly emerges with eggs which are unable to develop (Compare Fig. 11B, the ovary of the normal fly, with Fig. 12B, the ovary of an infected fly). By the time the fly is ready to oviposit the ovary is filled with spores (Fig. 13). Occasionally, there may be a few degenerate cells at oviposition (Fig. 9); but generally no eggs remain. Epon sections confirm that the ovarian follicles of the infected fly are completely destroyed as shown in Fig. 15 (compare with the normal ovary Fig. 14). Thus, it would be impossible for an infected fly to develop eggs again. The fat bodies of infected flies were always healthy. The development of the fungus in infected flies in the laboratory took between 3 and 5 days.

Longevity Experiment

The flies in this experiment were a mixture of *P. mixtum* and *St. mutata*. Only one fly, *St. mutata*, was found to be infected in the longevity experiment (Table 3). This reflected the low overall rate of infection for 1980. The fact that an infected fly

Figure 13. a) Photomicrograph of section of an infected *St. mutata* ovary showing massive numbers of spores.

Magnification: 174X

Stain: Heidenhain's iron haematoxylin and eosin

b) Photomicrograph of the part of a spore-packed ovary of an infected fly as seen on dissection.

Magnification: 128X (wet mount)

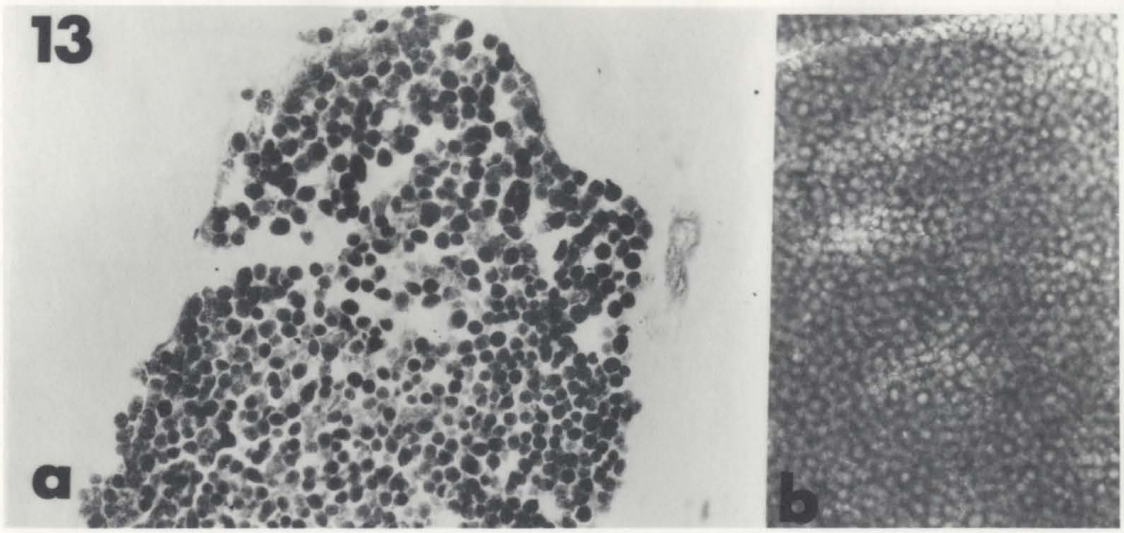

13
a
b

Figure 14. Photomicrograph of section of a normal *St. mutata* ovary (0.5 µm) showing healthy follicles with yolk granules - labelled in picture.

Magnification: 260X

Stain: Toluidine Blue and Sodium Borate

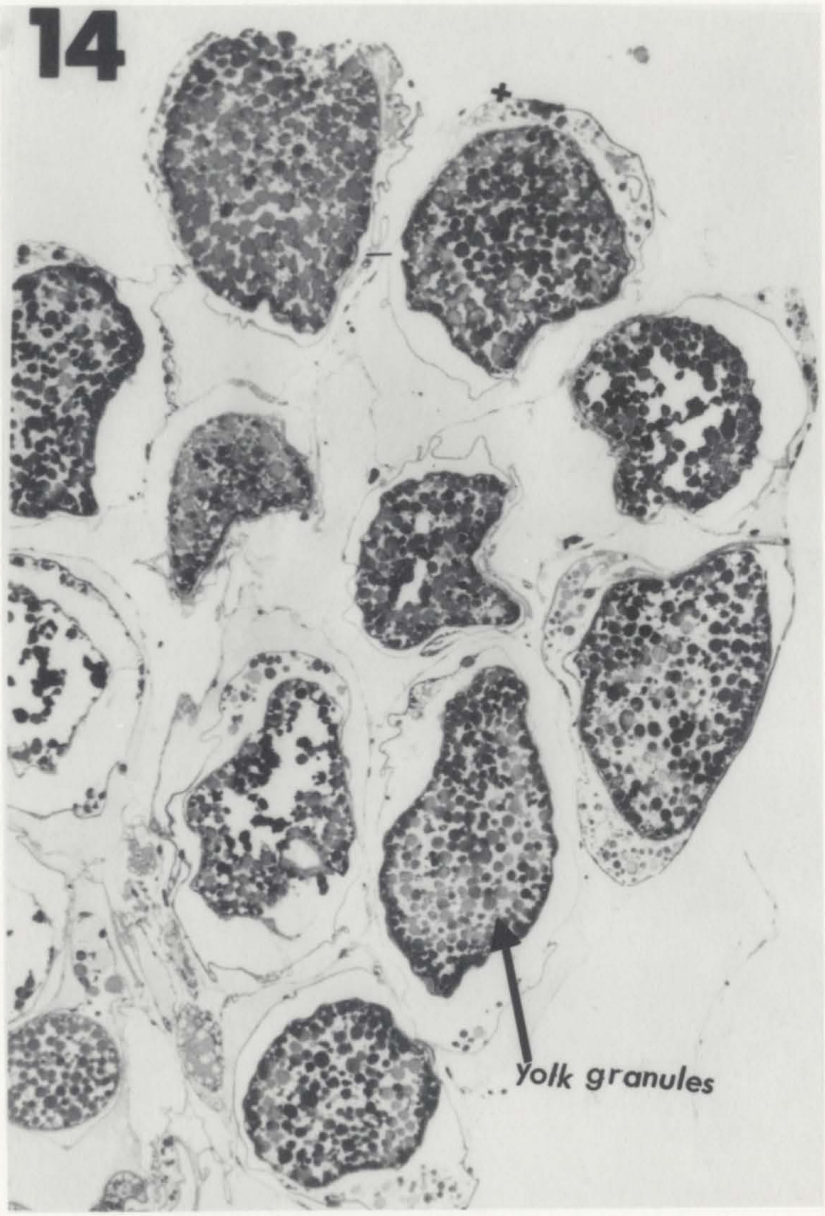

14
yolk granules

Figure 15. Photomicrograph of section through an infected *St. mutata* ovary (0.5 m) showing degenerate eggs and spores. Note the absence of yolk-filled follicles as in Fig. 14.

Three degenerate eggs are shown in A and C and spores magnified in B.

A) Magnification: 140X
B-C) Magnification: 558X

Stain: Toluidine Blue and Sodium Borate

de = degenerated eggs
s = spores

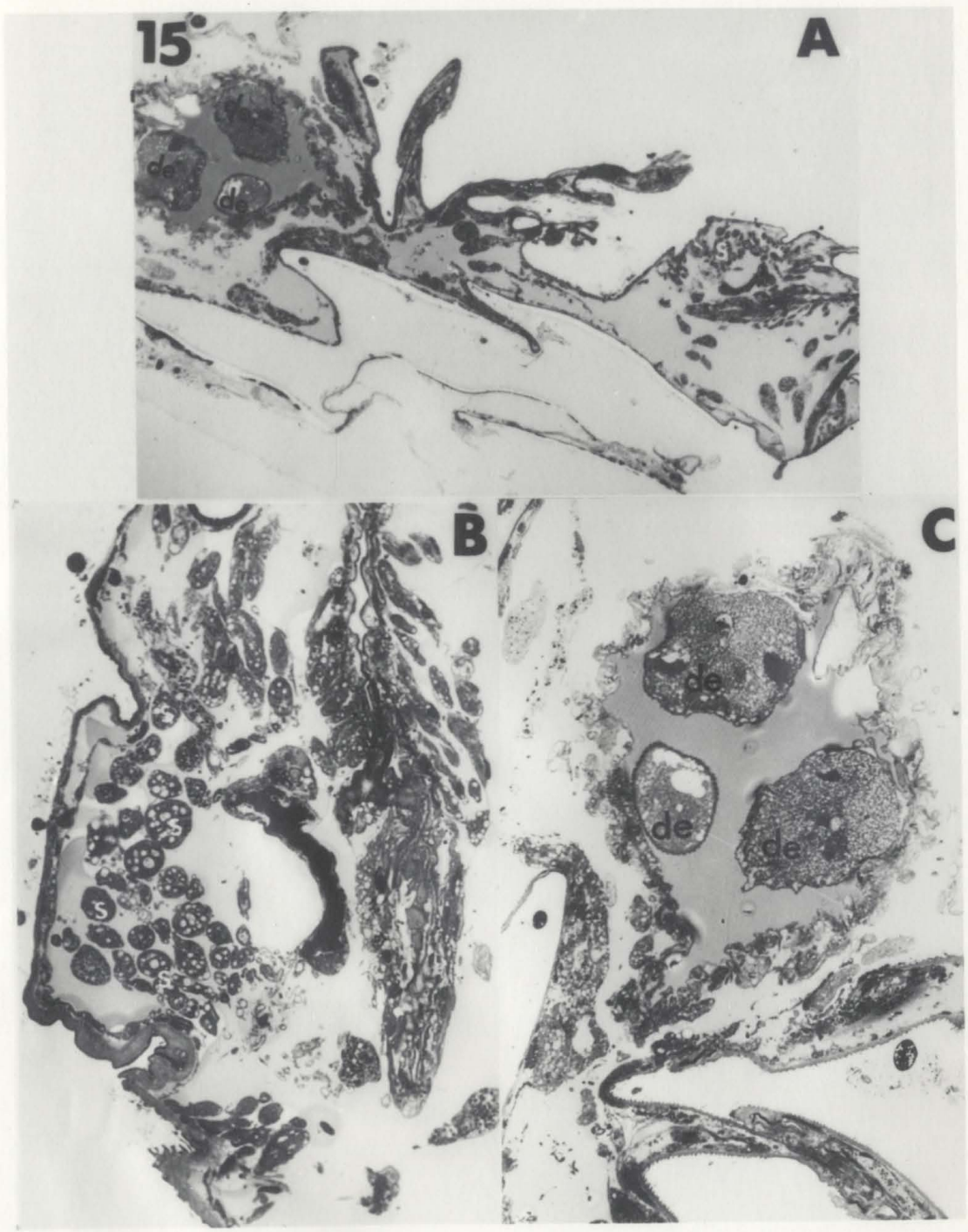

15
A
de
de
B
S
C
de
de
de

TABLE 3

Longevity of ovipositing flies in captivity.

Days in Captivity	No of Flies Died	SUMMARY OF MORTALITIES			
		INFECTED		NO. UNINFECTED	
		Oviposited	Not Oviposited	Oviposited	Not Oviposited
Initial number of flies = 35					
1	7*	0	0	4	3
2	4*	0	0	1	3
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	3	0	0	1	2
7	0	0	0	0	0
8	0	0	0	0	0
9	4	0	1	2	3
10	0	0	0	0	0
11	0	0	0	0	0
12	2	0	0	1	1
13	0	0	0	0	0
14	4	0	0	1	3
15	0	0	0	0	0
16	0	0	0	0	0
17	10+ 1 escaped	0	0	5	5

* Mortalities more likely due to trauma.
+ Flies 'knocked out' to terminate experiment.

caught at oviposition lived for 9 days indicated that the fungus does not kill the host. Allowing for 5 days between fly emergence and oviposition, then the infected fly in the experiment lived for at least 14 days which is considered normal (Davies, 1953; Mokry, 1979). A normal fly after oviposition retains the ovarian follicles and relics which indicate the number of gonotropic cycles (Fig. 16). However, because the infected fly in this experiment did not oviposit in the laboratory, the condition of an infected ovary after oviposition could not be ascertained.

Incubation of spores

Spores from dissected flies and those collected from the field could not be cultivated on the artificial media tested. However, partial success was achieved in water alone. The source of water, from the tap or field, did not affect the spore development into sporangia; however, temperature had a significant effect. At 4°C development was slow; between 4°C and 10°C the sporangia took 5-7 days to become completely developed. Above 10°C (maximum 20°C) the sporangia developed completely in 2-3 days. In the laboratory the spores developed one or more sporangia (Fig. 17 A-C); although it was usual to see development of single sporangium from spores collected on *S. verecundum* egg masses

Figure 16. Photomicrograph of normal *St. mutata* ovary after oviposition showing relics which indicate the number of egg cycles - one in this case. (wet mount)

The follicular relic, ovariole and germarium, and a'residual egg' are shown by labels.

Magnification: 500X

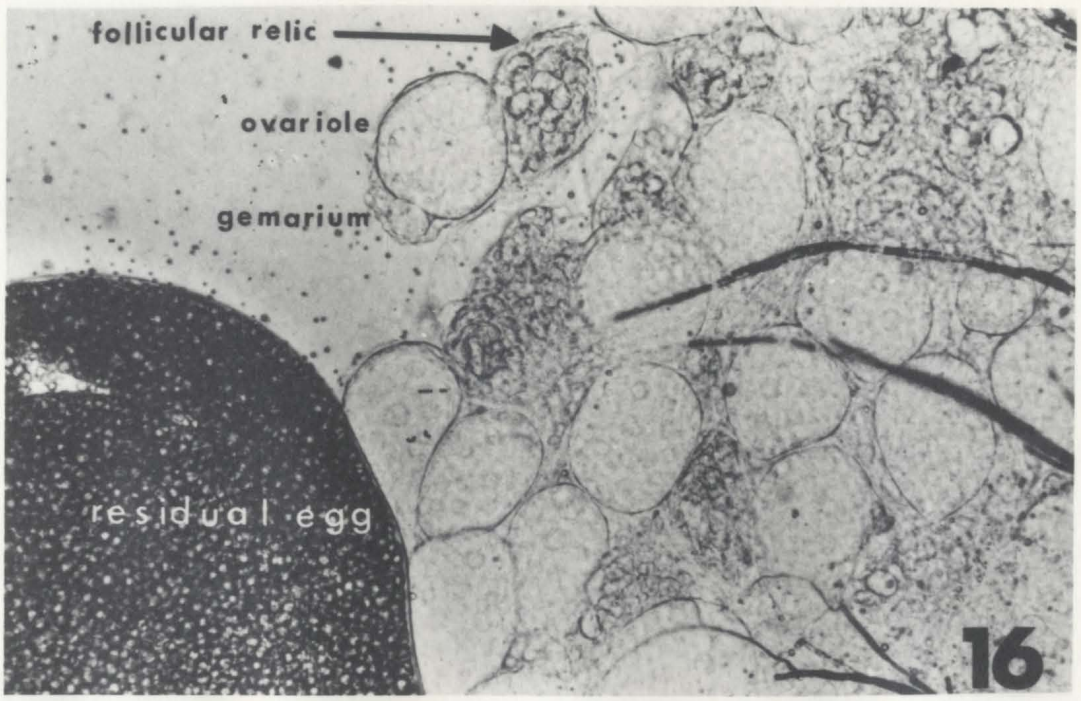

follicular relic
ovariole
gemarium
residual egg
16

Figure 17. Photomicrograph of stages of development of the fungus in water.

A-C) Spores collected from *St. mutata* and laboratory cultured.

D-F) Fungus collected from *S. verecundum* egg mass from which G-I developed in the laboratory.

A) early stages of sporangia formation (es). Magnification: 686X

B) Spore (sp) and spore with fully developed sporangia (s); a septum is shown on one sporangium. Magnification: 686X

C) Sporangium showing growing tip (gt); Sporangia separated from spore (Ss); Sporangia attached to empty "spore case"(Sc). Magnification: 268X Fixed: Bartch's method

D) Spores (sp) and sporangia (s). Magnification: 172X

E) Early stages of sporangia formation (es). Magnification: 172X

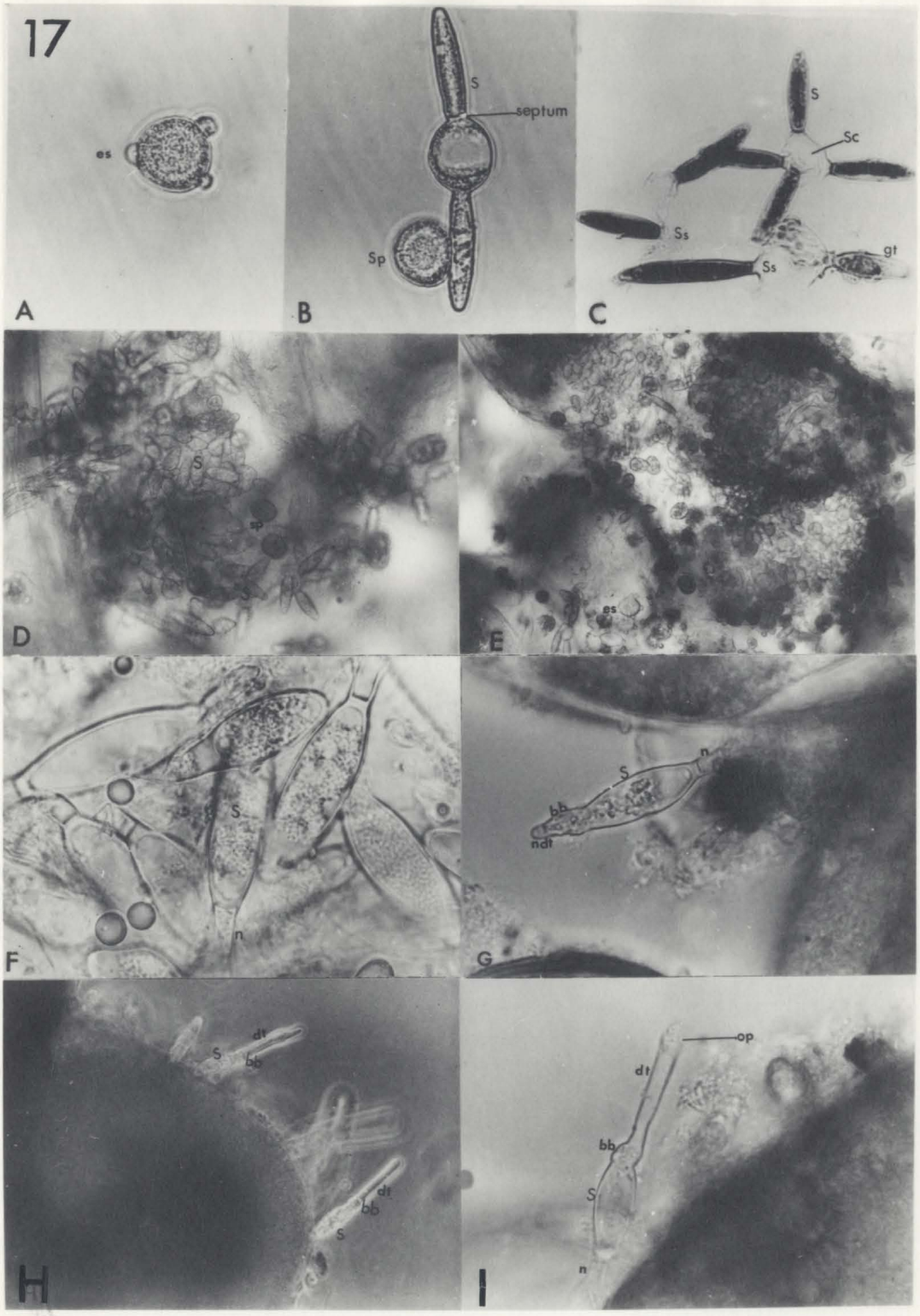
F) Sporangia showing neck (n). Magnification: 1072X

G) Early stage of discharge tube formation (ndt), and bulge (bb) at base of tube. Magnification: 686X

H) Fully developed discharge tube (dt) Magnification: 268X

I) An open discharge tube (op). Magnification: 686X

Only C was fixed; the rest were mounted wet.


17
es
A
S
septum
Sp
B
S
Sc
Ss
Ss
gt
C
S
sp
D
es
E
S
n
F
n
S
bb
ndt
G
S
dt
bb
S
dt
bb
S
H
op
dt
bb
S
n
I

in the field (Fig. 17F). As development proceeded sporangia appeared from the spore (Fig. 17A, E). A developing sporangium had a growing tip (Fig. 17C) which measured between 5 and 10.2 µm. Between the spore and the sporangium is a constricted region, referred to in this study as the neck (Fig. 17F). The neck measured between 15.3 and 25.5 µm in length and 4.5-6 µm in width. The sporangia continued to elongate until at maturity they measured 11.2 ± 1 x 38.2 ± 7 µm; $n = 10$, $\alpha = 0.05$ (Fig. 17B). The content of the spore then moved into the sporangia, leaving an empty "spore case" (Fig. 17C, Sc.). After evacuation of the spore, the base of each sporangium was sealed off by the formation of a septum (Fig. 17B). In some cases the "spore case" remained attached to the sporangium(a) for 3-4 weeks but eventually broke off (Fig. 17C, Sc). Development of the spores dissected from *St. mutata* and *P. mixtum* stopped at the sporangial stage, and after 3-4 weeks the sporangia started to die. There were also some instances of destruction by bacteria in the water. Some of the spores from *St. mutata* and *P. mixtum* were incubated with mature eggs dissected from flies caught at oviposition, but this did not affect the rate of development of the fungus. Development still stopped at the sporangial stage. The eggs did not hatch because they were in diapause and the sequence to break diapause was not followed.

The fungus collected from the field on *S. verecundum* egg masses were almost always in the sporangial stage at the time of collection, some days after oviposition (Fig. 17D); however, the process of development took the same form. Figure 17E shows the early formation of sporangia (es). On the *S. verecundum* eggs which hatched, the sporangia produced discharge tubes (Fig. 17G, H) within 48 hours of egg-hatch. The discharge tubes measured between 25.5 and 81.6 µm in length and 2.5 µm in width. Before the discharge tube formed the growing tip formed a bulge (Fig. 17G, bb) to mark off the sporangium from the discharge tube. The discharge tube eventually breaks open to release zoospores (Fig. 17I). The actual exit of the zoospores from the tubes was not witnessed. However, prolonged agitation of zoospores during which they moved slowly towards the tip of the discharge tube was observed. The fungus on *S. verecundum* eggs kept at 4°C did not develop the discharge tube. The spores stored dry did not develop when rehydrated in water.

Electron microscope studies of the spore did not reveal any additional information on the spore structures beyond what had been previously observed with light microscope.

DISCUSSION

The life cycle of the fungus is not completely understood; however, an attempt will be made, using the information so far obtained, to provide a hypothetical one. It would be expected that the life cycle of the fungus would coincide with that of the flies which they parasitize, and the evidence collected supports that hypothesis.

The life cycle of the fungus (Fig. 18), as observed outside the host, starts with the mature spore which is released as the fly attempts to oviposit. The fact that the spores did not develop on the artificial media tested but did develop in water suggests that free water is required for spore development. Also, as spores failed to develop after being dried, they must not tolerate desiccation which is also true for blackfly eggs.

Soon after its release into water the spore germinates, forming sporangium(a) which takes 1-7 days to develop. Undeen and Nolan (1977) observed germination after 3 days and up to 37 days of incubation in distilled water at 4°C. In this study germination was found to occur within 24 hours of incubation in water at 10°C and above (maximum, 20°C), and sporangia developed completely within 2-3 days. These temperatures are within the range normally recorded in the local streams.

Figure 18. Probable life cycle of ovarian "phycomycete".

A) Early stages of discharge tube formation
B) Fully developed discharge tube
C) Opening of discharge tube.

bb = bulge at base of discharge tube
dt = discharge tube
es = early stages of spore formation
esp = spore in early stages of sporangia formation
fs = fully formed sporangium
gt = growing tip of sporangium
ms = mature spore
n = neck
np = ? naked protoplasm stage of fungus in fat body
s = sporangium
sc = empty "spore case"
se = septum
sm = somatic cells in ovaries

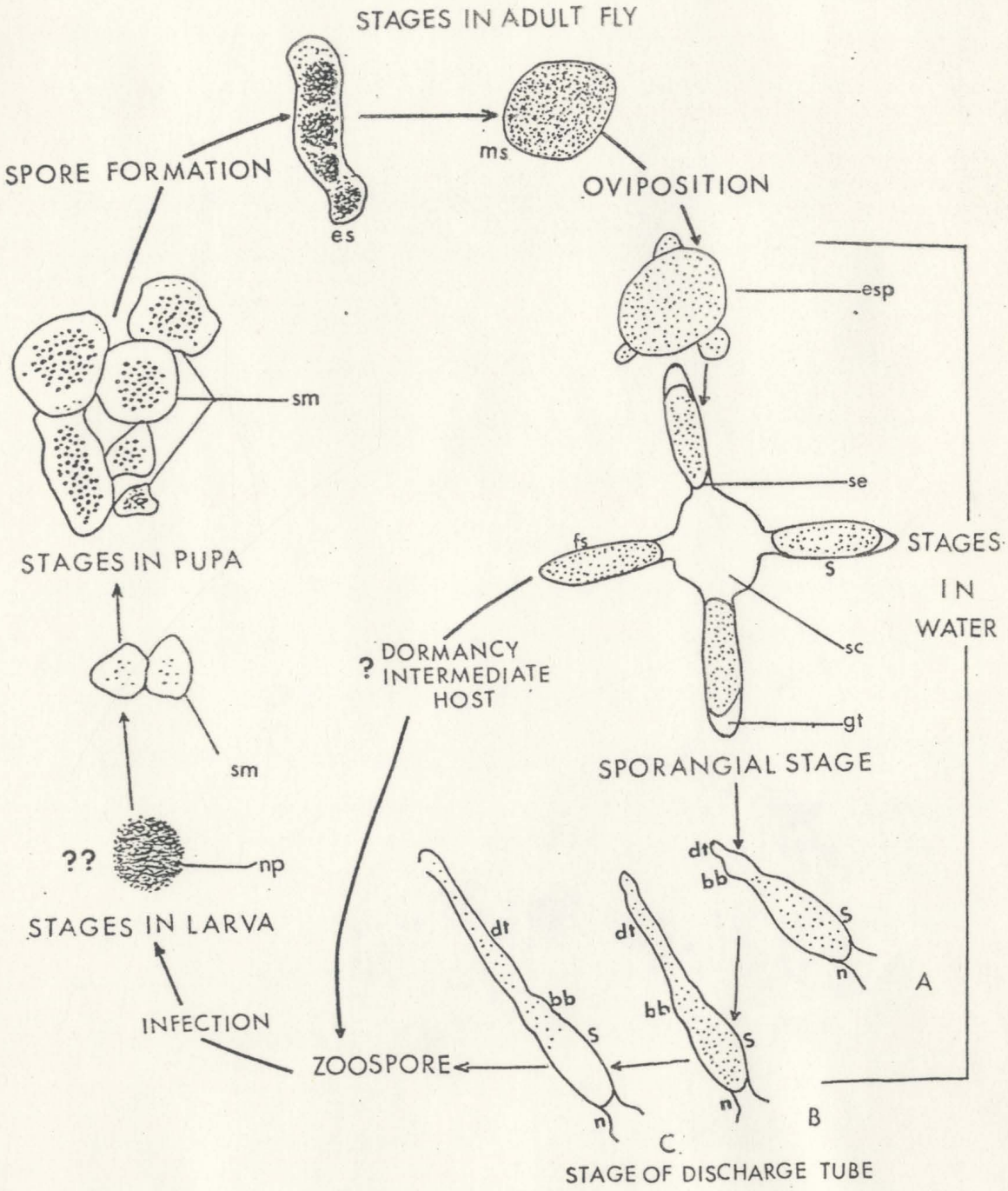

STAGES IN ADULT FLY
SPORE FORMATION
es
ms
OVIPOSITION
esp
se
fs
s
sc
gt
STAGES IN WATER
SPORANGIAL STAGE
sm
STAGES IN PUPA
? DORMANCY INTERMEDIATE HOST
sm
??
np
STAGES IN LARVA
INFECTION
ZOOSPORE
dt
bb
s
n
A
B
C
STAGE OF DISCHARGE TUBE

FIG. 18

At 4°C complete development of the sporangia took from 5-7 days; the development being retarded by these cold temperatures. During the process of development the cellular contents of the spore move into the newly formed sporangia which become sealed off from the empty "spore case" by septa. A developing sporangium has a transparent tip which bulges out at maturity and then develops into a discharge tube. The discharge tube eventually opens, possibly releasing zoospores which might infect newly hatched larvae. The nature of the zoospores, especially their flagellation, has so far not been elucidated. Development of the fungus from *St. mutata* stopped after reaching the sporangial stage. The discharge tube development was only observed in the fungus collected from *S. verecundum* egg masses. The discharge tube development appeared to coincide with egg-hatching, and perhaps, the egg-hatching process provides a stimulus that triggers tube development. The rate of development of the fungus therefore, appears to be determined by the rate of development of the species of the fly it parasitizes. This would be beneficial to the fungus. In the univoltine *St. mutata* and *P. mixtum* the eggs hatch in the fall but are oviposited in the spring, therefore having a summer diapause of about 4-5 months before hatching (Lewis and Bennett, 1974; Colbo, 1979).

The fungus parasitizing these species should have a life cycle that would coincide with that of the flies in order to survive. This may explain why development of the spores from *St. mutata* and *P. mixtum* was arrested at the sporangial stage which may be a resting stage for the fungus.

In contrast, *S. verecundum* hatches in the spring and starts oviposition in late June. It is multivoltine, with the early egg batches hatching soon after oviposition (Lewis and Bennett, 1974). One would expect the fungus to quickly develop beyond the sporangial stage if subsequent infection was to be achieved. In the laboratory the fungus on *S. verecundum* eggs developed discharge tubes shortly after the eggs hatched. From late summer the egg batches of *S. verecundum* are no longer seen on grass and overwintering diapausing eggs are laid (M.H. Colbo, personal communication). When *S. verecundum* eggs go into diapause in the fall, it would appear probable that, the fungus parasitizing this species would take an alternate pathway of development that could be similar to that of the fungus parasitizing *St. mutata* and *P. mixtum*. It is likely that during egg diapause the fungus also passes into a dormant stage or passes the next stage in an alternate host.

Coelomomyces, which is a "Phycomycete" parasitizing mosquito larva, has a resistant stage in the mosquito host and also requires a copepod to complete its life cycle (Whisler and Zebold, 1974; Whisler *et al.*, 1975; and Pillai *et al.*, 1976). It is, therefore, possible for the ovarian "phycomycete" to have a dormant and/or intermediate stage(s). In *Coelomomyces* infections, a zygote infects the host larva, finally developing thick-walled resistant sporangia (Whisler *et al.*, 1975). Under appropriate conditions the sporangia release zoospores of opposite mating type which infect the intermediate host. Each zoospore develops into a thallus and, eventually, gametangia. Gametes of opposite mating type fuse either in or outside of the copepod host to form the mosquito-infecting zygote. Zoospores of some of the lower fungi are known to encyst, germinate and release secondary zoospores (Alexopoulos and Mims, 1979). If the fungus parasitizing *St. mutata* and *P. mixtum* follows the pathway having an intermediate host, then development of the fungus after the sporangia have formed would be linked to the presence of the intermediate host. In the fall and winter the secondary zoospores will be released to infect the simuliid host larvae. Alternately, the fungus may follow the pathway of zoospore encystment; dormancy will then ensue until the simuliids hatch in the fall. However,

S. verecundum would seem to be infected soon after the eggs hatched in the summer; the infection would then be repeated through the two or three generations of the fly until the fungus eventually took an alternate pathway which coincided with the diapause stage of the host's eggs.

The life cycles of the flies involved suggest a possibility of two seasonal forms* of the fungus — summer and winter — each form having a life cycle that coincides with that of the blackfly species they parasitize. Additional evidence for their being two forms is that the infections in the winter and summer were found in different streams. The response of the fungus to the environment after the sporangia have formed may be critical for its survival. The absence of the appropriate stimulus in the laboratory environment may account for the death of *St. mutata* and *P. mixtum* spores in culture.

Although the infection process has not been observed, it is likely that, like some of the known forms of entomogenous fungi, it occurs by penetration of the host's cuticle. An early larval instar is most likely to be the stage infected by the fungus. Because the fungal spores are released at the same site at the time of oviposition, the larvae are most likely infected before they drift downstream. It seems unlikely that the pupa is invaded months after

---

* In this thesis "form" is used in the general sense and not meant to imply a definite taxonomic entity.

hatching and at a considerable distance downstream. Early larval infections will only be determined when the life cycle and biology of the fungus are well understood.

Development of the fungus in the larva is not fully understood. Some Chytridiomycetes are unicellular and holocarpic. These organisms have no mycelium and, in the early stages of their development, may lack cell walls (Alexopoulos and Mims, 1979). Also according to Fitzpatrick (1966), after some members of the Chytridiales effect penetration of the host, they live intracellularly — briefly as naked protoplasts. At maturity they possess a membrane and function as sporangia. The abnormal cells found in the fat body of a larva lacked cell walls as described above. However, it was felt that it would be inappropriate at this stage to attribute the mere presence of this kind of abnormality in the larval fat body to an infection resulting from the ovarian fungus. The ovaries are probably infected between middle and late larval instars of the blackfly host, with the earlier infections occurring most likely, in the fat body. Both ovaries were found to be infected in all cases observed. In the larval ovaries considered infected, all cells had cell walls; certain ones were nucleated which could possibly be ovariole cells, and there were also certain coenocytic ones which were probably the fungus (stage unknown).

Infection in the pupa can be seen clearly in the ovaries. The somatic cells of the fungus are unicellular and coenocytic. The infected ovaries at this stage begin to show signs of pathological changes. The eggs are reduced in number and do not develop well. It is likely that the fungus responds to the same stimulus that initiates transition from pupal to adult ovaries. The response of the fungus to the stimulus is probably induction or stimulation of the somatic cells to produce spores. The process of spore formation may be preceded by elongation of the somatic cells, aggregation, and separation of the cellular content. This stage of elongation or development of a rod-shaped body seems identical to that described by Garms (1975). The formation of spores is likely to begin in late pupa. The division of cells started in the pupa continues in the newly emerged adult. The immature spores have prominent nuclei (Fig. 11A); this seems to be the only stage in which the nuclei are so prominent. Development of the fungus in the blackfly host took 3-5 days under laboratory conditions. By the time the infected fly was ready to oviposit the ovaries had been taken over completely by the spores. There may be few eggs together with the spores at the time of oviposition, but these would be degenerate and not likely to be viable. The fungus thus completes its life

cycle at the time the fly is ready to oviposit.

It appears that the pathological effect of the infection is manifested in the adult fly when the final stage of development is reached. The result of parasitization is a complete destruction of the tissues of the ovaries and their contents. The eggs are completely destroyed and the ovaries become filled completely with spores. Another pathologic effect observed was fragility of the ovaries which burst in response to the slightest pressure from a dissecting pin. Lewis (1960a) reported a similar occurrence. He also concluded that most parasitized flies did not live long enough to bite a second time. However, because it was shown in the longevity experiment that the longevity of an infected fly was not affected by the presence of the parasite, the view expressed by Lewis cannot be supported here. The infected fly is rather rendered incapable of a second gonotropic cycle due to destruction of the ovarian tissues. In the present study no parous flies collected at the time of biting were found infected. Infected flies have no ovaries to develop after the first cycle and therefore do not return to oviposit a second time. The destruction of the ovaries probably results in the fly not exhibiting the normal blood-feeding behaviour after oviposition hence no parous attacking fly was infected. Therefore, the infection decreases

in the ovipositing fly population with time as only ovipositing nulliparous flies are infected. The important effect of parasitism on the host population is the loss of fecundity.

Based upon the species of flies examined so far, the infection appears to be established in *St. mutata* and *P. mixtum*. The infection rate in *St. mutata* was found to be 40%. The discrepancy between the rates of infection in larvae and pupae and the adults could be due to uneven distribution of the infection along the stream. It is known that *St. mutata* goes upstream to oviposit and that there is a general dispersal of larvae downstream with time (Colbo, 1979), therefore most of the spores would be deposited at the head of the stream and infection may be heavier there. Other reasons for the discrepancies in the infection rate may be because the fungal-infected flies emerged earlier than normal or the fungal spores matured faster than eggs so that parasitized flies oviposited earlier. A true infection rate in *P. mixtum* could not be determined because of the nature of the data obtained. *Simulium verecundum* is only a suspected host at present because the fungus was found on its egg mass; a survey of ovipositing flies of this species was not carried out. Also the abnormalities seen in the undeveloped ovaries

of laboratory-reared flies could not be positively attributed to the ovarian fungus.

Very few fungi can be identified if the nature of the zoospore, especially its flagellation, is not known. Because of the paucity of information about the ovarian "phycomycete" which made the initial work difficult, and also the limitations within which this study was undertaken, the specific identity of the fungus could not be elucidated. However, the rod-shaped, pre-spore stage and the spores observed in this study appear'to resemble' those reported by Garms (1975). Also from the illustrations shown by Lewis (1960a, 1960b), it appears he also worked on similar organisms. Therefore these fungi are probably widespread zoogeographically.

The ovarian "phycomycete" appears to have potential as a biocontrol agent for blackflies. The most important reasons for its practical use as a biocontrol agent are two-fold. First, because infected females do not produce eggs; therefore, manipulations of the fungus to achieve a higher infection rate in the field will certainly reduce the reproduction rate of the uninfected population. The second, and perhaps most beneficial, is the loss of vector potential of infected flies; because infected flies do not bite after oviposition, autogenous species do not blood-feed at all

and anautogenous species do not bite a second time. Therefore, a high parasitism by this fungus in a fly population would reduce the number of biting flies and the vector potential as well. This fungus thus, should be considered as a potential candidate for biocontrol against blackflies, at least, to the point of elucidating its complete life cycle and determining the possibilities for mass production for release.

SUMMARY

The ovarian "phycomycete" parasitizes *St. mutata* and *P. mixtum*. The maximum infection rate in *St. mutata* was found to be 40%, but the rate in *P. mixtum* could not be accurately determined. The fungus was also found on *S. verecundum* egg masses, although the infection was not positively identified in the adults examined.

The fungal spores deposited by the female flies develop in water. They did not develop on the artificial media tested, and they did not withstand desiccation under the conditions tested. In water the spore produces sporangia within few days of deposition. These in turn develop discharge tubes under certain conditions. The discharge tubes presumably open to release zoospores. The nature of zoospore flagellation, which is a key character in the taxonomy of the lower fungi, was not observed, and the fungus remains undiagnosed. Discharge tube development appears to coincide with egg development. The evidence gathered indicates at least, two seasonal forms — winter and summer — with each form parasitizing blackfly species in these seasons. The known portion of the life cycle of the fungus coincides with that of the host. It is possible that a dormant stage of the fungus equivalent to the diapause stage of the blackfly's eggs occurs, or that an intermediate host is involved during the host's egg diapause. It is presumed that the early

larval instar of the host is the stage of infection. This process is likely effected through the cuticle of the host. Infection in the pre-imaginal stages of the host can only be recognized by histological technique as no physical signs or symptoms are obvious. The fungal infection destroys the adult ovaries, resulting in the loss of fecundity. Also infected flies do not take blood meal following oviposition thus, eliminating infected flies as potential vectors of disease organisms. Therefore there is the possibility for the use of this parasite for the control of black-flies if additional work on the biology can elucidate methods of mass production and application in the field.

REFERENCES CITED

Alexopoulos, C.J. and C.W. Mims. 1979. "Introductory Mycology". Third edition. John Wiley and Sons, New York.

Beaudoin, R.L. and W. Wills. 1968. *Haplosporidium simulii* sp.n. (Haplosporidia: Haplosporidiidae), parasitic in larvae of *Simulium venustum* Say. J. Invertebr. Pathol. 10, 374-378.

Colbo, M.H. 1979. Distribution of winter-developing Simuliidae (Diptera), in Eastern Newfoundland. Can. J. Zool. 57, 2143-2152.

Collins, C.H. and P.M. Lyne. 1970. "Microbiological Methods". Third edition. University Park Press, Baltimore.

Couch, J.N. and C.J. Umphlett. 1963. *Coelomomyces* infections. In "Insect Pathology: An Advanced Treatise" (edit. by Steinhaus, E.A.). Vol. 2, Academic Press, New York.

Davies, D.M. 1953. Longevity of black flies in captivity. Can. J. Zool. 31, 304-312.

Debaisieux, P. 1920. *Coelomomycidium simulii* nov. gen. nov. spec. et remarques sur l'Amoebidium des larves de *Simulium*. La Cellule, 30, 249-277.

Fitzpatrick, H. M. 1966. "The Lower Fungi-Phycomycetes". McGraw - Hill Book co. Inc., New York.

Fuller, M.S. 1977. The zoospore, hallmark of the aquatic fungi. Mycologia, 69, 1-20.

Garms, R. 1975. Observations on filarial infections and parous rates of anthropophilic blackflies in Guatemala, with reference to the transmission of *Onchocerca volvulus*. Tropenmed. Parasit., 26, 169-182.

Garnham, P.C.C. and D.J. Lewis. 1959. Parasites of British Honduras with special reference to leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg., 53, 12-35.

Johnson, T.W. and F.K. Sparrow, Jr. 1961. "Fungi in oceans and estuaries." J. Cramer, New York.

Karling, S. 1977. "Chytridiomycetarum Iconographia." J. Cramer, New York.

Karnovsky, M.J. 1965. A formaldehyde-gluteraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol., 27, 137A.

Keilin, D. 1921. On a new type of fungus: *Coelomomyces stegomyiae* n.g. n.s.p., parasitic in the body-cavity of the larva of *Stegomyia scutellaris* Walker (Diptera, Nematocera, Culicidae). Parasitology, 13, 225-234.

Lewis, D.J. 1958. Observations on *Simulium damnosum* Theobald at Lokoja in Northern Nigeria. Ann. Trop. Med. Parasit., 52, 216-231.

Lewis, D.J. 1960a. Observations on *Simulium damnosum* in the Southern Cameroons and Liberia. Ann. Trop. Med. Parasit., 54, 208-223.

Lewis, D.J. 1960b. Observations on the *Simulium neavei* complex at Amani in Tangayika. Bull. Ent. Res., 51, 95-113.

Lewis, D.J. 1965. Features of *Simulium damnosum* population of the Kumba area in West Cameroons. Ann. Trop. Med. Parasit., 59, 365-374.

Lewis, D.J. and G.F. Bennett. 1974. The blackflies (Diptera: Simuliidae) of insular Newfoundland. II. Seasonal succession and abundance in a complex of small streams on the Avalon Peninsula. Can. J. Zool., 52, 1107-1113.

MacLeod, D.M. 1963. Entomophthorales infections. In "Insect Pathology: An Advanced Treatise" (edit. by Steinhaus, E.A.). Vol. 2. Academic Press, New York.

Madelin, M.F. 1963. Diseases caused by hyphomycetous fungi. In "Insect Pathology: An Advanced Treatise" (edit. by Steinhaus, E.A.). Vol. 2. Academic Press, New York.

McEwen, F.L. 1963. Cordyceps infections. In "Insect Pathology: An Advanced Treatise" (edit. by Steinhaus, E.A.). Vol. 2. Academic Press, New York.

Mendenhall, W. 1975. "Introduction to Probability and Statistics" fourth edition. Duxbury Press, North Scltuate, Massachussets.

Mokry, J.E. 1979. A study of some factors affecting blood-feeding, autogeny and fecundity of *Simulium vittatum* Zetterstedt and *Prosimulium mixtum* Syme and Davis. Unpublished M.Sc. Thesis, Memorial University of Newfoundland, St. John's.

Pillai, J.S., T.L. Wong, and T.J. Dodgshun. 1976. Copepods as essential hosts for the development of a *Coelomomyces* parasitizing mosquito larvae. J. Med. Ent., 13, 49-50.

Ross, I.K. 1979. "Biology of the Fungi." McGraw-Hill Book Company, New York.

Smith, D.S. 1968. "Insect Cells." Oliver and Boyd, Edinburgh.

Sparrow, F.K. Jr. 1960. "Aquatic Phycomycetes." Second edition. The University of Michigan Press, Ann Arbor.

Sprague, V. 1963. Revision of genus *Haplosporidium* and restoration of genus *Minchinia* (Haplosporidia, Haplosporidiidae). J. Protozool., 10, 263-266.

Steinhaus, E.A. 1967. "Principles of Insect Pathology." Hafner Publishing Co., New York.

Strickland, E.H. 1911. Some parasites of *Simulium* larvae and their effect on development of the host. Biol. Bull., 21, 302-338.

Undeen, A.H. 1978. Observation on the ovarian phycomycete of Newfoundland blackflies. Proc. Int. Coll. Invertebr. Pathol. and XIth Annual meeting for Invertebr. Pathol., Agricultural College Campus,

Prague. Sept. 11-17, 1978.

Undeen, A.H. and R.A. Nolan. 1977. Ovarian infection and fungal spore oviposition in the blackfly *Prosimulium mixtum*. J. Invertebr. Pathol., 30, 97-98.

Weiser, J. 1963. Sporozoan infections. In "Insect Pathology: An Advanced Treatise" (edit. by Steinhaus, E.A.). Vol. 2. Academic Press, New York.

Whisler, H.C. and S.L. Zebold. 1974. Alternate host for mosquito parasite *Coelomomyces*. Nature, 251, 715-716.

Whisler, H.C., S.L. Zebold, and J.A. Shemanchuk. 1975. Life history of *Coelomomyces psorophorae*. Proc. Nat. Acad. Sci., 72, 693-696.

Whittaker, R.H. and L. Marguilis. 1978. Protist classification and the kingdoms of organisms. Bio Systems, 10, 3-18.