

ENDOPARASITES OF SIMULIUM ORNATUM MG.  
IN SOUTH ENGLAND,  
WITH SPECIAL REFERENCE TO LARVAL  
PARASITIZATION

Thesis

submitted to the University of London for  
the Degree of Doctor of Philosophy

by

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## ABSTRACT

The thesis reports on investigations during 1967-69 of endoparasitism in Simulium ornatum Mg., with particular reference to the larval stage, from the English rivers Lea (Hertfordshire) and Tilling Bourne (Surrey). Methods included record of visual evidence of infections, dissections and smears of fresh material, and histological examinations of all material by light, and, by arrangement with Dr R.G. Bird of the E.M. Laboratory, L.S.H. & T.M., by electron microscopy, in the case of certain microsporidia. In preliminary observations no parasites were observed in either pupal and adult stages of Simulium ornatum; the study is concerned with larval parasites, of which several kinds were found. The Order Microsporidia of the protozoan Class Cnidosporidia was well represented by 3 genera, Thelohania, Plistophora and Nosema of the Family Nosematidae.

Pseudocysts from two forms of Thelohania, and one form of Plistophora, examined by electron microscopy, revealed the fine structure of sporogonic stages of the sporont and of the spore of Thelohania and, less intensively, of Plistophora.

Microsporidian spore size has been accepted by several investigators, with some controversy from others,

as a major criterion for specific classification. In this connexion, spore measurements carried out for Thelohania infections, from S. ornatum were, by statistical and graphical analysis, recognisable as composing four separate forms (A-D), with a minor form also, form E on the basis of spore shape. All Thelohania infections of larvae from the River Tilling Bourne are of form A, having the smallest spore dimensions. The other four forms, "B", "C", "D" and "E", occurred in Simulium larval populations of the River Lea. In the case of Plistophora another criterion in classification, in addition to spore dimensions, was notable, viz. the number of spores produced by the sporont. Two forms of Plistophora were thus distinguished. Nosema infections were of one kind only. All these forms, found in the Simulium ornatum larvae, are new records for microsporidia in the British Isles and, on taxonomical features different from those of other microsporidian parasites described from simuliidae from elsewhere.

Histological examination also revealed a light infection of a microsporidia-like organism in the gut epithelial cells of Simulium ornatum larvae from the Tilling Bourne only.

Fungal infections were also common. The pathogenic fungi, Coelomycidium sp. of the Order Chytridiales, occurred



larvae revealed 3 distinct larvae generations of Simulium ornatum per year, and the occurrence and abundance of the several parasites was noted in respect to these different larval populations.

Laboratory culture of "clean" material was desirable for the transmission experiments undertaken for Thelohania, and for Coelomyxidium. To obtain such a culture, it was necessary to affect both copulation of Simulium ornatum adults and to induce females to blood-feed. Repeated attempts at artificial insemination proved unsuccessful; blood-feeding was successful with only a low percentage of adult females. Failing to establish a "clean" culture resort was made to the use of apparently uninfected wild-caught larvae for experiments on the transmission of infections, but without success in inducing hatching of Thelohania spores or propagation of Coelomyxidium ingested by Simulium larvae. It remains still obscure whether or not, in nature, the Thelohania infections are transmitted transovarially through infected adults emergent from infected larvae; and the mode of transmission of Coelomyxidium is also not determined.

While the pathogenic parasites found must have controlled larva densities in the two streams, much remains to be studied and understood about their biology, transmission

and cultivation before their application in biological control could be envisaged.

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INTRODUCTION

In the present study of endoparasites of Simulium ornatum larvae from rivers in Hertfordshire and Surrey during 1967, 1968 and 1969, several organisms were found. It is appropriate to review first the literature for each kind of parasite separately.

1 - Protozoan parasites

(i) Class: Cnidosporidia Doflein

Order: Microsporidia

Family: Nosematidae

The earliest record of parasites in Simuliidae was of a microsporidian in S. ornatum in Europe, thought to be a new species of Glugea, G. varians (Leger, 1897). Lutz & Splendore (1904) assigned microsporidians in the larvae of Simulium venustum and S. ochraceum in Central America to Glugea, but later (1908) described the organism as 3 varieties of Nosema simulii, referring to them as Plistophora-type and Thelohania-type. Strickland (1911) wrote on "sporozoid" parasites of the larvae of Simulium hirtipes and other Simulium spp of N. America, and later (1913) designated them as three new species of Glugea, G. bracteata, G. fibrata and G. multisporea although expressing the doubt that they should, perhaps, belong to the genus Thelohania.



It is difficult to understand why Strickland first placed these organisms in the genus Glugea, which was erected by Thelohan in 1891 for microsporidians with two spores in their pansporoblast, since the organisms described by Strickland as G. bracteata and G. fibrata had octosporous pansporoblasts, a main taxonomic feature of the genus Thelohania (Henneguy, 1892), and Glugea multisporea had a multisporeous pansporoblast typical of the genus Plistophora (Gurley, 1893). Debaisieux (1919) described Thelohania varians but conceded that it was identical to Glugea varians discovered by Leger (1897). Further consideration by Debaisieux & Gastaldi (1919) of the three organisms of Strickland (1913) and parasites of some European species of Simulium larvae resulted in their designation as Thelohania multisporea, T. fibrata and T. bracteata. Although they included the multisporeous organism in octosporous Thelohania, they claimed their diagnosis justified their decision, albeit open to dispute. They also described material of what they called Plistophora simulii, noting its similarity to Nosema simulii described by Iutz & Splendore (1908).

However, Jirovec (1943) in a revision of microsporidian parasites of Simulium larvae classified an

organism similar to Nosema simulii of Lutz & Splendore (1904, 1908), - synonymously Glugea multisporea of Strickland (1913), and Thelohania multisporea of Debaisieux & Gastaldi (1919) - as Plistophora simulii. This parasite was from larvae of Simulium venustum Say, S. latipes Mg. and S. ochraceum Coq of C. America, and there were also two new species, which he named Plistophora debaisieuxi and Nosema stricklandi, in this Simulium material.

Although at first microsporidian parasites of Simulium were placed in the genus Glugea, later revisions and subsequent failure to find parasites in Simulium referable to this genus, show that Glugea does not parasitize Simulium.

In his 1911 report, Strickland mentioned his discovery of a parasite of Simulium larvae having numerous "ovoid" bodies bearing a flagellum-like organelle. Later, Weiser (1947a) described similar material as Caudospora simulii from larvae of some European Simuliids. This remained the only known species of this genus till Beaudoin et al (1965) described from the larvae of some N. American blackflies, C. pennsylvanica. Another microsporidian genus Octosporea, contains the sole species, O. simulii (Debaisieux, 1926). No further microsporidian species

were described from blackflies until 1960 when Weiser described a new species, Thelohania columbaczense, parasitizing pupae of Simulium reptans columbaczense in Europe.

There are two monographs on the microsporidia, Kudo (1924b) and Weiser (1961), as well as a revised key to the classification by Weiser (1966), yet the history and validity of earlier records of the microsporidian genera and species of Thelohania, of Plistophora and of Nosema is still confused with doubts about whether re-descriptions are actually of material identical with that formerly described. Characters relied on for differentiation were:

- 1- Host tissue infected.
- 2- Shape and dimensions of sporont
- 3- Number of spores produced by the sporont
- 4- Shape and dimensions of the spore

and occasionally:

- 5- Length of the polar filament extruded from the spore
- 6- A thin filamentous projection from the spore proposed as a new taxonomic feature of microsporidia (Vavra, 1963).

The value of the above criteria in speciation



requires comment: All the microsporidian species so far discovered as parasites of Simuliids were found to infect the fat body of, mainly, the larvae. The shape of the sporont of the parasites is usually more or less a sphere, although oval forms are not uncommon; the shape of the spore with few exceptions, is generally ovoid. Of the other criteria, and particularly in microsporidian parasites of mosquitoes, Walters (1958) ruled out spore dimensions as unreliable for taxonomic purposes. On the other hand, Thomson (1968), using an ocular micrometer, was able to measure microsporidian spores to the nearest  $0.1\mu$  and advocated strongly the validity of spore dimensions in microsporidian taxonomy. His measurements, it could be noted, were perhaps too remarkably precise.

Vavra (1964) outlined the possibility of error in measuring fixed and dried microsporidian spores by the conventional methods and devised a method of photomicrography of fresh spores in a monolayer.

Studies mainly with parasites from hosts other than Simuliids of other kinds, on spores, were pursued with a view to clarifying taxonomy. Farley's modification (1965) of Kudo's method (1924b) to demonstrate, by Ziehl's carbol fuchsin, the acid-fast nature of microsporidian



spores proved to be quite successful although this staining reaction of the spore remained unexplained.

Mercier (1908), Kudo (1918, 1924a, 1924b), Ohshima (1937), Trager (1937), Gibbs (1953), Dissanaïke (1955), Dissanaïke & Canning (1957), Walters (1958), Laird (1959), Weiser (1959, 1961), Kramer (1960), Canning (1962a, 1962b), Vavra (1963, 1964), Lom & Vavra (1963a, 1963b), Manier & Maurand (1966), Maurand (1967), Ishihara et al (1968), and others contributed useful biological, morphological and taxonomical studies on the microsporidian spore, for several different Orders of Insecta. Trager (1937) obtained hatching of microsporidian spores of Nosema in vitro and claimed a partial development of the spore in tissue culture. Experiments were made to cause extrusion of the polar filament of microsporidian spores with chemicals such as nitric acid, strong salts, hydrogen peroxide, etc. Concentrated  $H_2O_2$  was a most convenient reagent (Kudo, 1918) and as a 3% solution also (Walters, 1958). Ohshima (1937), successful with a mixture of  $H_2O_2$  and sodium chloride, obtained also a relation between osmotic pressure and extrusion of the filament. Gibbs (1953) first dried the spores and obtained extrusion of the filament on later immersion in normal saline. Dissanaïke (1955) extruded the polar filament by mechanical pressure under a coverslip,

a method also successfully used by Dissanaïke and Canning (1957), and Laird (1959).

Ohshima (1937) was the first to suggest that the sporoplasm passed from the spore case through the hollow everted filament. This concept was shared by others, including Gibbs (1953), Walters (1958) and Kramer (1960). Lom and Vavra (1963a) "definitely confirmed" that the sporoplasm may be injected into the host tissue through the hollow filament. A different view is held by Dissanaïke (1955) and Dissanaïke and Canning (1957) who suggested that the sporoplasm is withdrawn out of the spore case as the filament uncoiled. Later, Laird (1959) concurred with this view from his observations on rupture of the spore and discharge of the sporoplasm at the end of the extruded polar filament.

Being highly refractive and thick-walled, the microsporidian spore is undoubtedly difficult to examine for its internal structure by conventional light microscopy, even under phase-contrast, but much detailed information has been obtained by electron microscopy. Weiser (1959) first reported electron microscopy results, and described a vacuole that was formed anteriorly, within which the polar filament was not spiral but coiled irregularly. This interpretation of anterior irregular coiling may have

arisen from an artefact due to being air-dried before fixation, or just a misinterpretation, as later electron microscopy by Huger (1960), Lom and Vavra (1961), Kudo and Daniel (1963), Lom and Vavra (1963b), Lainson et al (1964), Vavra (1965, 1968), Lom and Corliss (1967), Ishihara (1968), Sprague & Vernick (1968a, 1968b), Sprague et al (1968), Stanier et al (1968), Vernick et al (1969), Sprague et al (1969) and Akao (1969) showed that the polar filament was coiled, but posteriorly and in a regular spiral. The organelle known as a "polaroplast" and first described by Huger (1960) has long been mistaken for a vacuole. Electron microscopy study on microsporidians of Simulium is limited, so far, to some comparative observations by Vavra (1965), on three species of Thelohania, two of Plistophora and also Thelohania corethrae from Chaoborus larvae.

I report below on five new forms of Thelohania, two of Plistophora and one of Nosema parasitizing larval Simulium ornatum. The features on which the classification was based and the incidence of the different forms will be described and discussed later, and some account given of fine structure and biology.

(ii) Class: Sporozoa Leuckart

Order: Gregarinida Lankester

Sub-order: Eugregarinina Doflein

Apart from a doubtful identification by Strickland



(1913), no gregarine has been recorded as parasitic in Simulium larvae, possibly due to their association with the host being harmless, as for most gregarines, and the absence of superficial evidence of infection in the whole insect. Those I report on below from Simuliid larvae, from histological studies, were found overlying the cells of the gut epithelium of the larvae and very rarely free in the space between the gut epithelium and the peritrophic tube. Garnham and Lewis (1959) found seven gametocytes of a gregarine in the body cavity of an adult female Simulium.

According to Weiser (Steinhaus, 1963), all "non-pathogenic" gut gregarines of insects belong to the Eugregarinina Leger. Keilin (1920) gave a description of what seemed to be the earliest record for a Eugregarine in the gut of certain terrestrial dipterous larvae; in 1923 he described a Schizogregarine from the same larvae. In 1955 Weiser proposed a new classification of the Schizogregarinina: I quote from him the statement that "Leger in 1900 separated the Schizogregarines from the Eugregarines on the basis of the occurrence of schizogony in the cycle of development of the schizogregarines only".

## 2- Fungal infections

(i) Class: Phycomycetes

Order: Chytridiales

Another little studied group represented amongst the parasites of Simulium larvae is the fungal order Chytridiales, the most primitive of the aquatic Phycomycetes. Some members of this order parasitic in Simulium larvae, lacking mycelia, have been mistaken for the protozoal Haplosporidia. Indeed, I was inclined on occasions to do so with an organism similar to the one described by Debaisieux (1920) and Nöller (1920) as a Chytrid.

At first, and before going into histological investigations, I placed this problematical organism in the fungal Order Blastocladales, Family Coelomomycetaceae, the Order being so closely related to the Order of the Chytrids that one of its families is given by some authors sub-family rank in the Order Chytridiales. The only record of a Coelomomyces in Simuliids is by Garnham and Lewis (1959), who found it in adults of Simulium metallicum of C. America.

Another organism from the larvae of the N. American Simulium bracteatum, somewhat similar to the organism I have found and elected to identify as a Chytrid, was considered by Strickland (1913) as probably a "gregarine".

Jamback (personal communication, 1967) still concurs with this view of Strickland, assigning an apparently similar organism, which he found parasitizing some N. American Simulium larvae, to the Gregarinida.

Confusion of these Chytrids with the protozoan Order Haplosporidia seemed unnecessary as Chytrids possessed naked flagellated spores only. Nevertheless, the spore is not yet described for all genera of Haplosporidia, particularly those affecting molluscs (Haskin et al, 1966). Moreover, a remarkable diversity of spore structure is to be found in one genus, Haplosporidium (Sprague, 1963). The Haplosporidia, however, generally produce spores that, although of the general appearance of microsporidia spores, lack a polar filament. The haplosporidian spore as described by Sprague (1963) has an orifice with a lid, or the spore membrane that raises like a lid, or, as described earlier (Sprague, 1940) and more recently (Woolever 1966), as having a bivalved wall.

No description is offered by Lewis (1960) and Lewis et al (1961) of what is identified as haplosporidia in adult Simulium damnosum from Africa, and Lewis's (1960) designation of one of the haplosporidia by the name of a Chytrid genus is further confusing. Jenkin (1964) also enumerated the Chytrids described by Nöller (1920) and

Debaisieux (1920) and the Trichomycete described by Leger et al (1932) with the protozoan Order Haplosporidia. In fact, the only certain haplosporidian reported from Simuliidae, is Haplosporidium simulii described by Beaudoin et al (1968), as a parasite of larval Simulium venustum of America.

(ii) Sub-class: Trichomycetidae

Order: Harpellales

Not all fungal parasites from Simulium larvae lack true mycelia rendering them difficult to distinguish from certain protozoa, nor are they all pathogenic to their host as indeed was the Chytrid of my material dealt with above. Leger and Duboscq (1929) described the first such non-pathogenic mycelial fungus from the mid-gut of Simulium larvae, and named it Harpella mellusinae, erecting a new Family Harpellaceae in the Order Entomophthorales to contain the organism. Later, when other apparently related genera were discovered and studied, all were combined together in the Order Harpellales of the Sub-class Trichomycetes, a group of primitive fungi.

Within this Order Harpellales of organisms endophytic in aquatic dipterous larvae, Leger et al



(1929) recognised the Family Harpellaceae for members with simple thali and, (1932), a Family Genistellaceae with branching thali. Three genera of the Family Genistellaceae were recognised as parasites in different species of aquatic dipterous larvae. One was Stipella with S. vigilans, an endocommensal in the hind-gut of Simulium larvae. The other two were from dipterous hosts other than Simuliids. Later, other Trichomycetes were reported by Tuzet et al (1955) from S. equinum of France, by Lichtwardt (1964) for Smittium simulii inhabiting the hind-gut of N. American S. argus and S. virgatum.

Whisler (1960) was able to culture a Trichomycete, usually ectocommensal, in tryptone-agar medium. Clark et al (1963) improved this technique by obtaining axenic culture of two endocommensal Trichomycetes from the larvae of Californian mosquitoes. A further success in axenic culturing of Trichomycetes from simuliids and chironomids was attained by Lichtwardt (1964).

I report below on Trichomycetes endocommensals which I have identified to the family Genistellaceae in the hind-gut of S. ornatum larvae, as well as on the pathogenic Chytrids which were obtained.



### 3- Nematode parasitism

Superfamily: Mermithoidea

Family: Mermithidae

Apart from protozoa and fungi, parasitic nematodes have frequently been noted from Simuliidae by many workers. However, knowledge of nematodes parasitic in insects is limited, and identification correspondingly difficult. Strickland (1911) discovered the first such worm in N. American S. hirtipes and had it identified as Mermis sp. Later discoveries were by Swinton et al (1913) of a nematode, Mermis sp., from an adult S. ornatum in Europe, and in Britain Mermis in Simuliidae (Edwards 1920) and, in a S. ornatum larva (Smart, 1934).

In Africa, Simulium damnosum, important because of its role in the transmission of onchocerciasis, has been much studied and many records of mermithids are incidental to biological researches on this fly, e.g. Lewis (1953) from the Sudan; Marr and Lewis (1965) from Ghana; and Lewis (1965) from Cameroon: In addition, records exist for other Simuliids elsewhere (Garnham et al 1959, British Honduras; Lewis et al, 1962 N. Venezuela; and Rubtzov (1964) for mermithids and other nematodes in Simuliids of the Leningrad region.)

Welch (1960) made important progress by describing

the life-cycle and bionomics of Hydromermis churchillensis from N. American mosquitoes. Later (Welch, 1963) a substantial work followed on generally most aspects of mermithid parasites of Simuliidae, discussed further by Petersen et al (1967, 1968). Before this, a monograph by Phelps and DeFoliart (1964) on nematode parasitism of Simuliidae, establishing<sup>ed</sup> valuable guidelines for the classification and biology of these worms, as well as reviewing most of the literature and considering culturing problems. A partial success in culturing was claimed by Phelps and DeFoliart, but, as yet, in vivo culture of mermithids of Simuliidae is still not solved in spite of the success in this respect with mermithids of mosquitoes (Muspratt, 1947, 1965; Petersen et al, 1968).

Contrary to the view of Hocking and Pickering (1954), who generally considered that mermithid parasitism prevented pupation, Phelps and DeFoliart (1964) showed that mermithids would readily pass into adult simuliids after infection took place in the larval stage. Garnham et al (1959) noted the destruction of the ovaries of infected simuliids.

I report below on the few mermithid worms parasitizing my material Simulium ornatum larvae.

4- Miscellaneous infections (Microsporidia-like infections)

I have encountered spores of indeterminate identity infecting the mid-gut epithelial cells of S. ornatum larvae; a brief description is included with the results.

The above review of literature relevant to the present thesis illustrates the limited information available over wide fields of diverse and different classes of endoparasites of Simuliidae, whether of animal and plant kingdoms. In view of this, generic or specific classification of endoparasites of Simuliidae is fraught with problems. The present work does not presume to solve these, but to determine identifications as accurately as possible as a contribution to the taxonomic issues concerned, and the endoparasite-host relationships involved. Indication is given of the seasonal incidence of infections which may be of value for other workers seeking material to pursue the problems which still await solution.

Mortality of the larvae in stream water in large stoppered glass jars was low in a 3-hour journey from the site of collection to the laboratory.

In the laboratory the larvae were placed in circular glass jars (8" diam. x 14") half-filled with a mixture of stream and tap-water at a room temperature of about 21° C. Air was bubbled by a "HY-FLO" air pump continuously against a rectangular glass slab immersed in the jar at an angle; this provided a surface for larval attachment which was readily removed for examination of the larvae as desired. This well-known principle was satisfactory and it was not necessary to use the water current aspirator developed by Yashida et al (1956). Diatoms and cellular algae brought back with the larvae in the stream water provided sufficient food for the larvae; dried yeast initially added was discontinued later.

Overwintering larvae never pupated in the laboratory, not even at the end of their diapause in spring, but larvae from the other generations readily completed metamorphosis, and produced adults within 3-5 days of their arrival indoors. Some pupated in transit.



The emerging adults were trapped in 0.75 ft cubic sleeved cages inverted on top of the breeding jars. These cages were then removed, the sleeves tied and kept in the laboratory at room temperature; glucose solution in blotting paper wicks was continuously provided. Adults survived for 2-7 days; at 6°C survival this extended to about 2-3 weeks. Longer than 3 weeks survival was obtained by keeping the flies in gauze-covered beakers, as devised by Wenk (1966), placed in a "Fison" climatic cabinet running at nearly equal day and night cycles with alternating temperatures of 15°C (day) and 10°C (night) and humidity of 70% (day) and 90% (night).

2- Examination for parasites

A. Larvae: Infection with microsporidia showed typically in live larvae at the time of collection as milky white lobes within the distended body cavity, and a shiny copper colour with honey-comb effect, often coupled by distension of the bulbous part of the body, was evidence of chytrid infection. Larvae showing these signs of infection as well as apparently non-infected larvae were collected in the field, but in the laboratory kept in separate jars for observation and experiments, as described below:

- a)- Examination of the apparently parasitized larvae
- i. Histological method

Larvae were fixed either in Carnoy or Newcomer's fixative for half an hour, or in aqueous Bouin for two hours, dehydrated, cleared in "Supercedrol" for 24 hours (3 changes), embedded in "Paraplast" wax at 60°C., without vacuum, for two hours, and then blocked. Blocks, each of 1 larva, were sectioned at 6 $\mu$ . Longitudinal serial sections spread on slides, were stained with one or other of a number of stains listed below, and microscopically examined for the type and extent of the infection, and for the structure of the infecting organism.

1. Alcoholic-based Gram staining technique for the demonstration of Gram positive organisms.
2. Farleys (1965) modification of Ziehl-Neelsen acid fast stain to demonstrate acid-fast microorganisms such as microsporidia.
3. Hess and Hollander's (1947) toluidine blue method for metachromasia.
4. Heidenhain's iron haematoxylin (Pantin, 1948).
5. Periodic acid Schiff reaction (McManus, 1946; Hotchkiss-McManus, 1948) of polysaccharides and muco-

polysaccharides which when positive is indicated by red or reddish-purple colouration characteristic of positive Schiff's reaction. DeTomasi's method for the preparation of Schiff's reagent was employed (deTomasi, 1936).

6. Giemsa's stain, after the hydrolysis with 1N-HCl at 60°C. for 6 minutes, to demonstrate the nuclei and nuclear division of the micro-organisms. It also gives the effect of acid fast stain.
7. Gridley's (1953) modification of the periodic acid Schiff reaction technique for the selective demonstration of different fungal structures, i.e. conidia are stained slightly differently from hyphae.
8. Feulgen reaction (Stockwell, 1945) - a specific DNA stain.
9. Toluidine blue-methyl green-orange G for nucleic acids (Korson, 1951).
10. Kurnick's (1955) and Papenheim's (Drury et al., 1967) techniques of methyl green - pyronin for the differentiation of DNA and RNA.
11. Himes and Morbider (1956) triple stain for DNA, polysaccharides and proteins, using Schiff's reagent which was prepared as for P.A.S. and Feulgen reactions.
12. Mallory's triple stain for the demonstration of

nuclei. It is based on acid fuchsin and aniline blue.

It was found that when a section of a microsporidian infection to be stained by Giemsa's solution was previously hydrolysed in 1N-HCl at 60°C for 5-6 minutes, a reaction similar to that given by Farley's (1965) acid fast stain was obtained; the nuclei were stained dark purple against a pale pink colour of the cytoplasm. This helped particularly in the demonstration of the grouping of the acid-fast mature microsporidian spores amongst the immature spores and other vegetative stages, and in the study of the pseudocyst of the parasite. The hydrolysis also served to show the nuclei of Chytrid sporangia, otherwise masked by the sporangial membrane that was normally coloured dark blue with conventional Giemsa's stain (Pl. XVI, figs. 4 & 9).

It was claimed by some workers that Heidenhain's iron haematoxylin was particularly useful in staining microsporidian spores and, although Weiser (1961) described it as one of the most beautiful and most lasting stains, it was not found to be so in this study.

Of the stains used to differentiate DNA from other constituents, toluidine blue - methyl green - orange G (Korson, 1951) was most successful in displaying the



general structure of the microsporidian pseudocysts; the methyl green gave the DNA a green colour and the toluidine blue stained the RNA blue; the cytoplasm was stained orange by the orange G with a variable amount of blue colouration according to its richness in RNA.

Papenheim's (Drury et al., 1967) method was not successful but Kurnick's method with methyl green - pyronin differentiated well between DNA (bright green) and RNA (reddish purple)

Feulgen reaction was another good alternative, especially for the Chytrid sporangia and their vegetative stages of development, but it rarely produced a strongly positive reaction with the microsporidian spores.

Schiff's reagent was prepared by deTomasi's (1936) method according to Stockwell (1945) and since most material was previously fixed in Carnoy fixative, the hydrolysis in 1N-HCl at 60°C usually took 8 minutes (Drury et al., 1967).

For demonstration of polysaccharides at all developmental stages of microsporidian spores and more effectively in the Chytrid sporangia, periodic acid Schiff's reaction was attempted following the Hotchkiss-McManus technique. Also, Gridley's (1953) modification of this technique was tried.

To assist in the identification of the fungal Chytrids use was made of chromic acid - methenamine silver reaction which, more or less, gave the same reaction of the P.A.S. test. The sections were oxidised in 5% chromic acid and then treated with methenamine silver till, if positive, the sporangia assumed a deep brown colouration which was differentiated until the background bleached with a solution of 1% gold chloride to leave black sporangia against a colourless background. The background was then counterstained with Ehrlich's haematoxylin and eosin.

ii. Dissections and smears:

Larvae recognised by eye as parasitized with microsporidia were dissected at intervals after each collection to remove the pseudocysts for various observations, as follows:

Structure of the sporonts and spores: The structure of the sporonts and spores in smeared fresh material from the pseudocysts, including phase contrast microscopy.

Development stages of parasites: For microscopical examination and comparative study of the stages of the life cycle of the parasites at different stages

of the year, pseudocysts were regularly smeared on slides, allowed to air-dry and, after a brief hydrolysis in 1N-HCl at 60°C, stained with weak Giemsa's solution overnight.

Spore measurements: The dimensions of spores and sporonts were first measured by the photomicrography method in both fresh and fixed-stained material, making use of Vavra's (1964) monolayer technique for fresh material but, later, most measurements were made with an "Olympus" filar micrometer from fixed Giemsa's stained smears.

Electron microscopy: Microsporidian pseudocysts of mainly Thelohania sp., and some Plistophora sp., were dissected out from infected larvae and, for electron microscopy study, immediately fixed in a 1.2% solution of potassium permanganate in saline in which it remained for 4 hours. After washing and the usual dehydration in ascending grades of alcohol, the material was embedded in araldite resin mixture, and blocked. Blocks were trimmed to a trapezium of 1-2mm dimensions, cut on a Cambridge-Huxley ultramicrotome into gold/silver coloured sections of 20-90µ thickness. Sections were viewed on a Zeiss EM9 electron microscope. The techniques following my dissection were carried out by staff of the Electron Microscopy Laboratory of the London School

of Hygiene and Tropical Medicine, by arrangement with the Director, Dr R.G. Bird.

iii. Trials on the transmission of infections :

Transmission of microsporidian and Chytrid infections was tried by exposing wild-caught, apparently uninfected larvae to parasitic material from infected individuals. Detail is included in later sections of the thesis.

b)- Examination of apparently uninfected larvae

i. Histological method

Out of every periodical collection of larvae from each river, 100 apparently uninfected larvae were prepared for sectioning and examination microscopically as described above for infected larvae. Alternate longitudinal sections were spread on two slides. One slide was stained with Giemsa's solution, or Mayer's haemalum and eosin. If a parasite, or inclusion body in the larval tissues was found, the other slide was stained with one of the appropriate stains enumerated above.

ii. Dissections and smears

At the beginning of the work and before histological techniques were routinely used, examination of larvae not obviously infected by superficial appearance was by



dissection in Ringer's solution or tap water, (about 4000 collected from the River Lea over five months), and microscopic examination for parasites of their tissues, gut and haemocoel contents both in fresh and dried-stained conditions. Later, with knowledge gained from histological investigations that followed, only selected larval tissues were isolated and examined. For gut parasites, the gut was gently pulled out intact after the head capsule as well as the last two posterior segments of the larva were snipped off. The peritrophic tube was then carefully removed thus leaving only the gut epithelium on the drop of fluid to be cut open longitudinally. Then, under a binocular stereoscopic microscope, the inner surface of the epithelium was carefully scanned and any fungal mycelia or other foreign bodies picked off for further microscopical examination in both fresh and fixed conditions.

B. Pupae: Except for the winter months when only larvae occurred and at the conclusion of emergence phases, about 50 pupae of Simulium ornatum were smeared on slides at the stream site every time a collection was made during the second year of the study. The smears were air-dried overnight and stained in Giemsa's solution for microscopic examination. No parasites were found in

pupae by this method. It was concluded, with reluctance, that the more intensive method of histological search for parasites in pupae as used for larvae, did not warrant the time which would be taken up within the overall period available for the present study. No other pupae studies were made.

C. Adults: About 25 males and 25 females of each batch of adults reared out in the laboratory from larval collections were fixed either in aqueous Bouin for two hours or in Carnoy for half-an-hour with subsequent dehydration and clearing processes, followed by embedding in "Paraplast" wax at 60°C and blocked. Blocks, each containing a single fly were sectioned at 4-6 $\mu$ , and stained with Giemsa's solution for microscopic examination. No parasites were found in adults by the histological method. No adults were dissected or smeared.

## RESULTS

Simulium ornatum: larval seasonal abundance:

There were marked seasonal fluctuations in the abundance of the S. ornatum larvae. Large numbers of overwintering larvae, hatched from eggs that were generally laid in the beginning of November, started to pupate in March of the following year and produced adults of the spring generation. By the end of April emergence of adults was complete; no larvae would be found.

Eggs were laid again in early May. The larvae hatched from these eggs had a much shorter life span than those of the overwintering brood, as adults of this summer generation would start to emerge in June, and by the end of July or the beginning of August, the emergence of adults had finished and only empty pupal cases remained in the breeding habitat.

The eggs of these summer adults were laid in August; they also developed rapidly through the aquatic stages to give a further autumnal generation of adults, all of which emerged by October to contribute to the November oviposition which started the overwintering larval generation.

There were, thus, three distinct seasonal

generations of larvae resulting in three distinct peaks of abundance and three periods of scarcity of larvae in the streams. A marked rise of water level or faster water currents caused by heavier than usual rainfall, constituted major factors in periodic reductions of the larval population which occurred at times.

### The parasites

Since no parasites were found either in pupae or in adult flies, this section deals exclusively with larval parasitization.

#### I- Protozoan infections

##### 1. Class: Cnidosporidia Doflein

##### Order: Microsporidia Balbiani

a)- General: The members of the Class Cnidosporidia possess, without exception, resistant spores, initially unicellular. Each spore may later contain 1-6 polar filaments and one to many sporoplasms (Corliss et al., 1963). In the Order Microsporidia, one of four in the class, there is a relatively small spore possessing 1-2 polar filaments and one sporoplasm, all enclosed within a single-valved shell, the Sub-order Monocnidina Leger & Hesse 1922 having a single polar filament and the Sub-order Dicnidina Leger & Hesse 1922 two polar filaments.



All microsporidia I found parasitizing S. ornatum larvae were of the sub-order Monocnida, possessing spores with one sporoplasm and one polar filament; and because the spores were ovoid or oval with length less than four times the width, they belong to the Family Nosematidae Labbe.

Three genera Thelohania, Plistophora and Nosema are represented in my material of larvae of Simulium ornatum.

Apart from larger pseudocyst formations, the sporont and the spore are the only stages I have observed for Thelohania and Plistophora, in spite of the many smears and sections examined. What I have termed as pseudocysts of the parasite have been referred to as parasitic masses by Canning (1953). Anderson (1968) also referred to them as parasitic masses and, in the case of Thelohania sp., he described them as having definitive borders which perhaps consisted of remnants of fat body and connective tissue of the host, larval mosquito.

b)- Morphology of the parasites

i. Pseudocysts: Pseudocysts were exclusively confined to the adipose tissue of the Simulium

larvae and, in advanced infections, are large creamy white opaque bodies which replace much or all of the parasitized translucent fat body (Pl. I and Pl. II).

In a light infection of Simulium ornatum larvae, which I presume to be an early stage of infection following ingestion of spores and their penetration, as planonts, of the gut to reach the haemocoel, cells of the fat body are seen packed with sporonts and spores (Pl. V, Fig. 8). In heavier infections parasitization is widespread in cells of the fat body; the cell boundaries eventually break down so that a fat body lobe may become a mass of sporonts, and spores, surrounded by a 2-5 $\mu$  thick multilaminated wall, the pseudocyst wall. The nature of this wall is obscure (Pl. V, Figs. 3, 4, 5 and 6). It stains reddish with eosin and pink with Giemsa, reactions similar to those I observed for insect connective tissue. In one instance I observed a band of 1-2 cells thick, surrounding the cyst. The cells of this band or wall, stained normally as for the host tissue, apart from being densely pigmented (Pl. IV, Fig. 6).

In electron micrographs (see EMG:1) a definite cellular wall is not evident, but an intricate structure of irregular discontinued membrane-bound cavities, other

structures similar to degenerated host cell mitochondria (M) and host cell nuclei (N) and peripheral to these features, dark round structures, 200-500  $\mu$  in diameter (B). All this suggests that the wall is usually derived from disintegrating fat body cells, rather than a cellular reaction to the parasitic mass.

At the late stage of infection, but only in Plistophora, reddish pigmentation is deposited on the pseudocyst wall and the membranous structures surrounding the sporonts and the spores. This is also reported by Canning (1953) for Nosema locustae in locusts and later, Canning (1962b), attributed to, probably, insectorubin a widespread insect redox pigment (Goodwin et al., 1950).

The generalised way in which the spread of these parasites occurs throughout fat body lobes is not yet clear; nor was I able to see convincing evidence of "germination" of spores to initiate extensive further infection of sporonts within an individual larva, as suggested by Kudo (1966).

In all parasitized larvae from the Tilling Bourne, both Thelohania and Plistophora occurred as one or two pseudocysts confined to the abdominal region of the larva (Pl. II and Pl. III, Fig. 1). On the other hand, all

parasitized Simulium larvae from the Lea have much of their haemocoel occupied by numerous septate pseudocysts. In light early infections small pseudocysts lie posteriorly and in heavier, presumed later, stages of infection they extend anteriorly until, in some instances, they reach the proleg and the head capsule of the larva (Pl. V, Fig. 3).

There seems also to be seasonal variation in the disposition of pseudocysts of Thelohania, which are packed close to each other in the haemocoel of the infected autumn larvae (Pl. V, Fig. 5) and are more discrete and apart from one another in the larvae of the following spring (Pl. V, Fig. 6).

In some pseudocysts the matured spores, which stain darker than other less matured stages, are peripherally situated (Pl. V, fig. 3). In others they are mainly situated in the centre of the pseudocyst, or grouped irregularly throughout the pseudocyst (Pl. IV, Fig. 7).

Although the spores were, in my experience, usually enclosed within the pseudocyst until the larval host perishes, yet there is one example of Thelohania spores breaking from the pseudocysts at a presumably later stage of infection, to disperse in the larval



coelomic cavity (Pl. V, Figs 1 & 2).

The nuclei of the parasitized larval fat cells:

Generally, the host fat cells including their nuclei, progressively degenerate during multiplication of the parasite and, finally, their cell walls. On the other hand, on occasions, there is persistence of the infected host cell nuclei in a hypertrophied state within the pseudocysts; this was seen in several samples of Thelohania infections (Pl. IV, Figs 5, 6, 7, 8 & 9). This feature has also been recorded by Debaisieux and Gastaldi (1919) for Thelohania bracteata, and Plistophora simulii. The shape and disposition of the hypertrophied nuclei within the pseudocysts in the larvae from the Tilling Bourne were similar at all seasons.

It was possible to dissect out intact pseudocysts from the infected larvae for smears and other purposes (Pl. III, Fig. 2). In spite of intensive examination of numerous Giemsa-stained smears representing infections of all seasons of the year, yet only two developmental stages of Thelohania, and of Plistophora, were recognised and further studied, the sporont and the spore. Earlier stages of development were not seen, except in Nosema in which a late schizogony stage prior to the production of sporonts could be observed (Pl. XIII Figs 1, 2 & 3).

The multinucleate sporont or "Plasmodium" described by Hazard & Weiser (1968) for Thelohania legeri in mosquitoes could not be matched nor was the infection of oenocytes evident in my observations.

ii. The sporont

A- In Thelohania

The sporont of Thelohania is generally spherical with a very thin wall, and diameter ranging from  $7\mu$  to  $20\mu$ . In one infection, oval sporonts were seen. The sporont as a uninucleate round body is the first recognisable stage of infection within the larval fat body cells. The highest number I observed within a single intact cell is 16 sporonts (Pl. IV, Figs 2 & 3). Stages observed seem to justify the following interpretation of spore formation. In its early uninucleate stage the sporont has a coarse granular cytoplasm and a large central nucleus. Later, the nucleus constricts deeply at the equator, followed by two further deep furrows at right angles to divide the nucleus into four sub-equal lobes (Pl. III, Fig. 4). Later the furrows deepen and join centrally to separate four daughter nuclei which either remain centrally, or move towards the periphery. In the latter case, they usually elongate to form a broken

circle of chromatin. Each nucleus divides further into two, to give 8 daughter nuclei, although sequential steps of this have not been seen in my material.

The mitotic figures produced by the nuclear division of the sporont, differed in one of a number of ways in different samples of the infected material I have studied (Pls. VII, VIII, IX & X and Pl. VI, Figs 1 & 2). In one infection, nuclear division shows distinct mitotic figures of thin chromosomes, as well as spindle formation (Pl. VI, Figs 1 & 2). Such detail was less clear in other infections (Pl. VII, Figs A1 (1-4) and A2 (1-4), Pl. IX, Figs C1 (1-4) and C4 (1-4) and Pl. X, Fig. D4 (1-4)), and even resembled merely nuclear fragmentation.

Other differences between sporonts occurred. Thus, in two infections (Pl. IX, Figs C2 (1-4) and C3 (1-4)) the nuclei round up after each nuclear division, in another infection (Pl. VII, Fig. A1(1)) they only round up at the 4-nucleate stage of the sporont, whereas in all others compactness of daughter nuclei follows the final nuclear and cytoplasmic divisions. In yet another infection, the daughter nuclei were of variable size, larger than those of the sporonts of all other

infections, stained darker and lacked signs of discrete chromosomes (Pl. XI, Figs 1 & 2, and Pl. X, Fig. D1 (1-4)).

The mitotic shapes of nuclear divisions of a sporont, as well as the shape, size and staining intensity of daughter nuclei in different infections, show some correlation with forms (?species), designated alphabetically A to D, on the basis of spore size which is discussed later below and tabulated (Tables VI & X). The relationship of these mitotic and other features to these alphabetically designated forms, A to D, based on primarily spore dimensions (see later) are summarised below.

Table I: Some features shown by the nuclear division in the sporogony of Thelohania



SPOROLOGY OF <u>THELOHANIA</u> INFECTIONS AS ILLUSTRATED ON:	MITOTIC FIGURES	DAUGHTER NUCLEI			APPARENT BI- NUCLEATE STAGE
		SHAPE	SIZE	STAINING	
<u>Plate: VII</u>					
Fig. A1(1-4)	intermed	regular	small	med	
A2(2-4)	intermed	irregular	small	med	
<u>Plate: VIII</u>					
Fig. B1(1-4)	in- apparent	irregular	small to med	med	sporoblast
B2(1-4)					sporoblast & spore
B3(1-4)					sporoblast & spore
B4(1-4)					sporoblast & spore
<u>Plate: IX</u>					
Fig. C1(1-4)	intermed	irregular	med	med	
C2(1-4)	inappar.	regular	small	med	sporoblast & spore
C3(1-4)	inappar.	regular	small	dense	spore
C4(1-4)	inappar.	irregular	small	med	sporoblast & spore
<u>Plate: X</u>					
Fig. D1(1-4)	inappar.	irregular	large	v. dense	
D2(1-4)	Inappar.	irregular	med	dense	
D3(1-4)	inappar.	irregular	med	dense	sporoblast
D4(1-4)	intermed	irregular	med	dense	
<u>Plate: VI</u>					
Figs 1 & 2	clear	irregular	med	dense	

The cytoplasm of the octonucleate sporont is understood to divide finally and condense around the nuclei, each of the eight uninucleate cytoplasmic bodies being a sporoblast, which develops subsequently into a spore of a distinctive shape and size. The sporont may now be referred to as a pansporoblast. The pansporoblast may have a limiting membrane that seems rigid and strong so that each spore is hard to separate from the compact 8-spore group, or very thin and easy to break so that each spore of the group could easily be separated.

Such differences were used by Kellen and Lipa (1960) and others as taxonomical characters. Kellen and Lipa reported for Thelohania californica, that the membrane of the pansporoblast remained intact for a considerable length of time surrounding the newly formed spores. I found the pansporoblast membranes from Tilling Bourne to be usually much weaker than those of the majority of larval infections from the Lea, but this was not constantly so.

B- In Plistophora (Pl. XII & Pl. XI, Fig. 3)

The sporont which is spherical when fresh has a weaker wall than that of Thelohania and in dry smears becomes either sub-spherical or elliptical, measuring about 31-33 $\mu$  on the long axis. Nuclear divisions are more numerous than in Thelohania. Because of difficulty in counting the numerous closely packed spores, numbers are not always certainly determinable but estimates were of 16-32 daughter nuclei aggregated centrally in one infection (Pl. XII, B) and 64-128 daughter nuclei dispersed throughout the cytoplasm in the infections of other larvae (Pl. XII, A). Accordingly, the pansporoblast contains finally a variable number, but more than 16, uninucleate sporoblasts, each finally developing into a spore. As will be noted, when dealing with spores below, 2 forms A & B of Plistophora are recognised

on the basis of the numbers of spores per sporont together with other differences (Table X).

C- In Nosema (Pl. XIII)

Nosema is the only genus of microsporidian parasites of Simulium larvae in which I observed a pre-sporont developmental stage. This stage produces, by schizogony, a spherical cyst enveloped within an extremely thin transparent wall, inside which up to 32 uninucleate sporonts are formed. Each sporont is in fact, one sporoblast which develops to give rise to a single spore. The sporont is similar in shape, but considerably larger than the mature spore.

The sporont (=sporoblast) has a comparatively large posteriorly situated nucleus which, as development proceeds, becomes more compact and finally assumes a central position. Its thin wall becomes thicker as it reduces in size, retaining its ovoid shape, to become a spore (see Table IV). Only one form of Nosema was seen (Table X).

D- Fine structure of the sporont and sporoblast of Thelohania.

In contrast to the usual smooth spherical appearance the sporont shows under light microscopy,



the sporont by electron microscopy has a lobulated outline which is most pronounced at the onset of nuclear division, and when deep furrows appear to divide the sporont externally into asymmetrical lobes (EMGs: 2, 3, 4 & 5). Such furrows as demonstrated in EMG: 4 might reach a depth of  $3\mu$  and an average width of about  $150-200\mu$ . The smooth, but wavy, sporont wall - a double membrane - is formed of electron-dense material having a thickness of about  $54-60\mu$  (EMG: 3, SW).

The cytoplasm of the sporont is extensively traversed by endoplasmic reticulum. At the periphery of the cytoplasm, and rather parallel to the outline of the nucleus of a sporont lobe, are dark wavy lines alternating with light bands of variable thicknesses (EMG: 2, PP). These membranes are possibly portions of endoplasmic reticulum, Golgi membranes or, following Sprague et al (1968), primordia of the polaroplast, an organelle that is described later in the mature spore. What look like secretion granules (SG) surrounded by dark membranes are seen in the central cytoplasm of the dividing sporont (EMG: 4).

The nuclei in the dividing sporont are large; the nuclear envelope consists of two membranes (EMG: 4, NM). The binucleation of the sporoblast that I have observed in some infections under light microscopy and



which was reported for electron micrographs of Thelohania by Vavra (1965) for what is called a "diplocaryon", is not evident in my electron microscopy studies.

Signs of a polar filament become evident after the final nuclear division of the sporont, and just before the separation of the lobular divisions to form sporoblasts (EMG: 2, PF). In the young sporoblast the polar filament shows 1-2 coils only (EMG: 5, PF). The uninucleate young sporoblast has an irregularly, though essentially spherical shape, its wall about the same thickness (50-60 $\mu$ ) and density as that of the mother sporont. Its shape later becomes broadly oval, or ovoid. The final number of coils of the polar filament becomes evident posteriorly, and the anterior straight end of the filament is seen surrounded by the newly formed polaroplast. The anterior base of the polar filament, at this stage, is a vesicular sac lacking the two side arms of the anchor-like shape it would assume later in the spore stage (EMG: 5, PFB).

All organelles of the ultimate spore are finally recognisable, except the two outer layers of the spore wall, the origin of which is, so far, unknown.

Degenerating nuclei and mitochondria of probably the parasitized host cell are seen lying in the mass of parasitic material (EMG: 3, M; EMG: 3, 4 & 5 HN).

iii- The spore

A- Morphology and reaction to stains

The general shape of the spore of Thelohania, Plistophora and of Nosema is ovoid. I have observed exceptions in Thelohania, one infection possessing ovoid-cylindrical spores in addition to the usual ovoid spores, both in fresh and in dried conditions (Pl. V, Fig. 9) and truncated oval spores (Pl. III, Fig. 3), or spherical spores (mean diameter  $5.5\mu$ ) in other Thelohania besides typical ovoid spores. The spherical spores possessed thick walls and large nuclei, and a diameter larger than the maximum length of the typical ovoid spores. Except the Thelohania infection with the truncated oval spores, all other infections have spores with a posteriorly situated conspicuous "vacuole". The area of this vacuole is less refractile than the rest of the spore. All spores have a greenish tint when freshly smeared.

All stains used show the presence of one large central nucleus occupying most of the internal space of the spore, except in one Thelohania infection, which

besides a small central nucleus, displayed a second nucleus pressed against the anterior tip of the spore (Pl. VI, Fig.5). In agreement with Sprague (1965), the Feulgen test gives a weak reaction with the nuclei which appear much smaller than with Giemsa. Giemsa proved best for showing nuclear details of the immature spores, particularly after a short hydrolysis with 1N-HCl at 60°C. The chromosomes and mitotic figures are readily seen, in some infections (Pl:VI, Figs 1 & 2) the sporoplasm does not stain. In Nosema spores a single large compact nucleus was present in the centre of the spore, in contrast to a dumbbell-shaped nucleus reported by Dissanaiké and Canning (1957) for Nosema helminthorum, or a double nucleus which shows clearly with Feulgen stain in Nosema locustae spores (Canning, 1962a). Sprague (1965) and Weiser (1965b) also reported binucleated spores of Nosema sp. and Nosema muris, respectively. Possible binucleate Thelohania spores (EMG: 7, N) may be section of two lobes of a dumbbell-shaped, or curved nucleus; complete tracing of ultra-thin serial sections would be necessary to establish binucleation.

Acid-fastness was claimed by Farley (1965), with Ziehl-Neelsen carbol fuchsin, as characteristic for

protozoan haplosporidian spores. In the present work, however, acid-fastness was also a feature of the mature microsporidian spore, which may explain their weak positive reaction in the Feulgen test: an unexplicable exception is that the spores from Nosema infections produce strongly positive reaction in the Feulgen test (Pl. IV, Fig. 4), as well as immatured spores of all other infections. This lack of reactivity to acids by fully matured spores is also evident with Giemsa staining following hydrolysis in HCl. The sporoplasm of the fully matured spore does not seem to be affected by hydrolysis and, contrary to the sporoplasm of the immature spore, it stains dark blue, masking the nucleus.

One of the constituents of the Gram +ve spore wall is a polysaccharide, which gives a weak PAS positive reaction for the wall, particularly at the anterior tip. There is also a weak positive reaction in the wall to the toluidine blue method for metachromasia.

Reactions of the microsporidian spores to stains used in this study are summarised in Table II.

Probably due to their thick walls and their highly refractive surfaces, these microsporidian spores do not reveal much of their internal structure, even under phase contrast microscopy. Electron microscopy has,



Table II

Reaction of Microsporidian spore constituents (in sections & smears) to different stains used.

S T A I N	IMMATURE S P O R E	M A T U R E S P O R E		
		W A L L	S P O R O P L A S M	N U C L E U S
Alcoholic-based Gram's Iodine	-	+++	-	-
Periodic Acid Schiff's reaction	-	+	-	-
CrO <sub>3</sub> -Methenamine silver reaction	-	+	-	-
Toluidine blue method for metachromasia	-	+	-	-
Carbol fuchsin * acid-fast stain	-	+++	+++	-
HCl-Giemsa-Colophonium stain	+++n	+	+++	+
Himes & Morbider triple stain	++	+	-	+
Toluidine blue-methyl green-orange C	++	-	+	++
Methyl green-pyronin	+ n	-	-	++
Feulgen reaction	-	-	+	-
	++ n	-	-	+

\* +ve reaction given in this test is characteristic for microsporidian spore.

n = nucleus

however, added considerably to the detail which can be observed especially in spores; this will be reported later below. Meanwhile, I will deal with features of my material which can be studied by light microscopy and, particularly, the dimensions of the spores which were found to differ in an interesting manner in different infections.

#### B- Spore dimensions

A comparison was made, for Thelohania, of the accuracy of a photomicrographic technique (see Methods) for measuring fresh and dry-fixed-Giemsa-stained spores against the filar ocular micrometer method of measuring dried-stained spores. Lengths and widths of 50 spores were measured by each method in smears taken from the same larval infection. The means for each group measured is given in Table III, viz., mean length x mean width, 6.3  $\mu$  x 4.14  $\mu$

Table III - Comparison of accuracy of photomicrographic and filar ocular micrometer methods of microsporidian spore measurements. (Thelohania sp.)

SMEAR	FRESH SPORES	DRY-FIXED-GIEMSA-STAINED SPORES	
	photomicrography	photomicrography	filar ocular micrometer
1	6.3 $\mu$ x 4.1 $\mu$	4.0 $\mu$ x 2.7 $\mu$	4.1 $\mu$ x 3.0 $\mu$
2	6.5 $\mu$ x 4.3 $\mu$	4.4 $\mu$ x 3.0 $\mu$	4.3 $\mu$ x 3.0 $\mu$

Both methods on fixed spores, while recording lower dimensions than fresh measurements, are comparable to each other. Although fixed material certainly underestimated the measurements, the filar ocular micrometer method was used for the measurement of fixed microsporidian spores in preference to the relatively expensive and time-consuming photomicrography. Furthermore, it was not possible to keep the fresh spores motionless long enough to take two micrometer measurements for each dimension. Fixed material was necessary and dry-fixation, with Giemsa-stain, was adopted as routine for the ocular measurements. In all, 68 smears were made and spores measured to the nearest  $0.1\mu$  using the filar ocular micrometer, at a magnification of  $\times 1000$ . For each of the 68 smears, each of a single larval infection, 50 spores were measured, and a mean of two readings for length and for width taken. Mean lengths and widths for 50 spores are given in Table IV for material of Nosema, Table V of Plistophora, and Table VI of Thelohania. The spore dimensions differed considerably, and were further analysed, statistically and graphically.

Table IV Mean lengths and widths of 50 spores, each of a single larval infection, of Simulium ornatum : also sporont dimensions. Arranged in size classes.

1- Nosema

GENUS	SIZE CLASS	SMEAR NUMBER	SOURCE	DATE	SPORE		SPORONT
					length:	width	1 x w
					$\mu$	$\mu$	$\mu$
Nosema	A	1	Lea	27/3/69	5.77 :	4.03	7-9 x
"	A	2	"	30/1/69	5.50 :	3.95	5-6
"	A	3	"	30/1/69	5.80 :	3.97	

Table V Mean lengths and widths of 50 spores, each of a single larval infection of Simulium ornatum: also sporont dimensions. Arranged in size classes.

2- Plistophora

GENUS	SIZE CLASS	SMEAR NUMBER	SOURCE	DATE	S P O R E		SPORONT
					length:	width	max.len
					$\mu$	$\mu$	$\mu$
Plistophora	A	1	Tilling	24/9/68	4.83 :	3.41	31-33
"	A	2	"	24/9/68	4.58 :	3.41	31-33
"	A	3	"	9/1/69	4.15 :	3.20	28-35
"	B	4	Lea	11/6/69	5.63 :	4.00	12-22



Table VI Mean lengths and mean widths of 50 spores, each of a single larval infection of Simulium ornatum: also sporont dimensions. Arranged in size classes.

3- Thelohania

GENUS	SIZE CLASS	SHEAR NUMBER	SOURCE	DATE	S P O R E		SPORONT	
					length μ	width μ	max. leng. μ	
<u>Thelohania</u>	A	3	Tilling B	24/9/68	3.16	2.81	10-13	
		4	"	"	3.24	3.01	11-13	
			30	"	11/10/68	2.52	2.33	7-13
			31	"	"	3.00	2.85	8-11
			36	"	9/1/69	3.17	2.80	10-12
			37	"	"	3.43	3.04	10-11
			38	"	"	3.42	2.97	10-11
			39	"	"	3.07	2.61	10-11
			40	"	"	3.51	3.06	
			59	"	24/4/69	3.25	2.96	9-10
			60	"	"	3.40	3.10	9-12
			61	"	"	3.65	3.30	9-11
	<u>Thelohania</u>	B	2	R. Lea	22/8/68	4.24	3.21	12-13
			8	"	3/9/68	4.10	2.97	11-12
			9	"	"	4.39	3.08	11-12
10			"	"	4.47	3.01	11-12	
11			"	"	4.15	2.99	11-12	
13			"	"	4.26	3.13	11-12	
14			"	"	4.34	3.01	11-12	
15			"	"	4.29	3.17	10-12	
16			"	"	4.11	2.86	11-12	
17			"	"	4.42	2.97	11-12	
18*			"	"	"	3.99	2.90	10-12
21			"	"	22/10/68	4.47	3.46	11-13
33			"	"	18/12/68	3.98	2.99	11-12
35			"	"	"	4.29	2.87	11-13
41			"	"	30/1/69	4.42	3.36	11-14
42	"	"	"	4.33	3.20	11-14		
43	"	"	"	4.20	3.11	10-14		
44	"	"	"	4.06	2.94	10-13		
45	"	"	"	4.25	3.30	10-14		
46	"	"	"	4.40	3.31	11-14		
47	"	"	"	3.90	2.99	11-13		
48	"	"	"	4.45	3.20	12-14		
51	"	"	27/3/69	4.48	3.49	12-14		

\*with truncate oval shape - all other spore forms have ovoid shapes

GENUS	SIZE CLASS	SMEAR NUMBER	SOURCE	DATE	S P O R E		SPORONT
					length $\mu$	width $\mu$	max. length $\mu$
Thelohania	C	5	R. Lea	22/8/68	5.10	4.08	10-13
		19	"	22/10/68	4.93	3.90	11-14
		20	"	"	4.97	3.54	11-12
		22	"	"	4.85	3.52	12-13
		23	"	"	4.66	3.69	9-13
		24	"	"	4.98	3.79	11-14
		25	"	"	4.94	3.86	8-14
		26	"	"	4.87	3.91	8-13
		27	"	"	4.80	3.57	9-13
		28	"	"	4.74	3.63	9-13
		29	"	"	4.68	3.42	9-12
		50	"	27/3/69	4.67	3.56	10-14
		52	"	"	4.80	3.63	12-15
		54	"	"	4.55	3.63	12-13
		56	"	"	4.59	3.69	11-14
		58	"	"	4.76	3.63	11-13
		Thelohania	D	1	R. Lea	22/8/68	5.50
6	"			"	5.83	4.35	10-13
7	"			3/9/68	6.11	3.47	12-16
12	"			"	6.01	3.37	12-15
32	"			19/11/68	5.90	3.54	14-20
34	"			18/12/68	6.10	3.73	11-15
49	"			30/1/69	5.76	3.70	13-18
53	"			27/3/69	5.90	4.00	13-18
55	"			"	6.15	3.95	14-17
57	"			"	6.12	4.10	14-18

C- Statistical analysis of spore measurementsThelohanian:

1- The mean lengths and mean widths of Thelohanian spores for each infection are tabulated (Table VI). For each mean spore dimension, variance, standard deviation and standard error were also computed (Table VII) as minimum and maximum ranges.

Table VII Thelohanian spore measurements

	MEAN SPORE LENGTH		MEAN SPORE WIDTH	
	minim.	maxim.	minim.	maxim.
	$\mu$	$\mu$	$\mu$	$\mu$
Variance	0.013	0.035	0.012	0.023
Standard deviation	0.115	0.189	0.112	0.153
Standard error	0.016	0.027	0.016	0.021

2- The Thelohanian spore measurements in Table VI are divided into four groups or size classes, A, B, C and D. When mean spore lengths were plotted against mean widths (Diagram No. 1), it was found that size classes B and C appear to coalesce to form one large group in the middle of the plot, whereas class A and class D formed two distinct groups, the first in the lowest range and the other in the upper range of measurements.

To test the validity of the grouping by spore dimensions as set out in Table VI and in the plot of lengths against widths (Diagram 1), a t-test was carried out between size classes A & B, B & C, C & D, and A & D. By the t-test, the difference between the two size classes or groups B & C, as well as between all the other groups was significant at the level of  $P < 0.001$  ( $P =$  probability) i.e. the probability that any one sample within one of these four groups belongs to any of the other three groups is less than 0.1%. Accordingly, spore size classes A, B, C & D of Thelohania parasites of Simulium ornatum larvae, are regarded as four distinct groups, each a separate form of Thelohania. These are described more fully later. For each form the mean spore dimensions are tabulated in Table VIII.

Table VIII Mean spore dimensions of Thelohania forms

<u>THELOHANIA</u> <u>FORM</u>	MEAN LENGTH	MEAN WIDTH
A	3.2 $\pm$ 0.08 $\mu$	2.9 $\pm$ 0.07 $\mu$
B	4.2 $\pm$ 0.03 $\mu$	3.1 $\pm$ 0.03 $\mu$
C	4.7 $\pm$ 0.03 $\mu$	3.6 $\pm$ 0.04 $\mu$
D	5.9 $\pm$ 0.06 $\mu$	3.8 $\pm$ 0.10 $\mu$

3- Spore measurements within each class (A, B, C or D) are grouped in a frequency (i.e. No. of spores



in each 0.2 $\mu$  dimension class) table for both length and width (Table IX).

Table IX Frequency table of Thelohania spore measurements recorded in size classes, A, B, C, & D.

LENGTH $\mu$	F R E Q U E N C Y				WIDTH $\mu$	F R E Q U E N C Y			
	A	B	C	D		A	B	C	D
2.0					2.0				
2.2	5				2.2	21			
2.4	17				2.4	31			
2.6	21				2.6	76	27		
2.8	29				2.8	141	158		
3.0	118				3.0	164	380		
3.2	136				3.2	82	266	38	40
3.4	106				3.4	28	147	171	65
3.6	80	10			3.6	5	65	251	72
3.8	27	63			3.8	2	7	220	97
4.0	11	226			4.0			110	122
4.2		285	23		4.2			10	74
4.4		259	83		4.4				20
4.6		171	190	4	4.6				7
4.8		34	277	8	4.8				3
5.0		2	182	22	5.0				
5.2			40	29					
5.4			5	32					
5.6				59					
5.8				80					
6.0				113					
6.2				38					
6.4				35					
6.6				24					
6.8				6					
7.0									

Graphical representation of the frequency of spore sizes within each form are given in Diagrams No. 2, No. 3, No. 4 & No. 5. It is evident, in general, that the highest

frequency values fall not only on or close to the means (Table VIII) for the form (cf. underlined figures in Table IX and mean in Diagrams 2, 3, 4 & 5) but notably, frequencies for values on either side of the mean decline progressively with increased departure from the mean. This indicates that the four forms have each characteristics of a normal distribution as regards length and width of their respective spore dimensions, confirming that they may be accepted as distinctive forms.

#### D Forms of Microsporidia

I will summarise here my conclusions about different forms of the microsporidian parasites taken from my simulium larvae material. Table X tabulates these and the characteristics on which they are differentiated; the few additional comments below emphasize certain aspects and note in which river the forms were found and, briefly, their seasonal occurrence.

Table X Classification of Microsporidian parasites of Simulium ornatum larvae as "forms", based on structural and developmental features.

GENUS	NUMBER OF SPORES PRODUCED BY SPORONT	NUMBER OF PSEUDO-CYSTS	SPORE SHAPE	SPORE WALL	SPORE SIZE		OCCURRENCE AND SHAPE OF HYPER-TROPHIED HOST-CELL NUCLEI
					length μ	width μ	
<u>Thelohania</u>							
A	8	1-2	ovoid	thick	3.2	2.9	fimbriated
B	8	many	"	thin	4.2	3.1	regular
C	8	many	"	med.	4.7	3.6	regular
D	8	many	"	thick	5.9	3.8	rare
E	8	many	oval truncate	thick	3.9	2.9	none
<u>Plistophora</u>							
A	64-128	2	ovoid	med.	4.5	3.3	none
B	16-32	many	ovoid	thick	5.6	4.0	none
<u>Rosema</u>							
A (only form)	1	6	ovoid	thick	5.7	3.9	rare - regular

i- Thelohania: On the basis of the spore dimensions, (Tables VI, VII, VIII and IX,) I recognised four "forms" of Thelohania - A, B, C and D. There is, however, one single infection that had oval truncate spores

(Pl. III, Fig. 3) and not the ovoid spores of the other forms. Although its spore size is similar to that of form B, I separate it as Form E.

The sporont size does not serve any additional taxonomical purpose; it is not as rigid as the spore and is liable to distortion in dried smears, to give misleading measurements. Furthermore, the sporont is in a continuous state of both nuclear and cytoplasmic divisions, which are coupled with variations in size and difficult to define. However, several other features (Table X) show an interesting constancy related to the particular forms, as based on spore dimensions, and help to confirm these taxonomic groups as entities and enable biological features to be presented, as below, for each form.

Thelohanian form "A"

Occurrence: This was the only form of Thelohanian found parasitizing larval Simulium ornatum in the Tilling Bourne (Surrey) during all seasons. It was never found in larvae of the River Lea.

Structure: All infections of this form formed 1-2 pseudocysts, which were situated latero-ventrally in the haemocoel at the bulbous part of the larva. They showed as opaque structures, without the milky colour of the other forms. The cytoplasm of the sporont stained darker, with Giemsa, than that of any of the other forms.



The pansporoblast membrane was thin and easy to break, in smears, so that separate spores were more regularly seen than groups of eight-spore. The spore showed a thick wall that stained blue with Giemsa, and a meta-chromatic polar granule. The cell nuclei of the infected larval fat body were always markedly hypertrophied, of fimbriated irregular shape, and variable size.

Mean spore dimensions:  $3.2 \pm 0.08\mu$  x  $2.9 \pm 0.07\mu$

Thelohania form "B"

Occurrence: About 46% of the incidence of Thelohaniasis in Simulium ornatum larvae of the River Lea only was of this form. It was found most of the year, but mainly in late summer and early winter.

Structure: Several, but comparatively large, pseudocysts were formed packed closely, throughout the larval haemocoel, particularly on the ventral side. They caused great distention of the bulbous part of the larva and, though milky-coloured at the posterior end only, some show a pinkish tint. The cytoplasm of the sporont is highly vacuolated, and the sporoblast, in general, has an appearance of containing double nuclei, which is evident in the spores.

Mean spore dimensions:  $4.2 \pm 0.03\mu$  x  $3.1 \pm 0.03\mu$

Thelohania form "C"

Occurrence: This was second in abundance, 34% , to form "B" in Simulium ornatum larvae of River Lea only. It was only found at the end of the summer and at the beginning of spring.

Structure: It forms small, numerous, pseudocysts that are markedly separated from each other. After each stage of nuclear division of the sporont, daughter nuclei round up in a typical manner. The pansporoblast has a rigid membrane, so that each spore was hard to separate from the compact 8-spore capsule. The ovoid spore produced shows numerous eosinophilic granules on its wall, as well as a polar granule in the sporoplasm looking like a second nucleus. The spore nucleus is comparatively small. The spore wall is moderately thick. Mean spore dimensions:  $4.7 \pm 0.03 \mu \times 3.6 \pm 0.04 \mu$

Infections of this form also produce, sporadically, spherical spores with mean diameter of  $5.5 \mu$  , in addition to the usual ovoid spores. These spherical spores possess thick shells and large nuclei.

Thelohania form "D"

Occurrence: This is a rarer form than B or C in Simulium ornatum larvae of the River Lea. Sporadic infections occurred throughout the year.

Structure: The sporont possesses comparatively large and dense nuclei that seemed to divide by fragmentation. The spores easily separate from the 8-membered capsule, presumably because of a thin delicate pansporoblast membrane. The ovoid spores are always uninucleate and have characteristic thick shells.

Mean spore dimensions:  $5.9 \pm 0.06\mu \times 3.8 \pm 0.10 \mu$

Thelohania form "E"

Occurrence: an extremely rare form that was only seen once in a Simulium ornatum larva from the River Lea.

Structure: The spores broke loose from the pansporoblast immediately after formation, so that 8-spore groups were rare to find in smears. The spore has a characteristic truncate oval shape and lacks the posterior "vacuole" shown by fresh spores of all other forms.

Mean spore dimensions:  $3.9\mu \times 2.9 \mu$

By comparison of spore shape and dimensions of previously described species (Table XI), it seems that all these five forms of Thelohania were entirely new to science, apart from being original records for Thelohania in Simuliids of the British Isles.

Plistophora form "A"

Occurrence: A rare microsporidian infection of Simulium ornatum larvae from the Tilling Bourne. The genus itself has never before been reported from Simuliidae of Great Britain, and this form is new. It occurred in autumn and winter.

Structure: It produces two opaque pseudocysts only, situated latero-ventrally in the bulbous part of the larva, a feature that so far seems typical of microsporidian parasites of Simulium ornatum larvae from the Tilling Bourne. The pansporoblast produces 64-128 ovoid spores that cohere in a compact group difficult to separate. Spore walls are moderately thick. Mean spore dimensions:  $4.5\mu \times 3.3\mu$

Plistophora form "B"

Occurrence: A single infection in June in a Simulium ornatum larva from the River Lea. It is also a new form.

Structure: Typical of microsporidian infections of Simulium ornatum larvae from the River Lea, this form produced numerous pseudocysts that were found throughout the larval haemocoel. But in marked contrast to all other white pseudocysts of microsporidia, this form produced reddish pseudocysts, which gave the infected



larva external reddish spots, particularly at the ventral side of the abdominal region.

The pansporoblast produces 16-32 thick-walled ovoid spores, easily separated from their grouping. The nucleus is rather large.

Mean spore dimensions:  $5.6\mu \times 4.0\mu$

Nosema form "A"

Occurrence: A rare infection, in January & March, of Simulium ornatum larvae from the River Lea only. Like Plistophora, this genus has never before been reported for British simuliids.

Structure: Only 6 pseudocysts were observed in the few infections found. The schizont may produce 16-32 sporonts enclosed in a thin membrane to form a spherical structure. The sporont, ovoid like the spore, is larger than the spore. Both sporont and spore are uninucleate. The spore, like that of Plistophora form "B" has a thick wall and a large nucleus.

Mean spore dimensions:  $5.7\mu \times 3.9\mu$ .

E. Spore fine structure

In part to confirm the identity of the parasitic material from the Simulium larvae, but also to study detail of the spores, material was prepared for electron microscopy. Unless otherwise stated my observations are on

Thelohania spores. It has been found, from the study of the electron micrographs produced, that a tri-laminate spore wall is evident by the time all the organelles of the sporoplasm are formed. The inner dense layer of the wall, which is about 13m $\mu$  thick, is in direct contact with the sporoplasm membrane (EMG 6, IC). This and an outer dense layer of about the same thickness (EMG 6, OC) are separated by a much thicker, third layer (EMG 6, ML) which over most of the spore is uniformly thick, about 165 m $\mu$ , but at the posterior pole thicker, about 200 m $\mu$ , and at the anterior pole much thinner, about 35m $\mu$ . There is controversy regarding the density of the middle layer of the spore wall. It is referred to by Huger (1960), Stanier et al (1968) and others, as an electron-transparent layer, whereas in this study it is found to be dense in one infection (EMG 6, ML), and electron-dense in the outer half and transparent in the inner half, in the other infection (EMG 11, ML).

Between the inner surface of the thin anterior arc of the spore wall and the outer surface of the polar filament base, the sporoplasm of the spore is 40m $\mu$  thick; except for Lom & Corliss (1967) who referred to it as a thin sheath of protoplasm, this zone has not been described in any previous electron micrographs by others. I refer

to it as a "sporoplastic cap" (EMG 6, SC) in distinction from the term polar cap of Lom & Corliss (1967) and other subsequent workers given to the base of the polar filament (EMGs 6 & 9, PFB), which lies immediately posterior to what I am now calling a sporoplastic cap.

The polaroplast is a complex structure lying under the base of the polar filament (EMGs 6, 7, 8, 11 & 12, P), first so named by Huger (1960). It is, in spores of these two particular infections, enclosed within a compartment, and composed of either straight or wavy convoluted lamellae disposed laterally to the central wider and straight part of the polar filament. It has a circular cross-section (EMG 8, P), which narrows posteriorly.

The polar filament (PF) arises from its base (PFB) with a diameter of about 200 $\mu$  (EMG 6). In section, the base of the polar filament looks like two arms of an anchor, stretching between the polar cap and the polaroplast. It extends back into the spore, at a uniform diameter of about 160 $\mu$  and, in the posterior third of the spore is in the form of 5-6 coils (EMG 6, EMG 7, PF) pressed hard against the inner surface of the sporoplasm membrane. Coiling varies, the polar filament of other infections examined containing 8-9 coils, also occupying the peripheral zone of the posterior half of the sporoplasm (EMG 11, PF). In both materials examined, the

polar filament has a circular cross-section. In one case (EMG 6) there is a central light core, 70 $\mu$  in diameter surrounded by a dense ring 25 $\mu$  thick, followed by a peripheral light layer of about 12 $\mu$  thickness. The whole structure is enclosed within a dense thick membrane about 10 $\mu$  thick, thus seemingly forming a hollow tube. This is contrary to the structure exhibited by the polar filament in the other form of spore. In the latter, the inner core is dense and looks a solid mass with a denser spot at its centre (EMGs 10A & 11).

The cytoplasm of the spore, referred to as sporoplasm, is finely particulate and traversed longitudinally and parallel to its outer surface by a series of tubules of the endoplasmic reticulum, which seem to arise from the vicinity of the polaroplast (EMG 6, ER). The general disposition of the tubules of the endoplasmic reticulum is parallel to the outer surface of the spore and to the outer surface of the nuclear membrane.

Although the nucleus of the spore, in both infections examined, appear under light microscopy as one unit, the electron micrographs reveal two large nuclei for <sup>some</sup> each spore presently studied (EMG 7, N). This is not a conclusive evidence for a binucleate spore, as the few ultrathin sections which show double-nuclei might



have been cut across a curved nucleus. The nucleus, enclosed within a wavy double-membraned envelope, which resembles the tubules of the endoplasmic reticulum (EMG 6, NM) within the sporoplasm and has a finely particulate and of variable density matrix. Intensely dark areas, endosomes or nucleoli such as those described by Sprague & Vernick (1968a) and Kudo et al (1963), or centrioles and spindle fibres as mentioned by Vavra (1965) are not apparent in this material. However, I have not even by light microscopy, observed any nucleoli in the nuclei of these microsporidians spores.

Some spores (EMG , Mp) show a thinning of the tapering anterior tip of the spore wall and of the sporoplasmic cap, presumably preparation of a weakness at this point for the eversion of the polar filament. This thinning of the spore shell at its anterior end has been referred to, by Lom and Corliss (1967), as a micropyle.

None of the material examined shows evidence of the polar granules described by Huger (1960), and suggested by him and some other investigators, as accounting at the anterior pole of the spore for its PAS-positive reaction.

The spore walls of the two Thelohania infections

are smooth (eg EMG 6) in striking contrast to the spore wall of Plistophora (form "B"), which appears to support an array over its entire surface of long villi-like processes, which intertwine with each other and those of adjacent spores (EMGs 13, &, 14). This has also been described by Vavra (1965) for the spore wall of Plistophora debaisieuxi. This peculiar structure requires further investigations; it is unlike the bristles on the surface of some Nosema spore walls (Sprague et al., 1968).

Apart from these processes in Plistophora, not seen in Thelohania, the basic structures of all microsporidian spores so far examined are similar.

F. Spore polar filament and emergence of sporoplasm

The length of the extruded polar filament of Thelohania spores is variable, between 40 $\mu$  and 60 $\mu$  (Pl.III Figs 7 & 8). Because of this it appeared to be unsuitable for taxonomic purposes. The Giemsa-stained filament shows, midway along its length a remarkably thicker part, about one-sixth of which stains like DNA. This was a constant feature in all everted polar filaments of some, but not all, of the Thelohania infections examined.

In one instance the majority of spores from a dead larva had their polar filaments everted; the sporo-

plasm and the nucleus were still within the spore case. In spores subjected to mechanical pressure to cause eversion of their polar filaments, the sporoplasm was, in a few spores, seen attached to the distal tip of the everted filament. The sporoplasm and polar filament in other spores either remained, despite pressure, within their spore capsules or, under sustained pressure, emerged together but the sporoplasm detached from the filament.

Under light microscopy, a minute orifice was seen at the centre of the broader, posterior end of the spore. Electron microscopy, however, revealed nothing of this posterior orifice.

## 2. Microsporidia-like infections

(Pl: XIV)

Infections of microsporidia spore-like bodies were revealed by histology in the epithelial cells of the mid-gut of Simulium ornatum larvae, from Tilling Bourne. Infection rates were usually low, except for May 1968 to January 1969, when the infection rate exceeded 1% with a maximum of 12% in September 1968.

The infection in individual larvae was always extremely light, only 2-3 epithelial gut cells being infected. The spore-like bodies, 12-24 in each cell were often concentrated round the cell nucleus which showed some hypertrophy (Pl: XIV, Fig. 6). The spore-like bodies were not grouped within a wall or membrane, but separate, though aggregated close together in the cytoplasm of the cell. Evidence as to the nature of spore production was not apparent. Their eventual fate remains conjectural although these so-called spores in some cells, seemed to be about to break into the larval gut lumen (Pl: XIV, Figs 4 & 5).

A spore measured about  $4-5\mu \times 2-3\mu$ , an oval or ovoid shape. It has a strongly Feulgen positive rod-shaped nucleus (Pl: XIV, Fig. 5). It possessed a



single-valved refractile shell, a single sporoplasm and an acid-fastness property: all features characteristic of Microsporidia. Many attempts to isolate these spores fresh, for microscopical examination, for possibly a polar filament, by dissection of the larval gut epithelium were not successful.

In sections, no signs of diseased condition in the infected larvae was evident, other than enlargement of the infected cell nucleus on occasions. Infected cells stained normally with the different stains used.

It is possible, from the different shapes and sizes exhibited by these spores (Pl: XIV, Figs 1, 2, 3, 4, 5, 6), that several entities may be involved in these infections.

3- Class: Sporozoa

Order: Gregarinida Lankester

a)- General: This Sub-order includes the majority of the so-called gregarines which are common parasites of arthropods (Kudo, 1966). When the spore is ingested by a suitable host, it germinates and a number of sporozoites are produced which partly penetrate the host gut epithelium. The end which leads in penetration develops to become an epimerite, an attachment of a certain

shape and size. Then growth is concentrated in the extra-epithelial region of the organism to develop into a 1-2 segmented trophozoite having a final length of 200-1200 $\mu$ . On the other hand, the sporozoite might completely penetrate an epithelium cell to spend its early developmental phase intracellularly as was observed by Kaschef et al (1966) for Pyxinia gibbii in its host, Gibbium psylloides, and others. Detachment of a trophozoite to become a sporadin or a sporont is effected when epithelial cells degenerate. Few gregarines undergo syzygy, following detachment; suitable sporadins conjugate (Harry, 1965). The anterior conjugant is known as the primate and the posterior, the satellite (Kudo, 1966), the deutomerite of the primate being attached to the protomerite of the satellite (Canning, 1956). The primate might, for some time during conjugation, remain attached to the gut epithelium (Kaschef et al, 1966). This association between two individuals is normally followed by cyst formation and the fusion of the gametocytes. The spores are then produced internally and, later, they escape through exit tubes. The majority of the gregarines, however, do not form this association or syzygy.

b)- Occurrence: For most of the time of this study, the occurrence of such organisms in histological preparations

of larval Simulium ornatum was overlooked, being mistaken for degenerate and discarded epithelial cells which pass into the gut lumen at the end of every moulting cycle (Pl. XV, Fig. 1). Later, the comparatively larger size and the characteristic gregarine vesicular nucleus with large central karyosomes, made it necessary to re-examine all previously examined larval sections. I have also examined more selectively the larval guts in later dissections for live trophozoites: a few of these were then observed (as section (d)).

c)- Organisms in larval sections: Trophozoites lie mainly in the posterior part of the larval mid-gut, lengthwise close to the epithelium. The epimerite is funnel-shaped with a long narrow stalk. Most parasitized cells are shrunken (Pl. XV, Fig. 3), probably because their contents are absorbed by the epimerites as suggested by Canning (1956). Unlike the Eugregarine Gregarina garnhami (Canning, 1956) but similar to the Eugregarine Iankesteria culicis Ross (Ganapati et al, 1949) the middle section. the protomerite, of this trophozoite is absent: thus the ~~anterior~~ <sup>posterior</sup> segment, the deutomerite, composes the whole trophozoite. The trophozoite is rarely seen detached and, only in sections of one larva, a few of these sporadins were seen free in the gut lumen lying lengthwise; two are in a state of pre-conjugation (Pl. XV, Fig. 6).

Each of these pre-conjugants has transformed to become shorter and broader,  $63\mu \times 33\mu$ , with a comparatively larger ovoid nucleus of about  $15\mu \times 22\mu$  dimensions.

The infection rate of the Simulium larval population was low and infection with these gregarines in individual larvae appeared always very light. No other stages of sporulation were seen.

d)- Live mature trophozoite (Pl. XV A): In its living condition the trophozoite exhibited what Wenyon (1911) termed as typical gregariniform movements of progression, flexion and constriction.. Few were recovered from the mid-guts of infected larvae by dissection.

The mature trophozoite is banana-shaped with only one segment, measuring a mean of  $200\mu \times 30\mu$ . Anteriorly, it is terminated by the scar of the epimerite attachment (ES). The ectoplasm is dense and granular, obscuring all internal details.

e)- Classification: Asexual reproduction or schizogony has not been observed for these organisms. Thus, on this basis, I have placed them in the Sub-order Eugregarinina. The trophozoite is not septate, therefore, the parasites belong to the Eugregarine superfamily Acephalinoidea Kolliker (Kudo, 1966). The mature trophozoite has a size and a spatulate shape similar to those



of the trophozoites of Parabesleria culicis Ross. But, because of the externally inapparent nucleus of this trophozoite and, also, the cyst and spore stages were not observed, generic and specific status remain uncertain at this stage.

## II- Fungal infections

### 1. Class: Phycomycetes

#### Order: Chytridiales

#### Coelomycidium sp.

a) General: Members of the fungal Order Chytridiales which mainly live as parasites in plants and animals, commonly termed Chytrids, are characterised by lack or only slight development of true mycelia. The simple thallus or vegetative body of the fungus which is described as holocarpic, in almost all cases originates from a motile unflagellated zoospore. These zoospores, which are naked, are formed asexually by the differentiation of the cytoplasm of a syncytial body called a sporangium which, in Coelomycidium sp. composes the entire thallus. A zoospore, following its escape from the sporangium and after a period of motility, loses its flagellum and develops a cell wall. When it reaches and penetrates the appropriate host cell, it enlarges with subsequent nuclear

multiplication to attain a form and size characteristic of the mature thallus or sporangium. Nuclear division by mitosis, finally followed by cytoplasmic division, produces further zoospores. Instead of producing sporangia, when infecting a host, zoospores may fuse, in a simple form of sexuality, to become zygotes. Because the method of formation of these 'zygotes' is usually in doubt, they are commonly termed resting spores.

Most of the known Chytrid species are parasites either in Arthropods, in roots, stems or leaves of higher plants and more often saprophytic in dead plant or animal material.

b)- Occurrence: 60% of a batch of Simulium ornatum larvae collected from the Tilling Bourne during the fourth week of November, 1967 showed an infection with what looked like a fungus. It was found frequently during 1968 and into 1969 though never in such high incidence as in November 1967. The "cysts" of the infecting organism occupied most of the coelomic spaces of infected larvae; in addition some larvae had a few of the fungal-like bodies in the head capsule and anal gills.

c)- Systematic position: I did several tests to clarify my view about the identity of these organisms;

whether they were protozoan Haplosporidians, the "waste basket" of protozoa or a fungus belonging to the Order of the Chytrids. In addition to establishing the presence of chitin and/or cellulose in high concentrations in the membranes of the zoospores, a main deciding factor in identification was recognition of the zoospore. Chitin, a mucopolysaccharide is a substance known to be present in the cell walls of most fungi, particularly the Phycomycetes, accounting for their +ve PAS reactions (Aronson, 1965). Cellulose, which is a PAS +ve simple polysaccharide, is also known to be present within the cell walls of some fungi. I found that in the two tests, PAS and silver, the intracellular sporangia gave negative reactions. It was only the extracellular sporangia with advanced cytoplasmic division, that produced strongly positive reactions in both tests. These positive reactions were found to be due to the membranes surrounding the newly formed zoospores within the sporangium. The strongly positive PAS and silver reactions became stronger in zoospores liberated from within the sporangial wall. Polysaccharides were absent from the wall of the sporangium itself.

The morphology of the motile flagellated zoospore, characterizes some Orders of the aquatic Phycomycetes, and the mode of escape of the zoospore from the sporangium has

also been accepted as the basis for primary classification within the Phycomycetes (Hickman, 1965). In the light of the above histochemical evidence for chitin and/or cellulose in the zoospore walls, in addition to the negative reaction given by all stages of sporangia in the acid-fast stain, and because of failure to find a spore other than the naked motile zoospore with a posterior single flagellum, characteristic of some phycomycetes, I elected to accept these organisms as fungi of the Order Chytridiales of the Phycomycetes. Accordingly, bodies or "cysts" found in the infected larvae will be described as sporangia and their derivatives as zoospores.

d)- Structure and development of sporangia

i)- Form "A" infections (Pls: XVI & XVII)

The earliest development stage of this parasitic form was revealed by histological means. It showed as small lobular bodies of about 7-10 $\mu$  in maximal length, parasitizing small groups of neighbouring cells of the larval fat body, particularly of the peripheral cells. These lobular bodies increased in size with accompanying enlargement of the parasitized host cells (Pl. XVI, Figs. 1 & 2). As in the case of microsporidia, the mode generalized distribution of the infection was not clear. Intensive examination of several hundred serially sectioned



larvae failed to reveal, conclusively, presence of hyphae, which could have provided an explanation for the inter-cellular spread of the parasite.

Details of nuclear division were not observed, but up to about 1000 daughter nuclei were found in a fully-grown sporangium of a mean diameter of  $88\mu$  (Pl. XVI, Figs, 3 & 4). There was always only a single multinucleate 'body' or sporangium in an infected cell. In the most advanced stage of the multinucleate condition all infected fat body and "connective tissue" disintegrated to fragments of fine membranes disposed around sporangia. In effect, a sporangium was thus free of the fat body cell tissue and completed its development extracellularly in the larval haemocoel.

Suspended in the haemocoelic fluid, sporangia of different sizes could be seen being transported by the general flow of the haemolymph, in a jerky oscillatory way, throughout the body cavity of the infected larva. There was usually a considerable aggregation of sporangia in the abdominal region of the larva, causing distention, infection was also evident by a shiny copper colour.

The regularly spherical sporangia measured  $30-90\mu$  in diameter with a mean diameter of  $88\mu$ . They were

refractile and some possessed peripherally, in their cytoplasm, highly refractile spheroid bodies, probably oil droplets. Rarely, a sporangium might show a large central vacuole (Pl. XVI, Fig. 6A, V) or might become surrounded by a horse-shoe-shaped multinucleate non-septate structure, (Pl. XVI, Fig. 9) possibly a rudimentary hypha or merely a spherical sporangium lodged within a cup-shaped depression of another. A few sporangia showed signs of slight budding, structures that might be interpreted as rudimentary exit tubes, or rudimentary mycelial buds.

The cytoplasm of the sporangium was enclosed within a thin membranous capsule, particularly well seen in empty sporangial capsules (Pl: XVI, Fig. 8), which, in Giemsa-stained smears, acquired a very dark blue coloration. This obscured the internal contents of full sporangia.

Zoospores: Cytoplasmic division was only seen to have taken place late in winter. Cytoplasm condensed round nuclei to give rise to pyriform uninucleate zoospores, whose limiting membranes produced very strong PAS and silver +ve reactions. The sporangia showed no definite exit tubes, but with the breakdown of the sporangial membrane, the zoospores were liberated into the haemocoelic

fluid (Pl: XVI, Fig. 7). Apart from the jerky movement of the zoospores, caused by the peristaltic "pulsation" of the haemolymph flow, zoospores remained quiescent for up to 4 days after liberation. Meantime, the infected larvae lose the honey-comb pattern and copper colour, which were previously external signs of parasitization. A persisting distention, posteriorly, could easily be dismissed as but the normal shape of a larva. On the 4th day after liberation, or thereabouts, all zoospores became highly motile in the haemocoel, with a flagellum which was then apparent. The zoospore has quick oscillatory pendulum-like movements due to the whipping action of its flagellum (Pl: XVI, Figs 10 & 11 and Pl: XVII, Figs 10 & 11). These zoospores are, presumably, the infective stage of the parasite.

The pyriform zoospore measured about 4-6 $\mu$  in length and possessed a flagellum 3 times as long as the body. It had a tiny anterior blepharoplast from which the flagellum arose. The large nucleus was surrounded by faintly-staining cytoplasm. Unlike that of the mother sporangium, the cytoplasm of the live zoospore showed a greenish tint similar to that exhibited by the microsporidian spore. A thin membrane, which was responsible for the

+ve PAS reaction of the divided sporangia, formed the limiting wall of the zoospore.

When the parasitized Simulium larva eventually died, it ruptured to liberate extremely large numbers of zoospores in the water. These swam actively for a few hours, and then apparently died and disintegrated. No encystment was observed.

ii)- Form "B" infections

These infections showed a very similar seasonal distribution to that of Form "A" sporangia. They also occurred as irregularly spheroid or lobular multinucleate bodies scattered in the fat body, each occupying one of a group of neighbouring cells in the posterior larval adipose tissue. The smallest sporangium was about 6 $\mu$  in diameter. Clear mitotic nuclear divisions of the young uninucleate sporangium were seen and a maximum of about 120 daughter nuclei; the multinucleate syncytial sporangium was then about 17-20 $\mu$  in diameter. Subsequent breakdown of fat cell contents and fat cell walls created cavities in the fat body bounded by outer layers of fat cells, peripherally. Within each cavity up to 15 sporangia were enclosed. These sporangia never developed any further, nor was cytoplasmic division ever seen. Parasitized larvae completed their life cycles in the laboratory normally through pupae to adults: infection was not recognised in this study in



sections of emergent adults.

iii)- Form "C" infections (Pl: XVIII)

Thick-walled sporangia

This form was also confined to larvae from Tilling Bourne. On rare occasions, larvae infected with Thelohania form "A" (see Table X) were found, upon sectioning, to harbour in addition a peculiar type of thick-walled sporangia, almost always posterior to the microsporidian pseudocysts (Pl: XVIII, Fig. 4). This I designate as form "C". They seemed to have originated and developed in the same way as the thin-walled form "A" sporangia (Pl: XVIII, Fig. 1). In spherical sporangia the thick wall was of uniform thickness, in section, but the ovoid or oval sporangia had their wall much thickened at both poles and narrowed laterally (see Pl: XVIII, Figs 3 & 5, W)

The maximum length was about 50 $\mu$ . Eventually the parasitized fat body cell-walls broke up to liberate the sporangia (Figs 4 & 5). At this stage, the more anteriorly sited microsporidian pseudocysts had attained a size which seemed to obstruct advance forward of these sporangia. They were always found in the posterior part of the infected larva. No zoospores or further development was noticed. Some of the mature thick-walled sporangia showed differentiation of cytoplasm into multinucleate

spherical bodies of various sizes; other sporangia also showed much vacuolation (Pl: XVIII, Fig. 6).

The biological significance of the association of these thick-walled sporangia, always in mixed infections with microsporidia, remains obscure.

e) Histological reaction of the sporangia

The sporangial cytoplasm of all these forms stained intensely with all stains used, thus masking the nuclei. It was, therefore, necessary to hydrolyse with N-NCl. With Giemsa after hydrolyses the nuclei were evident (Pl: XVI, Figs 1, 3 & 4). The nuclei of the form "A" sporangia produced weak +ve reactions in the Feulgen test, but form "B" sporangia and the thick-walled sporangia (form "C") possessed strongly Feulgen +ve nuclei.

The wall of the thick-walled sporangium (form "C") stained more intensely than the inner cytoplasm with toluidine blue and with pyronin, suggesting a higher content of RNA in the wall (Pl: XVIII, Fig. 5).

Form "A" sporangia in an advanced state of cytoplasmic division to produce zoospores showed strongly PAS-positive reactions on the newly formed zoospore membranes, and also with chromic acid-methenamine silver staining. Form "B" sporangia, form "C" sporangia and

young undivided stages of form "A" sporangia gave, however, negative reactions in both tests.

f) Classification

In all of the presumptive Coelomycidium material studied, no developmental stages or structures other than those described above were found. Some sections and smears showed fragments of what looked like parts of rudimentary hyphae. The structure already referred to as surrounding some sporangia looked like some kind of modified fungal hypha (Pl: XVI, Fig. 9). The undivided cytoplasm of some sporangia showed signs of shredding, and looked as if made up of irregularly coiled hyphae tightly packed within the sporangial membrane.

Although some protozoa, e.g. the pseudocyst wall of Toxoplasma, give a +ve reaction in the PAS test (Thompson, 1966), and the anterior tip of the microsporidian spore has been established as weakly PAS positive (Sprague 1965), yet it is on the intensity of these reactions, as opposed to the weak protozoan reactions, that fungi are differentially identified. The great intensity of reactions given by fungi in the PAS and silver tests should serve as an indication for the high polysaccharide contents of their walls, which characterises this group. It was partly on this, but mainly on the incidence of the flagellated

zoospores, exclusively derivatives of the sporangia of some Phycomycetes, that these organisms were not classed as protozoa. Also, following Weiser (1965a) in distinguishing between members of the protozoan Haplosporidia and members of the fungal Order Chytridiales, identification as chytrids seemed reasonable. The chytrids in most cases dissolve the tissue of the host before sporogony. This effect is not present in any protozoan infection. It is to be remembered that with two of the forms "A" and "C", the infected larval fat body disintegrated before sporangial maturation. Further development of the sporangia in forms "A" & "C" and their division to produce zoospores in form "A" took place extracellularly.

Accordingly, and in view also of the similarity shown by these microorganisms infecting S. ornatum larvae to the organism described from Simulium sp. in Europe by Debaisieux (1920), and, in the revision by Weiser (1966), as a chytrid, I elected to assign tentatively these Simulium ornatum infections as of chytrids, and to the same genus Coelomycidium as established by Debaisieux (1920). Specific designation is premature at this stage. I can but postulate that three distinct forms, "A", "B" & "C", of these infections exist.



2. Class: PhycomycetesSub-Class: TrichomycetesOrder: HarpellalesFamily: Genistellaceae

(Pl: XIX)

A)- General: The Trichomycetes, regarded as a group of uncertain affinities are mostly parasites of arthropods. Their thallus is filamentous, simple or branched, and is attached to the digestive tract or external cuticle of living arthropods by a holdfast, or basal cell. The mycelium is not immersed in the host tissue (Ainsworth, 1961). The hyphae are coenocytic and at first septate and slender. Later they develop occasional cross walls (Bessey, 1950).

b)- Occurrence: The presence of these endocommensals of larval Simulium ornatum was first discovered in histological preparations as masses of thread-like structures filling the posterior part of the rectum in a high percentage of larvae from both rivers (Pl: XIX, Figs 9, 10 & 11). Then, later, by dissection of larvae, bush-like structures reaching a length of about 1mm were recovered from larvae. They were found attached to the inner surface of the peritrophic membrane lining the hind-gut. When pulled by tiny jeweller's forceps, the whole mycelium detached very easily. Young unbranched hyphae were also recovered in the same way. The difference in the morphology of mycelia and the various

shapes of conidia suggested the presence of more than one form.

c)- The mycelia: In all "forms" or variants, the mycelium is composed of hyaline and non-septate, slender, and slightly to highly curved vegetative hyphae. The vegetative hypha is simple with a swollen base terminated by a pad-like structure possessing 6-8 lateral spines for attachment to the larval gut wall (Pl: XIX, Figs 1 & 2). It has either a bluntly tapering or a swollen apex with minimal granulation: the cytoplasm generally elsewhere along the hypha is highly granular which gives it a darker appearance (Pl: XIX, Fig. 2). As a vegetative hypha grows in length it narrows and becomes less granular until it reaches a length of about 600 $\mu$  (Pl: XIX, Fig. 6). Then single lateral branching takes place at about its mid-point to produce more than 8 secondary hyphae. The newly produced hyphae also, but to a much lesser extent, exhibit the coarse granular cytoplasm in addition to highly refractile oil droplets within the cytoplasm (Pl: XIX, Fig. 8). Later, cross septa appear and lateral alternate hyphal branches are produced to develop to a maximum width of about 4.2 $\mu$  - 4.7 $\mu$ .

d)- Conidia: These asexually produced unicellular indehiscent spores always originate termino-laterally, and are sparsely produced. They are hyaline, colourless and uninucleate, and of two distinct types. There is a pedunculate ovoid type measuring about 10 $\mu$  x 4 $\mu$  with a long stalk, approximately 4 $\mu$ , or conidiophore (Pl: XIX, Fig.4),

and an ampulliform sessile type measuring about  $27\mu \times 7\mu$  (Pl: XIX, Fig. 5).

The conidial appendages, which are long extremely fine filaments attached to the base of the conidium, described by Leger and Gauthier (1935), were seen in fresh preparations of the ovoid and the ampulliform conidia, but the number of appendages in each could not be correctly determined.

e)- Zygosporos: Formative stages of this sexually produced spore were not observed, but occasional gigantic spindle-shaped spores were recovered from mycelia with sessile ampulliform conidia. This one type of zygospore measured about  $340\mu \times 50\mu$  (Pl: XIX, Fig. 3). It apparently possessed chlorophyll which gave it a dark colour particularly round the single nucleus. The rest of the spore was hyaline.

f)- Classification: All characters mentioned above place these endocommensals of Simulium ornatum larvae in the family Genistellaceae. Mycelial branching and types of conidia produced differ from the morphological characters of Stipella vigilans (Leger & Gauthier, 1932), an endocommensal in the hind-gut of Simulium sp. in Europe, which produces unilateral long cylindrical conidia. The conidia on the other hand, show similarities in shape, size and arrangement to those of Smittium simulii (Lichtwardt, 1964), inhabiting the rectums of Simulium argus and S. virgatum in America but, according to Lichtwardt



(1964), this latter species differs from the present material by its non-divergent type of hyphal branching. The material herein observed certainly differs from the genus Genistella which produces unilateral "tufts" of conidia (Leger & Gauthier, 1932). Other species of Trichomycetes earlier found to inhabit the hind-gut of Simulium larvae, and which differ from the present material, are: Amoebidium sp. (Chatton & Raubaud, 1909), Paramoebidium simulii (Tuzet & Manier, 1955), Pennella grassei (Tuzet & Manier, 1955), Pennella hovasi and Rubettella simulii (Manier, 1963). One species, Harpella melusinae (Leger & Duboscq 1929), was described from the mid-gut of Simulium larvae. With the exception of Smittium simulii, all other species were described from European simuliidae.

All that can be decided at this stage, is that the endocommensal fungi seen in Simulium ornatum larvae in the present investigation are Trichomycetes of the Family Genistellaceae but designation to genera or species would require more study.



III-      Nematode infections

Superfamily:    Mermithoidea

Family:        Mermithidae

a)- General:    These worms are identified by the degenerate musculature of the oesophagus, the very long oesophagus, the presence of the oesophageal cells along its length and the development of the intestine as a food storage organ (Welch, 1963a). Welch further stated that little success has been achieved in associating immature stages found in insects by entomologists with free-living adult nematodes encountered by nematologists. The life cycle commences when the second juvenile stage penetrates the host cuticle and enters the body cavity where it grows and fills the body cavity of the insect host, within 12 days at room temperature, and up to 5 months in overwintering Simulium larvae (Phelps & DeFoliart, 1964). Inside its host the parasitic juvenile develops a thick cuticle which is drawn posteriorly into a spine or caudal appendage. This cuticle is found on the early post-parasitic juvenile together with its caudal projection and provides a diagnostic feature for identification of the worm. After emergence from the host, the post-parasitic juvenile gradually develops its internal structure until the moult to adult stage.

b)- Occurrence:    Nematode infestation of Simulium

ornatum larvae collected from the two rivers was not a conspicuous feature, being found only in exceedingly small numbers of the larvae, and only from the Tilling Bourne. Dissection of large numbers of larvae from the river Lea at the beginning of this study never produced a positive result for nematode parasitism.

c)- Systematic position: Comparison with diagnostic characters mentioned above placed these parasitic nematodes in the Superfamily Mermithoidea, Family Mermithidae Braun 1883.

d)- Parasitic and post-parasitic juveniles: (Pl: I & Pl: XX).

Infected Simulium ornatum larvae examined by eye showed slight distension at the abdominal region, with a faint greenish coloration reflected from the nematode in the distended part. Under the dissection microscope, the tail end of the worm was seen coiled in the bulbous part of the larva, the head end reaching just below the head capsule of the larva. The worm showed continuous coiling and uncoiling movements, yet the activity of the Simulium larva appeared normal.

Simulium larvae containing these large parasitic nematodes were put in aerated tap water-filled beakers. Then, about the 3rd day after collection of the infected

Simulium in the field, they fell to the bottom of the beaker and struggled vigorously for nearly 3 hours. The nematode then emerged through a hole it had burrowed in the abdominal wall of the Simulium larva.

The emerging worm or post-parasitic juvenile was greenish in colour and measured a mean length of 16mm and a mean width of 210 $\mu$ . All were females having a sigmoidal vagina. The raised vulva has a lumen of about 50 $\mu$  wide and 130 $\mu$  deep. All newly emerged post-parasitic worms had their cuticle drawn posteriorly into a tail projection about 700 $\mu$  long, under which there was bluntly rounded hypodermis.

These female post-parasitic juveniles were kept in small petri dishes full of distilled water changed daily. One week after emergence, signs of final moult were observed. The thin cuticle from the anterior half of the worm loosened and was shed. The posterior part of the cuticle, though loosened, was retained and looked at the tail end like a transparent sheath of a microfilaria. After loosening of the cuticle the tail region became rounded. The worms continued to live for about 1-2 weeks and then died. Just before their death, their trophosome became heavily vacuolated.

No male worms were seen.

e)- Classification: In view of the conflicting opinions regarding the taxonomy of these nemertean nematodes and of the limited material for studies, generic identification of these nematode parasites of larvae of Simulium ornatum is not here attempted critically. But some of the features exhibited by these nematodes, particularly the faint greenish coloration, resembled Gastromermis viridis Welch 1962.

#### IV- Seasonal incidence of the parasites

There are variations in the occurrence of the different types of infection observed in the Simulium ornatum larval populations of the two rivers, Lea and Tilling Bourne.

##### Microsporidia

##### Thelohania (Diagram 6 (1))

In the River Lea, Simulium ornatum larvae, when seasonally present were usually found infected with Thelohania, yet the infection rate was generally low, at most 9%. Four forms of Thelohania were taken in the Lea.

There was evidence of a seasonal cycle of abundance <sup>of</sup> Thelohania infections in larval populations of the Lea. Diagram 6 indicates a rise in infection rate from May 1968 to 9% in Sept. 1968, but with a drop in



July, due to the hatching of larvae of the summer generation, which were included in the sampling, and were either not yet infected or their infections were too young to be detected. Another decline in infection rate to 3% in October is explicable by an early hatching of overwintering larvae. In November all larvae collected were 1st stage of this overwintering population, with no parasitism. In December, infection again became apparent and persisted into January, 1969, with a sudden rise to another peak of 9% in February. This coincided with most of the overwintering larvae breaking their diapause and pupating, thus leading to an apparent increase of infected larvae since they do not readily, or fail to, pupate. With the continuing pupation of healthy larvae and emergence of adults, it was thought that a steady rise in the proportion of infected larvae might be evident in March and April, but the converse took place. This may have been because of a very high death rate in infected larvae. Infection was less than 1% incidence by April. By May there was a complete absence of Simulium larvae. In the previous year, 1968, the absence of larvae took place in April. Thus the cycle of abundance of Thelohania that followed, in the 1969 summer larval generation, started

one month later than in the previous year. Even so, the pattern of rising infection levels in the summer brood of larvae appears to be similar both years.

In the Simulium ornatum larval populations of the Tilling Bourne, the infection rate with one form of Thelohania (Form "A") was always less than 1%, and, usually, so infrequent as to be absent from most fortnightly samples of 100 larvae even by histological examination.

The rare Plistophora and Nosema infections have been adequately dealt with earlier.

The microsporidia-like infections, found in Simulium ornatum larvae at Tilling Bourne only show some similarity in distribution (Diagram 7) to that of Thelohania infections (Diagram 6) in River Lea larvae. They were abundant in September 1968, followed by a steady decline in October and November and another rise in abundance in January.

Gregarines: (Diagram 6(2))

An almost identical mid-summer peak of abundance of these endocommensals occurred in larval populations of both rivers. A gradual increase of incidence took place between the months of May and August, 1968, followed by a

steady decline in September, October and November.

Although regular fortnightly collections were not made from Tilling Bourne, there were indications of repetition of this cycle in 1969 - similar relative abundances in January and in July of both years.

Fungi:

1- Chytrids (Diagram 8)

Chytrids were found only in Simulium ornatum larvae of the Tilling Bourne. Three distinct forms were identified, A, B and C. When they were first observed in November, 1967, form "A" showed a high infection rate of 60%, whereas 17% of the larvae were infected with form "B". In both forms this rate declined steadily in December and January. By spring and early summer of 1968, both forms again showed an increased abundance over the previous January. Form "B" reached a peak by late summer (September), followed by a declining rate in October and November and a total absence of infection by January 1969. Form "A" had a later peak of abundance, in November, followed by a decline by January to less than 3%. The pattern for both forms is similar, though lower, to that of the decline in the winter of 1967-68.

Form "C" was rare.

## 2- Trichomyces (Diagram: 9)

These were found commonly in larval populations of both rivers. In the River Lea, peak abundance (30%-35%) was in summer larvae (May to July) of both 1968 and 1969, but infection, at a lower level, also occurred in the overwintering larvae in early winter months (up to December).

As for Thelohania, the spring-summer Simulium larvae in the Lea appeared a month later in 1969 compared to 1968, but parasitization with trichomyces developed similarly as soon as larvae were present.

The pattern of infection rate for the Tilling Bourne larvae is less certainly determinable since over 1967-1969 collections were not made monthly. Even so, trichomyces infections occurred throughout much of the year, including winter and summer months and at as high as about 25% to nearly 100% of larvae positive. The winter rise from October-December to about 15% infection rate in the Lea, may have its parallel in the very high winter rate (nearly 100%) by January (1969) in the Tilling Bourne.

### Permithid worms:

Though rare, these parasites showed a seasonal incidence, parasitizing larvae of the later summer and autumn broods mainly in September. They were recorded



only for the Tilling Bourne. Mermithid larvae were found usually singly in an infected simulium larva, but an infection of 2 mermithids was once observed.

V- Pathogenicity of different infections for the Simulium ornatum larval host.

Microsporidia: About 20% of infected Simulium larvae failed to develop histoblasts, and about 50% failed to develop any gonad rudiments or imaginal discs for legs and wings. Similar findings were reported also by Strickland (1911 & 1913). The fat body and connective tissue were infected. At the late stage of infection the translucent fat body lobes transformed into opaque pseudocysts of spores or spore masses, intermingled with some remnants of host cell membranes. The connective tissue disappeared. The parasite as it grew and multiplied in the larval host, destroyed the fat body progressively and hindered, by deprivation of essential nutrients, and perhaps other substances, the normal development of the host. In the case of Plistophora form "B", there was a deposition of a red pigment, probably insectorubin, on the remnants of fat body surrounding the parasitic spores. No hypertrophy of oenocytes took place, but there was a marked production of phagocytes or plasmotocytes (Wigglesworth, 1965). These plasmotocytes were found in aggregations surrounding the

posterior larval fat body lobes, particularly at the early stage of infection with microsporidia. The gonad rudiments, although developed, were always constricted by the surrounding parasite pseudocysts. Silk glands became reduced in size, and their silk production apparently reduced.

Either when the infection had reached its maximum development or shortly before this phase, the infected larva became sluggish and eventually died. Sometimes, before death, the larval integument ruptured, liberating the milky-white pseudocysts; these were seen on occasions on pebbles on the stream beds; smears of them revealed viable microsporidian spores mixed with bacteria and some ciliates.

In one form of Thelohania, also at the maximum stage of its development, the spores broke loose gradually from their pseudocysts and were dispersed in the general flow of haemolymph and finally lodged in such spaces as between the muscle fibres, within the head capsule and inside the anal gills.

In the course of rearing field collections of Simulium ornatum larvae, I have never known a larva with such manifestation of infection to reach the pupal stage. I have in mind that less obviously infected larvae might well pupate and adults emerge. However, upon histological examination of adults emerged from samples of an infected

larval population, none were found with microsporidian infection.

Chytrids:

Coelomycidium form "A": The effect on the host produced by these fungal parasites was somewhat similar to that of microsporidia. The sporangia of the parasite developed intracellularly in the larval fat body. As they grew, they did so at the expense of the host cells, which eventually disintegrated, thus depriving the host of essential nutrients. The sporangia completed their final stage of development and produced zoospores extracellularly in the larval haemolymph.

Again the gonad rudiments were missing or slightly developed and the silk glands were markedly shrunken in size, but less than in the case of microsporidian infections.

The infection was fatal to all infected larvae, often before the division of sporangia and production of zoospores.

Coelomycidium form "B": These infections never showed an appreciable effect on their larval host and, were it not for histological examination, they would have passed unnoticed. But they never passed into the adult stage of Simulium, as far as sectioning of adults revealed in this study.



Mermithid nematodes: In contrast to the view of Strickland (1911) effect of the mermithid worms on the development of the histoblasts of the parasitized larva was not obvious. Of the few larvae found infected by these worms, the majority developed histoblasts. The effect otherwise on the internal organs was similar to that produced by chytrids and microsporidia. The fat body and connective tissue disintegrated in the course of the development of the worms. The silk glands were also markedly shrunken. Due to loss of connective tissue and the constant movement of the worm in the larval body cavity, the internal organs were slightly deranged. The infected larva died immediately after the emergence of the nematode.

Gregarines: Although the gregarines must derive their nutrients from their host, they were harmless endo-commensals in the mid-gut lumen of the infected larva. Shrinkage of the epithelial cells underlying the gregarines was always made good by further production of new epithelial layers after every moulting cycle.

Trichomycetes (Fungi): Of least parasitic impact these fungal endo-commensals showed a similar host-parasite balance to that of gregarines. They were apparently harmless endo-commensals within the gut lumen. Even although there was a difference in the superficial attachment of the



fungus to the chitinous lining of the larval hind-gut epithelium as compared with the intracellular attachment of the gregarines to the epithelium of the mid-gut, and both types of attachments could have the function of drawing up nutrients from the underlying host cells, neither these fungi nor the gregarines appeared to cause damage to their larval Simulium host. The pad-like organ of adhesion at the base of the main thallus also acted as a sucker for providing the fungus with necessary nutrients from the host cell. When the epithelial cells were discarded during larval ecdysis and were replaced by newly-formed cells, the fungus thali were swept away with the discarded cells to which they were attached. Zygosporoes which possess appendages of adhesion to prevent them from being swept away as well, would seem to be designed to remain behind to germinate and reinfect the larva, or, if defaecated, to infect other larvae.

Other infections: No adverse effect was shown by any other micro-organisms observed in these studies, e.g. Coelomycidium form "C" and the microsporidia-like bodies. The incidence of these, however, was so low that conclusive views would not be justified.

VI- Trials on the transmission of infections:

Attempts to rear a 'clean' colony of S. ornatum in the laboratory were unsuccessful (see Appendix). Recipient larvae in the following experiments on transmission were apparently uninfected specimens from the Lea or Tilling Bourne rivers.

A- Microsporidia - Thelohania spp.

For each experiment, about 100 infected Simulium ornatum larvae were used as a source of infecting material, and divided into 3 lots, as follows:

1. Pseudocysts were dissected out from 20 infected larvae and crushed in distilled water in a tissue grinder. The resulting suspension of spores was added to a 1-litre beaker half-full of stream water and containing about 200 first stage larvae, apparently non-infected, from the River Lea.

2. 30 infected larvae were mixed with 200 apparently non-infected field larvae of mixed instars in a rearing beaker half-full of stream water.

3. 50 infected larvae were isolated in a third 1-litre beaker of stream water.

The beakers were aerated by bubbling air through the water by a "HyFlo" pump.

In lot (2) and lot (3) all infected larvae died by the third day of the experiments and their body-walls

ruptured, discharging spores into the water. The day after the beginning of the experiments, samples of about 25 apparently non-infected larvae from each of the first two lots, were dissected and examined for signs of new infections. Further samples of about the same number from lots (1) and (2) were examined by sectioning after 6, 10 and 15 days. None of these examinations revealed signs of possibly new infections in the fat body of the initially apparently "clean" wild-caught larvae, although intact Thelohania spores (without extrusion of polar filaments) were found in big numbers in the larval guts.

The experiment was repeated three times at 24°C, and again at 22°, 19° and 18°C, but without apparent success in inducing the development of spores in their own natural Simulium larval host.

Unsuccessful trials were also made to infect mosquitoes with Thelohania spores of these simuliid microsporidians, and for this, spores were placed with the eggs of Aedes togoi in their breeding bowls at 22°C. This was also repeated with young larvae, immediately after hatching from eggs.

The possibility of inducing the germination of the microsporidian spores by passing them through other



non-simuliid hosts was investigated as follows:

1. Two species of Cyclops, one from Regent's Park and the other from Osterly Park in London were bred in the laboratory and their internal morphology first studied by histology. Later, as in the foregoing trials on cross-infection between Simulium larvae, Cyclops were given an opportunity to ingest parasites, or otherwise acquire infection. Cyclops were then fixed in aqueous Bouin 15, 30, 45 minutes, 2 hours, 24 hours and 7 days after exposure to the Thelohania spores, processed for sectioning at 6 $\mu$ , and sections stained with Giemsa. No spore germination was evident in the Cyclops guts, although they had been ingested; they occurred unchanged in the mid-guts, hind-guts and in the excreta of the Cyclops. Masses of intact unchanged spores were also seen about to be discharged from the rectum of one sectioned Cyclops

To beakers of other Cyclops fed on Thelohania spores, some wild-caught apparently "clean" Simulium larvae of mixed ages were added. These larvae ingested most of the Cyclops; they were found in their guts. Then after 2, 6, and 24 hours of putting the Simulium larvae and the Cyclops together, the gut contents of the Simulium larvae were examined in smears but although spores



were identified in the Cyclops-fed Simulium there was no evidence that this double passage had promoted a capacity for the spores to infect the Simulium larvae.

2. The experiments were repeated with initial ingestion of spores by two species of Gammarus, and with leeches found associated with Simulium larvae in their stream habitat, but again without apparent success in inducing the Thelohania spores to "hatch" in Simulium larvae secondarily fed with these spores. This experiment was prompted by my observation of leeches ingesting Simulium larvae.

3. Some fresh Thelohania spores were added to a mosquito cell culture, kindly supplied by Dr M.G.R. Varma of the Dept. of Entomology, L.S.H. & T.M., and were later examined after 15 minutes, 30 minutes, 2 hours, 6 hours and 24 hours, in both wet and dry-fixed-Giemsa-stained conditions. This time, only a slight wrinkling of the spore-walls was evident in material examined 6 hours, and 24 hours after being immersed in the tissue culture. But no extrusion of the filament by the spores, as was observed by Trager (1937) for Rosema spores in tissue culture, or penetration of cells of the culture was observed.

I have, therefore, failed to induce development of Thelohania spores in Simulium larvae by procedures

apparently simulating natural, or various artificial conditions of exposure to infection.

B- Chytrids:

Coelomyxidium sp.

An aqueous suspension of sporangia and of zoospores of this fungal parasite dissected out from infected larvae from the Tilling Bourne, was fed to apparently parasite-free wild-caught Simulium ornatum larvae of mixed ages from the River Lea, following the same procedure described above for exposure to Thelohania spores. Since this chytrid infection was absent from Simulium ornatum larvae of the River Lea, these larvae provided a reasonably presumptive "clean" culture for exposure to potential infection with this parasite. The experiments were repeated monthly, for 4 months.

After being exposed to the parasite, larvae were examined after one, two and four days. There was no trace of sporangia or zoospores within the gut contents, or in any other part of the larvae. Examination under the microscope of the water containing the larvae, and to which the infecting material had been added, revealed neither sporangia nor zoospores, in suspension, even after the first day of the experiment. Thus, as for Thelohania, transmission of this chytrid was not obtained.



## S U M M A R Y

- 1- Simulium ornatum larvae were regularly collected from the River Lea (Hertfordshire) and occasionally from the Tilling Bourne (Surrey) from 1967-69. Preliminary surveys revealed no parasitism in pupal and adult stages of Simulium ornatum but several parasites, some common, occurred in the simulium larvae, and these were studied intensively by dissection, smears and histologically, including by electron microscopy.
- 2- Microsporidia were well represented by 3 genera - Thelohania, Plistophora and Nosema. Infection rates never exceeded 9% of larvae. Spore measurements statistically evaluated from different larval infections justify recognition of 4 main forms (A to D) and 1 minor form (E) for Thelohania parasites and 2 forms of Plistophora. Nosema, a rare infection, was of a single form. Specific names have not been given here to these several forms.
- 3- Intra-epithelial organisms referred to provisionally as microsporidia-like bodies caused light infections.
- 4- Chytrid fungal infections were found only in larval populations of the River Tilling Bourne at a considerably higher infection rate ( - nearly 60% positive larvae) than that of microsporidia. These

organisms were classified as three forms of a Coelomyxidium.

5- Mermithid worms resembling Gastromermis viridis was an infrequent (autumn) infection confined to larvae of the Tilling Bourne.

6- With the exception of the light microsporidia-like infections, microsporidia, chytrid fungi and mermithid worms were parasites of the body cavity or fat body, all causing disintegration of the fat body. Larval deaths, and delay in their growth and pupation, occurred.

7- Other organisms encountered were endocommensals in larvae from both rivers and were identified as (a) Eugregarines living in the lumen of the larval mid-gut, and (b) fungi belonging to the family Genistellaceae of the sub-class Trichomycetes, commonly found inhabiting the hind-gut of the larva.

8- It was found for the commoner parasites that seasonal cycles of abundance prevailed, including both winter and summer infections, which were related to the presence, necessarily, of summer or overwintering populations of Simulium larvae.

9- Transmission of infections was attempted by exposing apparently uninfected wild-caught Simulium larvae to spores of Thelohania and to sporangia and zoospores of Coelomyxidium but no evidence was obtained of active



infection being established in the exposed larvae.

10- Unsuccessful attempts were also made to induce mating and feeding of adult Simulium ornatum as a basis for a laboratory colony.

11- Although the pathogenic forms (esp. microsporidia and chytrid fungi) of parasites taken in this study must have exerted control on natural larval densities, too little is known of their biology, mode of transmission, or methods of culture to envisage their development, at present, for biological control of simuliids.

## D I S C U S S I O N

Protozoan Infections:1- Microsporidia:

The case for recognising several forms (?species) representative of 3 genera of microsporidia in my simulium larval material is fully stated in the foregoing text on the material, and only certain aspects are now considered in discussion.

Host susceptibility and transmission:

Some workers advocate host-specificity as a taxonomical feature for microsporidia. There are indications from the present work that the microsporidians in Simulium larvae are host-specific for Simulium. Thelohania spores were fed to different stages of Aedes togoi, besides to two species of Cyclops, to two species of Gammarus and to leeches from the same wild habitat as the Simulium larvae under consideration and also placed in mosquito-cell cultures. Different stages of Simulium larvae were fed on these spores. In all cases, however, no evidence of infection was detected; all spores found in the recipients were unchanged. The recipient hosts were examined for up to 7 days. These unrewarding attempts to induce the Thelohania spore to hatch cannot be regarded as conclusive

evidence for host-specificity; more trials with variable factors such as temperature, humidity, ages of the spore etc. included, are necessary. Furthermore, the spores failed to hatch in the larval host itself, indicating a missing link in stimulation. I adopt a conservative position at present in accepting that, probably, the microsporidians studied in my work are host-specific to Simulium and essentially tissue specific within this host (in its larval stage) to fat body.

We may take up here further the problem of transmission between Simulium hosts. The low rupture rate of apparently mature spores within larvae prompts the concept that either at that stage, or earlier, the Thelohania infection may pass, in larvae which survive their infection, through pupa and into the adult and, as reported for Thelohania in mosquitoes (Kellen & Wills, 1962b; Hazard & Weiser, 1968), invade the ovaries of the adult and so pass to the next larval generation. The considerable deaths amongst Thelohania-infected Simulium larvae under study may have involved selective deaths of male larvae, an effect reported by Kellen & Wills (1962b), by Kellen et al (1965), and by Hazard & Weiser (1968) in their work on mosquito infections with Thelohania. I do not know whether there was this kind of sex elimination



in Simulium larvae although, certainly, not all larvae of an infected batch appeared to be infected and not all died. Some pupated & adults duly emerged. Exploratory sections of a few of these adults revealed nothing that I recognised as a Thelohania infection, either of spores or of, possibly, a schizogony stage. So far as the present study permits conclusion, there is evidence then that larval infections of a sporogonic nature result in fully developed spores which have the capability, it seems, of initiating infection in a suitable aquatic host but experimental demonstration of this has been elusive. Whether another aspect of the host-parasite relationship involves transfer of infection from larvae through to adults and their ovaries, and possibly induces a schizogony cycle, remains unknown. It may be said that I had, on the other hand, fair success in infecting Aedes togoi larvae with spores of Nosema sp., infected larvae developing heavy infections. Material of Plistophora and Nosema was insufficient to undertake experimental work.

Pathogenicity:

A good description of the host-parasite relationships between Thelohania fibrata, Plistophora simulii and P. debaisieuxi and Simulium larvae was given by Rubtsov (1966).



Parasitism was restricted to the fat body. But the larva showed other adverse consequences in that larval life was prolonged with delay in development of pupal and imaginal organs, disintegration of the fat body, appearance of epithelial intestinal cells in the lumen of the intestine, deterioration of other organs, especially spinning glands, but also Malpighian tubules, muscles, gut, nervous system, culminating in the death of the infected larva. I find this closely resembles my observations except for the appearance of the gut epithelial cells in the gut lumen, which is not necessarily due to the infection, but is a normal phenomenon of the moulting in the development of the larva.

#### Speciation:

This present study showed that ranges of spore measurements from different Simulium larval infections of Thelohania fall into 5 distinctive forms (with mean dimensions of  $3.2\mu \times 2.9\mu$  for spore size Form "A",  $4.2\mu \times 3.1\mu$  for "B",  $4.7\mu \times 3.6\mu$  for "C",  $5.9\mu \times 3.8\mu$  for form "D" and a fifth form "E", with an unusually oval-truncate spore). Other features (See Table X) correlated well with spore size. It was possible also to recognise 2 forms in the genus Plistophora, dependent on both spore dimensions

and numbers of spores per sporont. Nosema, a rare infection, showed only one form. Whether these should be assigned as species, sub-species or by other taxonomic designation is premature to decide. I have elected in accord with Tsai et al (1969) in their mosquito studies to specify what I recognised as distinctive "forms" by alphabetical designations, A, B, C, D, and the additional form E with other special features for Thelohania and A and B for Plistophora (Table X). It is to be noted that these "forms" have not hitherto been reported in the literature. The genus Plistophora has not been reported for simuliidae of Great Britain, so that both forms are entirely new to British records. So far, only two species of Plistophora are reported parasitizing simuliids of Europe and the U.S.A (Table XI). One of the new British forms (Plistophora B, Table X) resembles Plistophora simulii (Debaisieux & Gastaldi, 1919) in that the sporonts of both give rise to a mean number of 20-30 spores; but they differ in their spore sizes, viz. Plistophora simulii has mean spore dimensions,  $4-5.5\mu \times 2.5-3.5\mu$  and the material of Plistophora form "E"  $5.6\mu \times 4.0\mu$

Table XI tabulates previous records for spore dimensions of material examined by other workers.

Table XI Spore dimensions of species of microsporidian parasites of Simulium, as measured by different workers.

Species	Spore dimensions reported by:			Spore Shape
	Debaisieux et al, 1920	Jirovec 1943	Weiser 1966	
<i>Thelohania varians</i>	6.5-8 $\mu$ x 4.5-5.5 $\mu$	5-5.5 $\mu$ x 3-4 $\mu$	5.5-8 $\mu$ x 3-5 $\mu$	ovoidal & pyriform
<i>Thelohania fibrata</i>	7 $\mu$ x 3.5 $\mu$	6-7 $\mu$ x 3.5-4 $\mu$	5-7 $\mu$ x 3-4 $\mu$	oval
<i>Thelohania bracteata</i>	3-4 $\mu$ x 2.5-3 $\mu$	3-4 $\mu$ x 2.8-3.6 $\mu$	2.5-4 $\mu$ x 2.3-3.6 $\mu$	broadly oval
<i>Thelohania columbaczense</i>			3-3.5 $\mu$ x 1.5-1.8 $\mu$	pyriform
<i>Plistophora simulii</i>	6-8 $\mu$ x 3.5-5 $\mu$	4-5.5 $\mu$ x 2.5-3.5 $\mu$	4-5.5 $\mu$ x 2.5-3.5 $\mu$	oval
<i>Plistophora debaisieuxi</i>		7-8 $\mu$ x 3-4.5 $\mu$	7-8 $\mu$ x 3-4.5 $\mu$	oval
<i>Nosema stricklandi</i>		5 $\mu$ x 2.5 $\mu$		pyriform

cf. Tables IV, V & VI of measurements of the spores of microsporidian parasites of Simulium ornatum in the present study.



Identification:

One may summarise distinctive features of the microsporidia recognised in the Simulium larvae under study by a key for their identification, on the basis of Weiser's (1961) key, as follows:

- |    |  |   |
|----|--|---|
| 1- | One, or two large pseudocysts _____  | 2 |
|    | Several smaller pseudocysts _____  | 3 |
| 2- | Sporont producing 8 spores : <u>Thelohania "A"</u>                                     |   |
|    | Sporont producing 64-128 spores : <u>Plistophora "A"</u>                               |   |
| 3- | Single spore per sporont : <u>Nozona "A"</u>   |   |
|    | 16-32 spores per sporont : <u>Plistophora "E"</u>                                      |   |
|    | Maximum 8 spores per sporont _____   | 4 |
| 4- | Spore longer than 5 $\mu$ : <u>Thelohania "D"</u>                                      |   |
|    | Spore shorter than 5 $\mu$ _____   | 5 |
| 5- | Spore truncate oval in shape,<br>no apparent posterior vacuole : <u>Thelohania "E"</u> |   |
|    | Posterior vacuole apparent _____   | 6 |
| 6- | Spore ovoid in shape _____   |   |
|    | several large pseudocysts packed<br>close together : <u>Thelohania "B"</u>             |   |
|    | Small, numerous discrete pseudo-<br>cysts : <u>Thelohania "C"</u>                      |   |

Fine Structure:

Electron microscopy is a valuable technique for the elucidation of protozoan structure; and I will comment



on certain aspects of microsporidian fine structure.

Lom & Vavra (1963b) advocated a tube-like polar filament which might seem solid, however, from compression or, due to electron-dense material in the lumen (Hudo & Daniel, 1963). Akao (1969) concurs with the latter view and conceives that the substance is driven out of the filament at extrusion, followed by the sporoplasm. Much earlier, Ohshima (1937) had concluded that the sporoplasm of Nosema bombycis passed from the spore case through the hollow evaginated filament. In this study, electron micrographs of Thelohania show a tubular polar filament in one form and an apparently solid fibrillar core to the filament in another, resembling findings by Huger (1960), Ishihara (1967) and Sprague & Vernick (1968a). The solid formation may represent contents within a tubular filament, as interpreted also by Lom & Vavra (1963b) and Akao (1969).

The swelling of the anterior part of the polar filament to form two arms, anchor-shaped in section or a mushroom head in reality, is another source of controversy. Sprague & Vernick (1969) described it as the polar sac containing the polar cap of Lom & Corliss (1967), and attributed to it a PAS positive reaction. In agreement with Vavra (1968) I think, however, that the PAS positive material is located in the spore wall and, also, that the

mushroom head acts as an attachment or anchorage of the filament to the sporoplasm. It could conceivably also be concerned in rupture of the anterior weaker part of the spore wall, prior to extrusion of the filament and emergence of sporoplasm. A point of morphological interest from the present work is that there is a narrow band of sporoplasm separating the polar filament base from the spore wall. This band of sporoplasm which I have termed a sporoplastic cap. Sprague & Vernick (1969) on the other hand suggested that a sheath to the filament, continuous with the polaroplast, was the essential part of the device for anchoring the polar filament to the sporoplasm. Erickson et al (1968), supported by an earlier postulation of Sprague & Vernick (1967), even claimed that their study produced strong evidence that the coiled filament of the spore terminated in a sac containing the "germ" of the spore, explaining this as a mass of material containing the genome of the spore.

It is not yet clear exactly, how the sporoplasm of the microsporidian spore emerges from the spore. The general belief so far is that it is expelled out through the tip of the extruded polar filament, and injected into the host tissue. Lom & Vavra (1963a) believed that the



sporoplasm passed out of the spore case through the hollow tubular polar filament and was injected into the host tissue. Sprague & Vernick (1968a) who supported this view, suggested that the sporoplasm may not be capable of invading the host cell without mechanical aid and concluded that the polar filament played the role of an inoculating needle.

I have not been able to satisfy myself about these conflicting views from my electron micrographs. In this study, however, the extrusion of the polar filament of Thelephania spores by mechanical pressure, recalled the view held by Dissanaiké & Canning (1957) regarding the emergence of the sporoplasm, which showed as a globular refractile mass at the tip of the extruded filament. Kudo & Daniel (1963) described the dull appearance of the empty spore shell as compared with the refractility of the intact spore; they attributed the loss of high refractility of the spore to the eversion of the filament. In this

I found that spores that had extruded their filaments naturally, still showed a high refractility and there was no sporoplasm at the end of the everted filament (Pl. III, Figs 7 & 8). It appears that extrusion of the filament, at least by artificial mechanical pressure, need not lead to simultaneous discharge of the sporoplasm but

this may be so when filaments are extruded under natural stimuli.

The polaroplast, first described by Huger (1960) from electron micrographs, was earlier regarded as a "polar capsule" or a vacuole. The lamellae that constitute the basic structure may be tightly packed as in the spores of a Nosema (Stanier et al, 1968), or widely spaced as in my Melohania material. As in the case of the endoplasmic reticulum generally, the absence of ribosomes from the surface of the membranes may be an artefact due to use of potassium permanganate as a fixative. Lom & Vavra (1963b) regarded the polaroplast membranes as granular and also Sprague et al (1968) who suspected the occurrence of high concentrations of RNA in the polaroplast laminae. On the other hand, Vernick et al (1969) described the membranes of the polaroplast of a spore of Glugea as smooth and devoid of ribosomes.

Sprague (1965) reported DNA type staining by the polaroplast but later, Sprague et al (1968) failed to confirm DNA with the Feulgen test.

The polaroplast of Nosema nelsoni spore has a distinct outer zone of cisternae and an inner zone of vesicles like a Golgi complex, with a possible extension of the polaroplast to become associated with a posterior



vacuole (Sprague & Vernick, 1969). Akao (1969) also reported this posterior extension of the polaroplast. I have not noticed this posterior extension in the spore of Thelohania although the polaroplast primordia in the sporont of my material resemble certainly Golgi apparatus. This could be derivative from the endoplasmic reticulum as suggested by Sprague et al (1968). The polaroplast was always surrounded by a definite wall in my Thelohania material and was confined to the anterior third of the sporoplasm. A similar limiting wall, or membrane, is reported only by Sprague et al (1968).

Lom & Vavra (1963a and 1963b) attributed to the polaroplast a capability of enormous swelling, by imbibing water, and subsequent supply of energy and pressure as high as 60 atmospheres for the extrusion of the polar filament. The extrusion of the filament, they claimed began at certain levels of this pressure. Richness in RNA attributed to the polaroplast by Sprague et al (1968) indicates that it is a centre of high metabolic activity.

I failed to see a posterior vacuole in the spore of Thelohania as reported by Lom & Vavra (1963b), Sprague & Vernick (1969), and Akao (1969). However, Erickson et al (1967) could not decide whether this posterior structure was really a vacuole, and they together with Sprague &

Vernick (1968a), supposed it might be a nuclear vesicle.

The position of the posterior vacuole corresponds with the region the polar filament coils; this may well give rise to the vacuole-like appearance. This region of coils is, moreover, less refractile than the rest of the spore. The refractility of the spore may not be due to the polar filament as mentioned earlier for Kudo & Daniel (1963), but to the sporoplasm itself.

A binucleate condition, from electron micrographs, is typical for the spore of Nosema Locustae (Huger, 1960) and Ishihara (1968) reported, also from electron micrographs, not only two but three nuclei in spores of Nosema bombycis. Dissanaïke & Canning (1957), by light microscopy observations, described uni- or bi-nucleate spores in N. Locustae. Indications of binucleation were seen in smear preparations of sporoblasts and spores of the present Thelohania material (e.g. Pl. VI, Fig. 5) and in electron micrographs (EMG. 7).

Under light microscopy, metachromatic granules may simulate an extra nucleus (Dissanaïke, 1957, in Nosema helminthorum; Petri, 1969 in N. cuniculi). Double nucleation might also be a result of failure in autogamy of a binucleate sporoblast or be the diplocaryon of Vavra (1965). In ultrathin sections of spores, a section may



cut through two arms of a curved nucleus, or of a dumbbell-shaped nucleus or parts of an annular nucleus, as described by Dissanaiké & Canning (1957) in N. locustae. Binucleation would be, then, an artefact.

Elucidation of binucleation would require study of serial ultrathin sections.

Finally, it was noted by Hudo & Daniel (1963) and Sprague (1965) that the internal structure of microsporidian spores differs much among different species of the micro-organism. Yet in this study and by comparison with all other previous electron microscope studies, it appears that, basically the main structure of the spore is remarkably similar in all microsporidia, with only minor differences in details. One striking difference, however, is the occurrence of tubular processes on the sporoblast and spore walls of Plistophora form "B" of this study, a feature also reported by Vavra (1965) for Plistophora debaisieuxi from Simulium larvae, and not so far seen in any other species of microsporidia.

No mitochondria were seen in the electron micrographs of the microsporidian spores in the present work.

## 2- Other microsporidia-like bodies:

Spores found within the gut epithelial cells of Simulium larvae showed affinity to both microsporidia and

haplosporidia. In their fixed-stained condition some showed the posterior vacuole-like zone characterising microsporidian spores. If they were microsporidian, then by virtue of lack of grouping of spores they should belong to the genus Nosema. On the other hand, one form showed refractile granules in association or grouped around the nucleus of the spore, recalling a description of a haplosporidian spore by Sprague (1940) from the Malpighian tubules of a cockroach. My material resembles that claimed by Maurand (1967) to be an intracellular stage of Thelohania bracteata and/or Plistophora simulii. If Maurand's assumption is true, then one might expect these organisms to be found in simulium larvae from the River Lea as well, where there was a much higher representation of microsporidia. But they were found only in larvae from the Tilling Bourne.

### 3- Gregarines:

Survey of gregarines in the Simulium ornatum larvae was difficult. There was no correlation between the rare live gregarine trophozoites I could recover from larval guts, by dissection, and what seemed to be a common occurrence of trophozoites in sections of the larvae,



confusion arising from gut cells, and their degenerate nuclei, shed at periodic larval moultings, resembling gregarines and their nuclei. Harry (1965) also recognised this process of mid-gut epithelial cell breakdown as a source of misinterpretation of such cells as stages in Eugregarine development in Schistocerea gregaria.

This degeneration in my Simulium larvae was frequently evident as pedunculate gut cells, particularly suggestive of attached gregarines, at the posterior part of the mid-gut. Misleading degenerate epithelial cells were also seen in lumens of the gastric caeca and Malpighian tubules, although only at certain times of a year.

If cyclical changes in the gut epithelium of moulting Simulium larvae were better understood, this source of confusion in the gregarine recognition would be less difficult to overcome. The only report on the physiology of the Simulium larval gut is by Pacand (1950), who deals only with the localization and functional significance of glycogenesis in the gut epithelium of fourth-instar larvae of Simulium costatum. More precise study of the nature of gut epithelial-cell degeneration and its replacement in Simulium larvae during moulting cycles is needed. There would be need also to determine the origin and nature of apparently degenerative epithelial cells in the lumen of

Malpighian tubules, which may also create difficulty in the recognition of gregarine parasites.

The majority of acephaline gregarines (Ray, 1933, Ganapati & Tate, 1949) as well as some cephalines (Maschef & Noshdy, 1966), parasitic in insect guts, possess early intracellular stages of development. I have not observed other than the microsporidia-like spores I have previously mentioned, any intracellular stage or inclusion body in the larval gut tissue of Simulium ornatum which seemed to be gregarine in form. In this connexion, it is of interest that, apart from the immature gametocytes of a gregarine reported by Garnham & Lewis (1959) from a female Simulium adult, no other record is known in the literature of gregarine parasites in Simuliidae. The several indubitable gregarine organisms observed in the present study of Simulium larvae, are interesting additions to knowledge of gregarine parasitism in Simuliidae.

### Fungal Infections

#### 1- Chytrids:

It appears that organisms described from Simulium by Strickland (1913), Debaisieux (1920) and Noller (1920) are identical and that my chytrid material is similar and is justifiably placed, as I have done, as a chytrid



fungus of the genus Coelomyxidium. I am hesitant to be more specific although my material fits well with Coelomyxidium simulii Debaisieux. The genus is, otherwise, represented as an insect parasite only by C. ephemeræ (Weiser, 1947b). Admittedly, chytrid classification is very difficult. Debaisieux (loc.cit) recognised, for example, that C. simulii had developmental stages resembling those of another chytrid species, Olpidium viciae, an intracellular parasite of plants. More complicating, workers have not always been clear in their differentiation of fungal chytrids from protozoa Haplosporidia. Thus, a chytrid parasite, Coelosporidium periplanetae of the Malpighian tubules of cockroaches was first designated as a microsporidian, Nosema sp. (Schaudinn, 1902), later as a haplosporidian (Sprague, 1940) and referred more recently (Woolever, 1966) to species as the haplosporidian Nephridiophaga blattellae. This last work noted that both a haplosporidian and a microsporidian might possibly inhabit the Malpighian tubules of cockroaches, giving a mixed infection, a concept that did little to clarify confusion between the two Orders. Ganapati et al (1964) described a Coelosporidium from the body cavity of a marine copepod which resembled Coelomyxidium in its development and to this extent these two genera appear to

have close affinities. Weiser (1965a) proposed assigning the genera Coelomyxidium and Coelosporidium, together with some other 12 genera, to the fungal Order Chytriales on the basis that "Chytrids in most cases dissolve the tissue of the host body before sporogony. This effect is not present in any protozoan infection. Protozoa are intracellular or epicellular; Chytrids grow in haemolymph". This viewpoint not only enables me to disregard a haplosporidian identification for my material but also eliminates the intracellular chytrid Olpidium as a satisfactory identification. The Coelomyxidium which I have studied is certainly extracellular at certain stages.

Diagnostic staining for fungi requires comment in relation to this question of recognition of problematical organisms. The periodic acid Schiff's test is said to be diagnostic for fungi, but the protozoan Toxoplasma gives a positive reaction by this test. The positive reaction given by fungi in the chromic acid-methenamine silver test is equivalent to that of the PAS, yet the anterior tip of the protozoan microsporidian spore, particularly that of the genus Nosema was PAS positive (Sprague, 1965) and I observed a slight positive reaction to this silver stain in my related Thelohania spores. These observations cast doubt on the usefulness of these two stains as selectively



staining for fungal bodies as opposed to protozoan organisms. Even Gridley's (1953) could not be regarded as conclusive for fungi. What remains of value, for diagnostic differentiation between fungi & protozoa, in these two tests appears to be the intensity of the reaction produced by an organism, intense for fungi, slight for protozoa. This is not a very satisfactory criterion for organisms of uncertain status. A good example of this is Pneumocystis carini, a rare pathogen in human lungs, which is classified by some investigators as a fungus, but most regard it as a protozoan. Yet, it gives a strongly positive reaction, deemed indicative of fungi, with both the PAS and silver stains (Callerame & Nadel, 1966). Notwithstanding these problems about the validity of specific stain reactions, the chytrid sporangia (and the thali of the Trichomycetes) I have examined produced intense reddish-purple coloration in the PAS test and intense black coloration in the chromic acid-methenamine silver reaction, consistent with accepting these organisms as of fungal nature, rather than as protozoan organisms.

Shredding of the cytoplasm of the full-grown sporangium to resemble irregularly coiled hyphae within the sporangial membrane suggests also a fungal nature apart from the production of zoospores. More conclusive

of the fungal, and chytrid nature, is the formation of zoospores within the sporangium as a result of asexual reproduction, the absence of true mycelia and absence of a nuclear cap or side "body" (Bessey, 1950) in the posteriorly uniflagellate zoospore.

Having accepted a chytrid identification as valid, I turn to development. Debaisieux (1920) describes the young stages of summer forms of sporangia of Coelomyxidium simulii in Simulium larvae as markedly variable in size,  $3\mu$  to  $30\mu$ , with one or more nuclei; many were free in the haemocoel, others partially enclosed in the fat body cells. He concluded that the plasmodia (the term he gave to the sporangia) could move from one part of the host to another before finally producing zoospores. This description of "summer forms" agrees well with what I have described as form "A" of sporangia, which started their development intracellularly but completed their development extracellularly, and were then about in the general flow of the larval haemolymph. What Debaisieux (1920) described as a winter form of this same species, C. simulii is similar to what I have termed "thick-walled" sporangia, form "C". He suggested that the winter forms might have developed freely in the water during spring as during a transitional period from summer to winter, Simulium



larvae were found to harbour both types of sporangia. There was no mention, incidentally, by Debaisieux (1920) of the constant association between the thick-walled sporangia (or what he called winter forms) and Thelohania sp., as was evident in my observations.

Strickland (1913) and Debaisieux (1920) also observed, as I have, the zoospore of this chytrid, the latter noting a pear-shaped nucleus, a posterior accessory nucleus and a 20 $\mu$  long flagellum, associated with the pear-shaped nucleus, first rolled around the protoplasmic body and free later on. This description agrees fairly well with my material from Simulium ornatum larvae, except that what Debaisieux referred to as a nucleus I accept as a blepharoplast with its association with the flagellum; his accessory nucleus was the actual nucleus of the zoospore. According to Debaisieux, the zoospores were produced only by the "summer form" of sporangia and it was in my form "A" of the Chytrid that zoospore formation was also seen.

The life cycle of chytrids in Simulium larvae is still incomplete. Growth of sporangia is completed within a single larva; zoospores are produced all at the same time. The mode of intercellular spread of the infection

is not clear; the uninucleate young sporangia reported by Debaisieux (1920), as responsible for this, were not seen in the present study. Their mode of transmission in Simulium populations has not been worked out. The characteristics of the zoospores suggest a function as the transmission stage but I was unable to show this to be so.

## 2- Trichomycetes:

These endocommensals of insect guts constitute a heterogenous group of widely distributed fungi. The Trichomycetes which I found in the hind-gut of larval Simulium ornatum, appear to fall into the Order Harpellales Family Genistellaceae. They resemble, moreover, Smittium simulii (Lichtwardt, 1964), endocommensal of N. American Simulium larvae, but a noteworthy difference was the type of hyphal branching. The parasites of the Simulium ornatum larvae show alternate divergent branching, whereas Smittium simulii as described by Lichtwardt (1964) has opposite non-divergent branching. The number of conidial appendages, a feature of importance in final classification, has not been ascertained for the Trichomycetes of the S. ornatum larvae under consideration in this study. In view of this, the genus Smittium is probably not applicable to my material.

Tuzet & Manier (1950) suggested that the morphology of the base or holdfast in the Family Genistellaceae



offered features for generic classification. According to my observations, the holdfast of my material of fairly constant shape, rather like the simple ball-like formation specified by Tuzet & Manier, as one type of holdfast in contrast to other types composed of 2-3 short filaments or a forked foot in a mucus cup. The classification of these Trichomycetes is, however, not less problematic than other groups of parasites I have found represented in parasitism of larval Simulium ornatum.

#### Nematode Parasitism:

The immature parasitic and non-parasitic stages of mermithid nematodes have few obvious taxonomic characters. A green colour of the cuticle was a striking external feature of the parasitic juvenile mermithids I encountered parasitizing Simulium ornatum larvae.

Phelps & DeFoliart (1964) reported that from the time of development of a thick cuticle, juvenile mermithids assume a colour characteristic for each genus. They went on to say that "observations of delicate blue-green nematodes in Simulium vittatum by Twinn (1939) and a huge cabbage-green snake-like creature emerging from an adult S. ornatum described by Swinton et al (1915) suggest that these authors may have been dealing with a species related to Gastromermis viridis". Earlier, Welch (1962)

claimed that Gastromermis viridis, a parasite of simuliid larvae was easily recognized by the green colour of the adult worm.

The characters common to the post-parasitic juvenile female I found in the larvae of Simulium ornatum and the post-parasitic juvenile of Gastromermis viridis (Welch, 1962) may be summarised as follows:

Average length range 11-25mm, average width at vulva 0.14-0.27mm, green-coloured cuticle, formation of a 70 $\mu$  - 140 $\mu$  long spine with a swollen base by the cuticle of the tail, projection of cuticle is retained posteriorly by the post-parasitic juvenile, mouth is slightly ventrally displaced, hypodermis is bluntly rounded posteriorly, trophosome nearly fills the whole body cavity of the juvenile and is bluntly rounded posteriorly, S-shaped vagina.

I conclude that the mermithids in my material were Gastromermis viridis. All S. ornatum larvae died after emergence of the parasitic nematode or, apparently from loss of fat body reserves, before escape of the worm.

In mosquitoes as well as in simuliids, the mermithid parasitic nematodes start their life cycle as pre-parasitic juveniles which hatched from the eggs laid by free-living adults. The juveniles may penetrate the



abdominal cuticle of their arthropod host, or through the gut route after ingestion, to settle and develop in the haemocoel of the larva. Petersen et al (1967) reported on the former way of entry by Agamomermis culicis in larval mosquitoes and on the persistence of the developing mermithid throughout the aquatic stages of the mosquito host, without affecting any of those stages, except in the case of heavy infections, i.e. more than 8-9 nematodes per first-instar larva. Otherwise, the mermithid is still present in the adult mosquito from which, later, it escapes by penetrating the abdominal cuticle. Earlier, Welch (1960) described a species, Hydromermis churchillensis and more recently Petersen et al (1968) described another species, Romanomermis sp., both completed their life cycles in the haemocoel of the larval mosquitoes. In those cases, the mermithid emerged at the larval stage causing death of the larval host, usually as a fourth-instar larva. This pattern of mermithid development is similar to that of the nematodes I found parasitizing Simulium ornatum larvae. Although the mode of first entry into the larval haemocoel was not established in this work, it is relevant that Welch (1962) described for Gastromermis viridis, entry of the host, by ingestion. Although development involving escape of juveniles from larval hosts occurs with mermithid



parasites of Simuliidae, Welch (1963b) maintained that, a majority of observations report in fact on parasitism of adult flies as well. Obviously, escape from adult flies could lead to development of the free-living adult mermithid and subsequent production of progeny. Welch comments that adult fly infections would promote good dispersal of the parasitic juvenile.

The absence of male parasitic juveniles in my Simulium ornatum larvae may have several explanations. They may have been overlooked because of their small size. Possibly, they are particularly rare - even the female juveniles were infrequently found in my samples. Poinar & Welch (1968) described a mermithid Filipjevimermis leipsandra parasite of Chrysomelid larvae which reproduced without males, and they reported the scarcity of normal males in that species.

Welch (1963b) advocates the concept of host-specificity for mermithid parasites of blackflies. On the basis of the numerous observations of Petersen et al (1967, 1968 and 1969) that mermithid parasites of mosquitoes have a wide host range in Aedine, Culicine and Anopheline mosquitoes, I attempted infecting Simulium ornatum larvae with a mosquito mermithid, Romanomermis sp., kindly supplied by Dr Reynolds of I.S.H. & T.M. I placed a number of newly hatched larval nematodes with about 10 first-instar

S. ornatum larvae in a small beaker filled with aerated stream water. No penetration of larval nematodes into the simulium larvae resulted, although the nematodes kept hovering around the Simulium larvae as if trying to pierce their cuticle. This continued until the larval nematodes died about 2 days later. None was ingested by the Simulium larvae. One can say that a mosquito-infecting mermitid did not readily penetrate the Simulium larvae; specificity was probably confined to mosquitoes as opposed to simuliids.

## C O N C L U S I O N S

Parasitism of Simulium ornatum larval populations of the Rivers Lea and Tilling Bourne may be regarded as of two distinct categories; pathogenic and non-pathogenic infections. Of the pathogenic infections, the most important group includes three genera of Microsporidia, Thelohania, Plistophora and Nosema, the members of which I have grouped, according to spore sizes and other structural features, into several forms (? species). In the Tilling Bourne, fewer forms of Microsporidia produced limited and less important infections than those in larval populations of the River Lea, a feature that is counterbalanced by higher incidence of the pathogenic fungus Coelomycidium as opposed to its complete absence in Simulium larvae of the River Lea. Both these microsporidian and fungal infections adversely affected the Simulium larvae, particularly causing loss of fat body and deterioration of silk glands. Deaths of larvae occurred. The mermithid nematode, showing affinities to Gastromermis viridis Welch was found only occasionally parasitizing the larval body cavity and although extracellular, it also, like microsporidia and Coelomycidium, caused disintegration of fat body and, with its constant movement, derangement of the larval internal organs and deaths of larvae.



A second and less important category of infections included endocommensals in the larval gut; protozoan gregarines in the lumen of the mid-gut and fungal trichomycetes in the rectum of the larva. Of interest is that the commonest infection was of these harmless fungal trichomycetes, which gave infections rate of 30% to nearly 100% of larvae positive; this correlation between non-pathogenicity and incidence may apply to gregarines but the difficulty of establishing that gregarines exist make this a matter of conjecture; it was certainly not obviously so.

It was observed for some infections that they showed seasonal cycles of abundance. Some, like microsporidia, fluctuated between low levels of incidence, others like fungi (both pathogenic and non-pathogenic) showed higher peaks of incidence. Infections of overwintering larvae were quite common as well as of summer larval populations. In general, certain infections could be found whenever the simulium population was currently in a larval phase.

It was noticeable that the Simulium ornatum larval populations of the Tilling Bourne were infected by a wider variety of plant and animal parasites. This may be related to definite ecological differences between larval habitats in the two rivers. Larvae collected from the Tilling

Bourne were found attached to vegetation trailing, or growing, in the water of the river, whereas larvae collected from the Lea were found attached to pebbles at the bottom of the river. The vegetation of the Tilling Bourne may provide a more favourable habitat for stages of the several parasites spent outside the Simulium larval host, ensuring continuity of more varieties of infections.

The lethal effects of microsporidia, Chytrid fungi, and mermithid worms on their Simulium larval hosts suggest that they may have applications for biological control of this group of biting flies. Clearly, natural infections of the kind studied in this work must be subject to some degree of larval reduction, partly from deaths directly due to loss of fat body or escape of organism by rupture of the larval integument but also, possibly, from larvae with sub-normal silk-glands failing to avoid being swept away to unsuitable sites, or stranded, during floods or other disturbances. However, the prospect of introducing such parasites in a planned programme of control, to supplement natural low levels of parasitization or to initiate infection in parasite-free water courses, must depend on an adequate understanding of the life cycle and biology of the parasites, and an ability to cultivate a parasite in bulk under controlled conditions preparatory to

using it in a logically organized control project. The present study shows that much remains obscure in these respects. The life-cycles are not understood in detail nor means of cultivating the organisms known and, in particular, transmission even using apparently infecting forms is by no means certain of success.

The present work contributes to a wider knowledge of Simulium parasites, at least of Simulium larvae, particularly for the British Isles and points to numerous unresolved problems not only in speciation, and at higher taxa levels, but concerning the transmission and ecology of the parasites which were discovered.



## A P P E N D I X

Simulium colonization

To study different aspects of endoparasites most commonly taken in the present work on Simulium ornatum, a "clean" laboratory-reared colony of the flies, particularly of larvae, was desirable. Breeding of Simulium ornatum in continuous laboratory colony is still, however, an unsolved problem; one still has to rely on material collected from the wild. Blood-feeding and successful copulation by adults was essential to establishing a colony. The adult flies fed satisfactorily on glucose solution. A short account is given here of the unrewarding attempts to induce the S. ornatum to take blood-meals and to obtain successful copulation of these flies.

McMahon (1968) using apparatus set up in constant temperature of approximately 21°C and a relative humidity of 65%, providing ranges of colour intensities through different colour filters, was able to induce adult S. ornatum to blood-feed through a membrane at a reasonable rate. He obtained a maximum of 48.5% fed by using a yellow filter. Wenk (1966) working with Simulium erythrocephalum was able to induce a high percentage of

flies to take blood from human hosts and rabbit ears, without intricate techniques. In this study, I have used guinea-pigs, suckling rats, mice, as well as chicken membranes containing guinea-pig blood and using the basic McMahon's apparatus, to induce the S. ornatum adults of different ages to blood-feed, with little success.

Although blood-feeding was discouragingly difficult with S. ornatum, I also investigated the problem of mating the flies, no less essential to colonization than blood-meals. Artificial insemination of insects was developed in studies with mosquitoes by McDaniel et al (1957) and Baker et al (1962) who were able to produce remarkable results in both Aedine and Anopheline mosquitoes. In a very brief report, Field et al (1967) claimed to have succeeded in inducing copulation of adult Simulium vittatum, but none of the mated females survived long enough to oviposit. They have not confirmed, however, presence of sperms in spermatheca of supposedly mated flies, which makes their claim for success unfounded. Wenk (1965) illustrated the right position of a mating couple of S. erythrocephalum and he described a swarming dance prior to mating. I made many trials by the technique of Baker et al (1962) to induce adult S. ornatum to copulate, without appreciable success. A preliminary part of this aspect of the work was to examine detailed structure of the male



and female genitalia of S. ornatum of my collections. Earlier studies of the structure and function of the different parts of the male terminalia of simuliids were done solely for taxonomical purposes, as it was found that those structures gave reliable specific characters. Lundstrom (1911) has first described the male terminalia of simuliidae and then they were briefly redescribed by Edwards (1915). Although Gibbins (1935) gave a detailed and well illustrated description of those structures with particular reference to Simulium nili, Freeman (1950) produced the best study by describing the male terminalia of 3 species of Simulium and by clarifying the terminology, previously very much confused. For my own investigations, dead and dried adult Simulium ornatum were collected from the cages into which they had emerged from wild-caught larvae and were hydrated by being passed into graded alcohols down to distilled water, followed by boiling in 10% potassium hydroxide for about 15 minutes, rinsing in tap water and passed up again through graded alcohols to absolute alcohol. Then they were either embedded for sectioning, or cleared in xylene to be mounted whole, mounting the 8th and 9th segments with the attached terminal appendages, or the terminal appendages only. The aim was to interpret the structure of the chitinous parts of the



external terminalia.

The adults embedded in wax were sectioned at 12-20 $\mu$  and stained using Bertram's (1939) modification of "Bethe's stain". The modification consisted of using saturated solutions of the reagents, which were allowed to act for long periods. The stain should have become green to blue at the chitinous parts, in tap water, but none of the sections were colourised in any of five attempts.

In addition to whole mounts of dried specimens, female genitalia were also examined by sectioning at 6  $\mu$ , stained with either Giemsa or Mayer's haemalum counter-stained with eosin.

(a)- Male terminalia (Pls: app. I & app. II)

The external genitalia of both sexes are situated beneath the tip of the 9th segment. In the male simuliids, the shape of the ventral plate (V) is the main character used in interspecific differentiation. Before the terminology of Freeman (1950), the common name given to this structure was the "anterior part of the phallosome", the "posterior part of the phallosome" being the parameral hooks (PH). Freeman (1950) suggested that the function of the ventral plate was merely for support and the parameral hooks for retaining the grip of the male on the female, by fixing the hooks into the wall of the female genital duct. Each of the two parameres carries

a cone-like structure armed with chitinous hooks and spines with their tips almost meeting at the apex.

In Simulium ornatum, the ventral plate (V) is typically shield-like, covered with dense hairs and resting between the two coxites (Cx), posteriorly supported by a pair of tong-like highly chitinized arms (Vs) attached to the dorsal side of the coxites. Anteriorly, it has a small pair of arms forking from the junction of the posterior arms to meet the bases of the two paramers (P) at the dorsal part of the inner margin of the coxites (Ps). These supporting arms of the ventral plate were termed "apodemes" by Gibbins (1935).

The coxites (Cx) are large and meet the mid-line basally and having the styles (St) articulating with them, they all form a pair of claspers. The style which carries one or more apical spines (Sp) varies slightly in shape in different species of Simulium, thus offering two possible features for specific classification. That of Simulium ornatum, which is terminated laterally by two small apical spines (Sp) of differing sizes, is fairly long and slightly curved inwards.

The triangular 9th segment (9) carries all terminal appendages ventrally, holding them in a vertical position. Whereas, the 10th sternite and 10th tergite (10s & 10t) are reduced to small membranous structures with the cerus



(C) situated between them; they all lie immediately posterior to the terminalia.

b)- Female genitalia (Pl: app. I, Figs 4 & 5, Pl: app. II, Fig. 7)

A striking feature is the presence of a single highly chitinized light brown spermatheca in the posterior part of the abdomen. This has a spherical shape, but might get compressed to look ovoid, in sections. It has an S-shaped slightly chitinized duct which opens exteriorly at the genital opening (GO), between the bifurcation of the genital fork (GF). The cerci (10) are large and rounded distally with slight marginal chitinization and dense short setae. Lying ventral to the cerci, are the two moderately chitinous lobes of the paraproct (Pp). These are also large with sparse long setae. The gonopophyses is long and tapering to a spatulate, the processes of the two sides lie one above the other (Gp).

c)- Mating and artificial insemination

Efforts to obtain copulation and insemination were repeatedly tried by caging the sexes together, and by artificial insemination for adult Simulium ornatum summer generation. Flies from period 8th June - 20th August, 1967.

i- Cage mating: Flies emerged from weekly larval collections were placed in 1-litre beakers; each



beaker had its floor covered with a 2-3cm thick layer of slightly wetted cellulose, overlaid with filter paper and fitted with a platform cut from a card to prevent the flies injected into the beaker from dropping directly onto the wet base to which the wings might stick, and also acting as a resting place for the flies. A small petri-dish containing wicks of blotting paper, impregnated with glucose solution and placed inside the beaker. The beakers, covered with fine netting were placed inside a "Fison" climatic cabinet running at alternating equal day and night cycles of temperatures of 15°C (day) and 10°C (night) and a relative humidity of 70% (day) and 90% (night).

The females were either dissected at the following intervals: 2, 3, 6, 7, 8, 9, 10, 11, and 14 days and had their receptacula seminis examined for evidence of sperms, using Wenk's (1965a) technique, or they were longitudinally sectioned at the same intervals to observe any signs of a mating plug in their atria. A number of descriptions of mating plugs formed in newly mated female mosquitoes are available; the most recent is by Giglioli et al (1966). In both cases there was no positive result for presence of sperms in spermathecae or mating plugs in the atria of female adult flies.

ii- Artificial insemination (after McDaniel et al (1957) & Baker et al (1962)): Males and females emerged from wild-caught larvae were separated, and each sex was placed in a beaker by itself and stored in the climatic cabinet.

At certain intervals, several males were removed and, after being anaesthetized with ether, half of them were decapitated, and in addition to the other half glued by the dorsal side of the thorax on small drops of "Durofix" arranged over the rim of a small 3" petri-dish. Then by turn, they were presented with lightly anaesthetized females held by the thorax with a vacuum micro-pipette, till a responsive male was found. About 70% of decapitated males were responsive, raising their claspers at right angles to the abdomen, causing the parameral hooks to be levered upwards in a vertical position, thus enabling them to be inserted in the genital opening of the female prior to the introduction of the cerus. In spite of repeated attempts with long exposures, nearly all the females showed no response to this act.

Half of the females were fed on glucose solution prior to attempting to induce copulation; the other half was kept without feeds for 24 hours before the experiments. Different combinations of ages of males and females were

used and, altogether, attempts were made with 660 females and 243 males. But on only 3 occasions did copulation appear to take place. The spermathecae of these three females were examined, using Wenk's (1965a) technique. In only one female, the spermathecae showed the "cotton-wool" appearance of sperms, as described by Wenk for inseminated female simuliids.

There is the possibility that blood-feeding in the laboratory is not only essential for development of eggs, but necessary to stimulate the female Simulium adult to accept the male in copulation. Since I had virtually no success in blood-feeding, this factor was not pursued. It remains to say that laboratory rearing of Simulium ornatum is still an unsolved problem, and that, as in this study for experimental infection of this insect with its endoparasites, resort has to be made to material collected in the field.



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