A STUDY OF SPOROZOAN PARASITES OF STORED PRODUCTS COLEOPTERA

By

R.W. ASHFORD, B.Sc.(Lond.), A.R.C.S.

A thesis submitted for the Degree of Doctor of Philosopy in the Faculty of Science of the University of London.

Dept. of Zoology and Applied Entomology,

Imperial College Field Station,

Ashurst Lodge,

Sunninghill,

Ascot, Berkshire.
FEBRUARY, 1967

ABSTRACT

A new species of neogregarine protozoan, Lymphotropha tribolii
Ashford(1965) is described from the haemocoele of the red flour beetle,
Tribolium castaneum (Herbst). The trophozoites, which live extracellularly
in the haemolymph are large, and have longitudinal striae. There is only
one type of schizogony, in which about eight merozoites are produced, and
this is rarely seen. Gametogony produces 32 gametes, but instead of the
expected 16, an average of 9 or 10 lemon shaped spores (oocysts) are
formed.

Young larvae only of T. castaneum were susceptible, and a dose of 10^5
spores per gm. of culture medium caused 50% mortality. Larvae of four
other species of Tribolium were also susceptible, but T.confusum proved
refractive to infection.

The parasites produced a pronounced host reaction in the form of
encapsulation. However, as only encysted gametocytes were affected,
little benefit could be had by the insect.

The parasites had remarkably little effect on the physiology of the
host; no gross changes could be discerned in larval oxygen uptake,
susceptibility to D.D.T., or activity. The disease caused severe
reduction of the food reserves in the fat body, and slowed the rate of
larval gain in weight.

Death was thought to be caused by the physical obstruction of the
host's system, and by a form of tissue starvation.

Adults, which were not susceptible, sometimes survived larval
infection. About 25% of the survivors had a decreased longevity and female
oviposition rate, but the remainder were apparently unaffected.

The taxonomy of Farinocystis tribolii Weiser, and Triboliocystis
garnhami Dissanaike has been investigated, and it is concluded that
only one species is involved, which must be called Farinocystis tribolii
but that the original description requires considerable amplification.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

INTRODUCTION

Insect pests regularly cause heavy losses in stored produce throughout the world. Control of pest outbreaks usually depends on preventive measures such as the cleanliness of warehouses, and the rapid turnover of produce. Insecticidal methods of control depend mainly on fumigation, as solid or liquid insecticides tend to leave undesirable traces. Whatever preventive or control methods are used, they are expensive and require skilled operation.

Stored products insects, particularly the beetles of the genus Tribolium are well suited to mass laboratory rearing, and are commonly cultured for use in studies on such basic subjects as genetics and population dynamics, as well as for insecticide assays.

The importance of pathogens in these insects is thus two-fold. First, they may constitute a potential biological accessory method for the control of pest outbreaks, and secondly, laboratory cultures must be kept disease-free or misleading experimental results may be obtained.

The widespread rearing of stored products pests for laboratory purposes had led to the discovery of many pathogens which might have passed unnoticed in nature.

In 1953, a neogregarine pathogen of Tribolium castaneum (Herbst) was discovered in cultures in the Laboratory of Insect Pathology, Prague. This parasite, Farinocystis tribolii Weiser, was subsequently found destroying cultures in many other insectaries, and also in wild populations of T.castaneum. A rather similar protozoan was described from the same

host species by Dissanaike (1955) as _Triboliocystis garnhami._

The original purpose for the work presented in this thesis was to sort out the taxonomic position of these two organisms, and to investigate the nature of their pathogenicity in _Tribolium_ spp.

However, early attempts to produce infections were diverted by the discovery of a third, and new, neogregarine in the Imperial College cultures of _T.castaneum._ This protozoan, which was described as _Lymphotropha tribolii_ Ashford was easily cultured and after elucidating its life cycle, it was decided to investigate various aspects of its pathogenicity.

Infective material of _F.tribolii_ later became available for study, and it was possible to clarify some points in its life cycle. The conclusions drawn from the life cycles of the two species studied have general relevance to the taxonomy of the neogregarines, which is discussed at the end of this thesis.

INTRODUCTORY REVIEW

A fine review of the history of insect pathology is given by Steinhaus (1956a), in which the predominance of early observations on fungal and bacterial diseases is stressed. The first record of a protozoal insect disease probably came from Aristotle, who observed honey bees, Apis mellifera L., dying as a result of infection with what may have been Nosema apis Zander. Recognition of the importance of protozoa as insect disease agents came with Pasteur's work in the mid 1800's on pébrine, the disease caused by Nosema bombycis Nägli in silkworms, Bombyx mori L.

The first discovery of a pathogenic sporozoan infecting a stored products beetle came at the turn of the century when Léger, publishing a series of important works on the gregarine parasites of insects included the description of Ophryocystis mesnili from the larvae of the yellow meal worm, Tenebrio molitor L.

Since that time, stored products insects have been the subjects of intensive research. Cotton (1956) gives a review of the species of importance in the United States, and Hinton (1945) extensively reviews the beetles of 12 families occurring in stored products. The completion of this monograph would represent a milestone in stored products entomology. Munro (1966) has given a popular review of the most important stored products pests, in which he mentions 47 beetles belonging to 15 families. The most important families are the Dermestidae and Tenebrionidae, though species of major importance belong also to the Anobiidae, Ptinidae, Cleridae, Nitidulidae, Cucujidae, Mycetophagidae, Bruchidae and Anthribidae.

Good (1933, 1936) has summarised the biology of the flour beetles, Tribolium spp. (Tenebrionidae), and has shown how the preadaptation of T.confusum and T.castaneum has led to their becoming the most serious pests of stored grain products throughout the warmer parts of the world. Even in such a well known group of beetles, there has been very little work on their protozoan parasites.

The most widespread sporozoa associated with insects are the eugregarines, which have been reported as non-pathogenic parasites from most orders. Steinhaus (1949) found in a brief survey of the literature that 180 species of Coleoptera have been described as harbouring gregarines. This number compared with 67 from the Orthoptera, and smaller numbers from 13 other orders. It is probable that many more beetles are hosts to gregarines, but that only a small proportion of these have been described.

Foerster (1938a,b) made an extensive survey of the gregarine parasites of Silesian insects. Of the 24 species of stored products beetles which he investigated, he found 17 to be parasitised by 14 species of gregarine. The parasites belonged mainly to the large genus Gregarina, and to the genus Pyxinia.

The species of Gregarina infecting Tenebrionid beetles have been listed by Théodoridès (1955). Among the members of this family infesting stored products, Tribolium confusum harbours G.minuta Ishii and G.polymorpha Hammerschmidt; T.castaneum is infected by G.minuta, and Tenebrio molitor L. is host to G.steini Berndt, G.polymorpha, and

G.cuneata Stein.   Steineina ovalis Stein is a less common parasite
of this last beetle.   Ishii(1914) described three species of
eugregarine from Tribolium ferrugineum Fabr. (=T.castaneum (Herbst)
in addition to G.cuneata Stein.   Watson (1916) split one of his
species, and questioned the validity of the others.   Laird (1959)
pointed out that all the descriptions are incomplete, and that further
work is required before the species of eugregarine parasitising
Tribolium spp. can be enumerated.

Ghöre (1943), in a classic study of the gregarine parasites
of T.molitor found that each was distributed within the host's gut
according to the pH. of the region.   He also showed histochemical
differences between associating sporonts (gametocytes) of G.cuneata.

Sumner (1936) experimented on the rate of growth and longevity
of larvae of T.molitor infected with G.steini, and claimed that the
gregarines were essential for the normal growth of the larvae.   Her
observations were however made on very small numbers of insects in
poorly controlled conditions, and her conclusions are open to further
examination.

The species of the genus Pyxinia have been summarised by
Vincent (1922), who showed that all the 6 known species parasitise
stored products beetles (Dermestes spp. and Anobium paniceum (L.)).
P.crystalligera Frenzel has been extensively studied in Dermestes
vulpinus Fabr. by Kozloff (1958, Kozloff and Brown, 1963) who succeeded
in obtaining in vitro hatching of sporozoites both in media prepared
from the host gut and in preparations containing the enzyme trypsin.

Beetles infected with eugregarines may also contain neogregarines, as has been shown by Finlayson (1950) who found *Gregarina laemophloei* Foerster in stocks of *Laemophloeus minutus* Olivier infected with *Mattesia dispora* Naville, and Tyler (1962) who found eugregarines in *Tribolium confusum* infected with *Triboliocystis garnhami*.

The schizogregarine parasites of insects (Schizogregarina, Léger (1900) in part; Neogregarina, Grassé (1953)) are commonly pathogenic and therefore prospective candidates for use as biological control agents (Weiser 1962, 1963)

Apart from *Tenebrio molitor*, *Ophryocystis mesnili*, mentioned above, has been found by Steinhaus and Marsh (1962), and Lipa and Steinhaus (1962) also to infect *Trogoderma granarium* Everts, and four other beetles of the same genus. Canning (1964) described another neogregarine, *Mattesia trogodermae*, in *T.granarium*, and indicated its differential pathogenicity in different strains of the host. This parasite was not infective to the hosts of *Mattesia dispora*, which include two moths, and the beetles *Cryptolestes pusillus* (Schönherr) (=*Laemophloeus minutus*) and *C.ferrugineus* Stephen, but not *C.turcicus* Grouve, from the stored products medium (Finlayson, 1950).

Weiser (1953a) described the first neogregarine parasite from *Tribolium castaneum*, which he named *Farinocystis tribolii*, and which he found to decimate laboratory cultures of the beetle. The life cycle of this parasite was somewhat anomalous in that the gametocytes were plurinucleate on association, and apparently homologous with the macronuclear schizonts of many other neogregarines.

F.tribolii has subsequently been found in the U.S.A. by Steinhaus and Marsh (1962). Jafri (1961, 1964) found that T.castaneum adults exposed to infection with F.tribolii were hypersensitive to treatment with gamma radiation. He supposed that the parasite and the radiation acted synergistically to destroy the host's fat body.

Dissanaike (1955) described another neogregarine, Triboliocystis garnhami from the same host. T.garnhami was morphologically very similar to F.tribolii, but differed in that the second cycle of schizogony was complete, giving rise to uninucleate pregametocytic merozoites, and that 8, not 32 oocysts were produced in each gametocyst.

Tyler (1962) reported T.garnhami causing heavy mortality in English cultures of T.castaneum, and infecting 11 strains of T.castaneum, T.confusum, T.madens (Charpentier), and T.destructor Uyttenboogaart. He also found infected beetles in grain imported from China, Kenya and Jamaica. Laird (1959) and Stanley (1961, 1964) have repeatedly found T.garnhami in Canadian laboratory cultures of T.castaneum. In the last of the three works cited, a perfected method for cleaning eggs in order to remove any contaminating spores is described, involving washing the eggs in H Cl, and also in diastase. The treatment has little effect on the viability of the eggs, and allows the rearing of disease-free cultures of the beetles. Laird (1959) noting the disastrous effect of the parasite on laboratory cultures suggested its potential as a biological control agent.

In 1963, Weiser stated that T.garnhami was synonymous with F.tribolii, but did not amplify this statement. In the same article,

he noted, also without reference, that F.tribolii caused a tenfold
increase in the susceptibility of T.castaneum to D.D.T.

Confavreux (1961) attempted to differentiate between F.tribolii
and T.garnhami, but was unsuccessful as the large T.castaneum larvae
used proved resistant to infection, and her infective materials were
mixed.   She did, however, establish these mixed infections in the
following stored products beetles: T.castaneum, T.confusum, T.madens
T.anaphae Hinton, T.destructor, Latheticus oryzae Waterh., Palorus
ratzeburgi (Wissm.), P.subdepressus Wollaston, Gnathocerus cornutus
(Fab.) and G. maxillosus (Fab.).

Among the coccidia, the genus Adelina contains most of the
monoxenous insect parasites. Bhatia (1937) described A.tribolii, a
highly pathogenic parasite of the larvae of T.confusum.   Park (1948)
in his important work on competition between T.castaneum and T.confusum,
found that in the presence of A.tribolii, the former beetle was at a
considerable disadvantage, owing to its higher susceptibility to the
disease.   Adelina tenebrioides Sautet is a parasite of Tenebrio molitor.

Regarding other coccidal parasites of stored products insects,
Weiser (1963) states – "Another group of Coccidia infect insects living
in plant debris, detritus and mud...... This group is closely
associated with stored products insects, but our present knowledge of
all these organisms is not adequate for a profound evaluation or class-
ification of the many species.   There are undoubtedly many synonyms
among those which have been named, and there are other problems that
require experimental work with living specimens for their resolution".

The inclusion of the Microsporidia with the Sporozoa has recently been disputed (e.g. Grassé 1953, Honigberg et al. 1964, Levine 1961, 1966), and they are usually classified nowadays as an order in the separate sub-phylum Cnidospora.   Thompson (1960) has catalogued the known insect-parasitic species, but for the sake of completeness mention will be made here of the two species described as infecting Tribolium spp.   Weiser (1953) and Dissanaike (1955) each accompanied their neogregarine descriptions with records of spores of microsporidians which they named Nosema whitei and N.buckleyi respectively.   These infections were not common, and life cycle studies were not attempted. Thompson (1960) rejected both names owing to the incompleteness of the descriptions.   West (1960) described the life cycle of a Nosema species from T.castaneum, but did not attempt to identify or name it.   Fisher and Sanborn (1962) found that a Nosema from T.castaneum also infected newly moulted larvae of Tenebrio molitor, Bombyx mori, and Galleria mellonella (L.).   Other insects could be infected from implants. They considered that the absence of a peritrophic membrane in T.castaneum was responsible for the high susceptibility of the beetle to the micro-sporidian.

The study of the systematics of the neogregarines has been assisted by the fact that in order to place an organism within the group with certainty, it is usually essential to know most of its life cycle.   In spite of this, the taxonomy of the group has been the subject of a number of conflicting reviews.   Since Léger and Duboscq (1908) divided the Schizogregarines into two tribes characterised by

the production of one (Monosporea) or more than one (Polysporea) spore,
Doflein and Reichenow (1953) Weiser (1955a,b) and Grassé (1953) have
produced important contributions to the subject.   Doflein and
Reichenow merely listed the known genera without attempting to relate
them phylogenetically.   While our present knowledge of the group is
insufficient to warrant any definitive classification, the works of
Weiser and Grassé are useful tentative systems which may form a basis
for discussion.

Weiser divides the suborder Schizogregarina into two families,
Caulleryellidae and Ophryocystidae, on the basis of the number of
distinguishable schizogonic cycles undergone in development.   He
divided each of the families into sub-families largely on the number
of oocysts (spores) produced in sporogony.

Grassé, in wider review of the gregarines as a whole,
divides the class Gregarinomorpha into three orders: the Archigregarina
which are parasites of marine invertebrates exhibiting primitive
schizogony; the Eugregarina, parasites mainly of annelids and arthropods,
in which schizogony has been lost, and the Neogregarina, parasites of
arthropods (mainly insects) in which a variety of secondary mechanisms
of schizogony have evolved.   While it is not absolutely clear how he
differentiates between the primitive schizogony of the archigregarines
and the secondary schizogony of the neogregarines, this classification
offers certain advantages, particularly as it separates the marine and
terrestrial schizogregarines, and as it implies the possible poly-
phylogeny of the neogregarines.   Grassé's system is the one accepted
at the supra-famial level by the Committee on Taxonomic Problems of
The Society of Protozoologists (Honigberg et al., 1964).   Largely on

the basis of the morphology of the trophozoite and spores, as well as on the habitat of the parasites within the host, Grassé divides the neogregarines into five families.

Joyet-Lavergne, (1926a), Ghöre (1943) and others have reported histochemical differences between gametocytes of eugregarines, associated with differences in redox potential, and regarded as indicating a sort of cytoplasmic sexual differentiation.   Such observations have not been repeated in the neogregarines, where sexual differentiation is only known in Schizocystis, which is anisogamous, and perhaps in Farinocystis, where one gametocyte encloses the other.

Other observations on the histochemistry of the gregarines have only rarely been made.   The reserve food substance, paraglycogen is characteristic of the group (Bütschli, 1885) and is thought to be a stabilised polymerisation product of glycogen (Ghöre, 1943).   This substance reacts strongly with the periodic acid-Schiff technique, and Jennings (1962) found this reaction useful in the detection of parasites at low infective levels.

The Golgi apparatus of some gregarines has been described (e.g. by Joyet-Lavergne, 1926b, Tuzet 1931) as consisting of a variable number of small "dictyosomes".   Cytoplasmic granules of volutin (Reichenow, 1935), and globules of lipid material have been reported. Daniels (1938) used ultracentrifugation to separate the cytoplasmic components within the unbroken cell, and was able to distinguish fats, Golgi body, nucleus, mitochondria, paraglycogen and chromidia

(=volutin granules). Grassé (1953) has summarised the subject of the cytology of the gregarines.

Steinhaus (1947, 1949) shows that many insect diseases cause characteristic symptoms in their hosts. Fungi may sometimes be identified specifically by the external appearance of the dead host, and viral and bacterial diseases may commonly be identified as such by the liquefaction of the host tissues on death. Protozoal diseases, on the other hand cause less distinctive changes. Insects which are infected with microsporidia may show diagnostic external symptoms, but those which have died from neogregarine infections are not readily distinguishable from those which have died from other causes (e.g. Weiser 1953a, McLaughlin, 1965).

A comprehensive study of a protozoan insect disease was made by Huger (1960) who described the degeneration of the fat body of Agrotis segetum (Schiff) larvae infected with Nosema perezioides Huger. The histopathological effects of neogregarine diseases are usually mentioned in conjunction with descriptions of species. Authors such as Weiser (1953a) Dissanaike (1955), Canning (1964), show how only infected organs are visibly affected. Hypertrophy of the nucleus of infected cells has been observed with various species of Nosema, and eugregarines, but gross nuclear changes have not been reported from neogregarine infections.

In order to infect an insect, a parasite must overcome or
avoid the natural resistance of the host. Salt (1963a) has shown
that the most important resistive mechanism in insects appears to be
encapsulation, and has summarised the present knowledge of this
process in the case of metazoan parasites of insects. The haemocytes
of the affected insect agglomerate around the parasite under certain
conditions, and may either secrete an inner layer of impermeable
substance, or harden, and themselves isolate the parasite from the host
tissues. This process has been called "giant cell formation" by
Steinhaus (1947) and others, but "encapsulation" is the most commonly
accepted term. Encapsulation of microbial parasites has been summarised
by Stephens (1963). Huger (1960) described the process in the
infections mentioned above, and found that groups of parasites and even
heavily infected lobes of fat body were isolated in capsules. The
chemical nature of capsules in Pristophora parasitised by the hymenop-
teron, Mesoleius was found by Bronskill (1960) to be a form of muco-
polysaccharide.

Salt (1963b) has shown that a parasite artificially placed
in an unnatural host may stimulate encapsulation, though it is unaffected
by its normal host. He also showed that inert foreign bodies are
encapsulated by many insects. The implication drawn from these facts
is that some property of the surface of a parasite in its normal host
prevents it from stimulating encapsulation. This surface property,
which might be physical or chemical must be part of the delicate
balance between host and parasite, and is probably responsible for
many cases of host specificity.

Where this has been studied, most neogregarines appear to be capable of infecting a wide range of hosts. Examples found by Confavreux (1961) and Finlayson (1950) are mentioned above. However, Canning (1964) was able to reinforce her views on the specific nature of Mattesia trogodermae when she found Anagasta khūniella (Zeller), Plodia interpunctella (Hübner) and Cryptolestes pusillus, which are hosts of the related Mattesia dispora, to be refractive to infection. Thus, while host specificity is sometimes fairly strict, the discovery of a parasite in a new host does not imply a new species of parasite.

The problems involved in estimating dosage-mortality curves for insect diseases have been pointed out by Bucher and Morse (1963), who emphasised that the many variable factors involved in this type of assay compared with chemical insecticide tests make considerable replication necessary. Variation between assays on one strain of Pseudomonas aeruginosa (Schroeter) Migula in Melanoplus bivittatus (Say) was greater than that which would be expected between related strains. Fisher (1963) summarising the methods used in the assay of commercial preparations of bacterial insecticides again emphasised the inherent variation and need for replication.

Benz (1963) has reviewed present knowledge of the physiology of insect diseases. A good indication of physiological effects may be obtained by comparing the respiration rate of infected and uninfected insects. Sussuman (1952) found that Platysamia cecropia L. pupae infected with the facultative fungal parasite, Arpergillus flavus Link

had an enormously increased oxygen quotient (QO_2). Pupae which had been killed, and whose enzymes had been inactivated by heat did not show this high oxygen quotient when the fungus was grown on them. The increase in QO_2 was therefore interpreted as being due to the host and not the parasite.

Lysenko and Slama (1959) found that the respiration rate of diapausing pronymphs of the sawfly, Cephalcia abietis L. rose similarly when they were inoculated with suspensions of the bacterium, Serratia marcescens Bigio. The interval between inoculation and the onset of the rise in oxygen uptake was inversely related to the dose administered. The authors concluded that the prepatent period was inversely related to dose, and that this supports the theory that the development of pathogenicity in an infection depends on stresses such as the pressure of a heavy infection. No attempt was made to differentiate between the respiration of the host and that of the parasite.

Apart from Jafri (1961, 1964) mentioned above, various workers have studied the effect of disease on the susceptibility of insects to injurious stimuli. Steinhaus (1956b) stated that microbial and chemical insecticides are commonly compatible, and sometimes synergistic. Rosicky (1951) found that beetles, Otiorrhynchus ligustici L. infected with Nosema otiorrhynchi Weiser were approximately four times more susceptible to D.D.T. than healthy ones. The disease also caused acceleration of knock down and death.

Veber and Jasič (1961) found that sublethal doses of <u>Nosema bombycis</u>. a generalised parasite, infecting many organs including the ovaries, greatly reduced the fecundity of surviving females of <u>Bombyx mori</u>. The normal average egg production of 453 was reduced to 341 by an infection of 5×10^3 spores in 5th instar larvae, and to 209 after the same dose had been administered to 4th instar larvae. Similar results were obtained using <u>Hyphantrea cunea</u> Drury. They concluded that for the proper evaluation of the effect of insect infections, not only mortality, but also the general reduction of biological potency should be considered.

It will be seen from the above notes that many aspects of insect pathology have been studied, each in a few instances, but there have been few broad studies of specific host-parasite associations. It is to be hoped that the present work rectifies this situation to some extent.

MATERIALS AND METHODS

1. Origins of the Parasites and Beetles.

The T.castaneum cultures first used in this work came from the Imperial College Insectaries, and were found to be heavily infected with L.tribolii. Disease free insects were obtained from the Pest Infestation Laboratories, Slough (P.I.L.). The T.confusum cultures at Imperial College were infected with Adelina tribolii, a microsporidian (probably Nosema whitei), and a eugregarine (probably Gregarina polymorpha). Stocks free from pathogenic organisms, but still infected with the eugregarine were obtained from P.I.L., as were cultures of T.anaphae, T.madens and T.destructor. The presence of the eugregarine was not thought to affect experimental results, and most of the work was done on T.castaneum which was rarely parasitised by this organism.

Other infective material was supplied as follows : T.garnhami from old infective flour held at Imperial College, originating from the Cooper Technical Bureau; F.tribolii in dead adults and larvae of Tribolium sp. from the Laboratory of Insect Pathology, Prague; F.tribolii in groundnuts originating from Gambia, from which infected Tribolium adults had been removed; F.tribolii in dead adults and larvae of T.castaneum, preserved in salt, from the Department of Primary Industries, Brisbane, Australia.

Professor A.S. Dissanaike was kind enough to give me some of his original slides of T.garnhami, and Professor P.C.C. Garnham lent some similar slides from his personal collection.

2. Culture of the Beetles.

Good (1933, 1936) has summarised the biology of Tribolium spp., and Howe (1956, 1960, 1962) has determined the optimal conditions for development of T.castaneum and T.confusum. The conditions maintained in the present work were adapted from those recommended in these papers, and from Kane's (undated) instructions.

(a) Medium.

All the species of Tribolium used in this work were cultured satisfactorily in whole meal flour. The flour was first passed through a 50 mesh sieve to separate the bran, which was then ground to the same particle size as the flour in a Christy-Norris Junior centrifugal mill. After mixing, the bran and flour was sterilised overnight in a shallow tray at 110°C. The flour was not used for culturing until at least two weeks after this sterilisation, in order to allow its moisture content to equilibrate with the atmosphere.

Yeast was not added to the culture medium as the keeping properties of the flour would be reduced by this addition, and the beetles were found to live and reproduce satisfactorily in flour alone. Also, in natural infestations, the beetles live in unadulterated flour.

(b) Containers

Stock cultures were maintained in 2 lb. jars containing about $\frac{1}{2}$lb. of flour. A piece of 9 cm. filter paper was stood vertically for the beetles to crawl upon, and the jars were covered by a similar piece of paper sealed in position with vaseline. Many hundreds of insects could be maintained in each jar, but breeding was not successful under

these conditions owing to overcrowding and cannibalism.   Cannibalism
was reduced by keeping insects of uniform age together.

Smaller numbers of beetles for experimental purposes were
kept in 2 × 1 in. flat bottomed glass tubes with cork tops.

All cultures were kept in an incubator at 28°C, with no
humidity control.   All containers were sterilised overnight at 110°C.

(c) Procedure.

In order to obtain eggs, a large number of adults were ex-
tracted from a culture using a 20 mesh sieve, and placed in fresh flour.
After the requisite number of days, allowing about 5 eggs per adult per
day, and allowing for the fact that the first eggs hatched after 3 days,
the adults were removed in the 20 mesh sieve, the flour was passed through
a 50 mesh sieve to remove the eggs.

The eggs were not washed, but loose material was removed
by running them down a sloping piece of paper.

Larvae or pupae were isolated as required, by sieving.   Larvae
were cleaned by allowing them to crawl on newspaper, and blowing off
any debris not removed by the sieve.   Sieving was not observed to
damage eggs, larvae, pupae or adults.   Only first instar larvae passed
through the 50 mesh sieve, but these were rarely required, and could be
obtained in sufficient numbers by allowing them to crawl on newspaper,
and tipping off the surplus flour.

(d) Precautions.

In general, all equipment was sterilised; the working bench
was swabbed regularly with 90% alcohol, and covered with newspaper which

was rejected after use.   Although all cultures were kept in close proximity, the only accidental infections which occurred could be accounted for by lapses in the above procedure.

(e) Sexing.

Larvae could not be sexed.   Pupae were distinguished by examination of their sub-terminal abdominal sternitor, as described by Halstead (1963).   The sexing of pupae was easy and rapid, and could be carried out without risk of injury.   Adults were more difficult to separate, on the presence or absence of a hair-lined pit on the inferior face of the fore femur (Hinton, 1942).

(f) Timing.

As all the cultures were maintained at 28°C, in similar constant conditions, the life cycles were of almost constant duration. Eggs of T.castaneum hatched after 3-5 days.   The larval stage, which included an indefinite number of moults ended between 22 and 28 days after laying, and adults hatched after 33-40 days.   A small proportion of larvae took longer to mature, but adult eclosion rarely extended to beyond 45 days.

Other species of Tribolium were not cultured in large numbers : T.confusum developed in a slightly longer time than T.castaneum; T.destructor took about 3 months, and the other species developed in intermediate times.

3.   Culture of Parasites.

(a)   General.

Farinocystis and Lymphotropha were cultured similarly.

Infection takes place as the result of ingestion by larvae of spores
of the parasites.   Larvae which have died from one or other of the
diseases are packed with infected spores which remain viable for a long
time.

(b) Procedure.

Dead larvae were separated from the living, and from the culture
medium by a combination of sieving, blowing off unwanted larval skins
and debris, and manual separation.   The dead larvae were kept until
required, at room temperature.

New infections were established by lightly grinding dead
larvae and adding the powder to a culture in the required quantity.
A sample of the powder was first examined after soaking in a drop of water,
under negative phase contrast (×800) to confirm its purity.

(c) Standardisation of infective doses.

When accurately standardised powder was required, it was first
ground very finely, then a sample taken to be weighed and estimated.
This sample was ground further in 10% KOH using an emulsifier.   As the
spores tended to bunch together, this process had to be very thorough.
The suspension thus obtained was made up to a measured volume, and samples
of it were estimated in a haemocytometer.   The number of spores per
unit haemocytometer volume was extrapolated to show the number of spores
per unit weight of spore powder.   Spore powder usually contained in the
order of 10^8 spores per gram.

When less accurate standardisation was required, remembering
that the powder contained approximately 10^8 spores per gram, powder and

flour were mixed roughly, as required.

It was assumed in the experiments that the infective dose taken up by larvae was in some way proportional to the concentration of spores in the flour.

(d) Precautions.

All materials and equipment were thoroughly sterilised by dry heat before and after use.   Before any infection was established the spore powder was examined to confirm the absence of contaminating spores of unwanted organisms.

As no method was found for accurately measuring the proportion of viable spores in any sample, infective material was not used for standardised doses when more than two months old.   Unless otherwise stated, no spores were used after storage for more than a year.

(e) Mass production of infective material.

In order to produce the maximum quantity of infective material, either light infections were given, or larvae were not infected until they were about ten days old.   Thus larvae were large at the time of death, and produced a comparatively large amount of spore powder.   When eggs were allowed to hatch in highly infective flour, the resulting dead larvae were very small, and besides being difficult to isolate, produced comparatively little infective material.

4. Preparation of Sections.

(a) Fixation.

After trying a variety of histological fixatives, Heidenhain's Susa was found to produce the best results. The fixative was prepared according to Pantin (1946). Larvae were immersed in fixative, and their heads and terminal abdominal segments were then cut off. The tube containing the larvae was placed in a flask and the pressure reduced with a water jet pump until the fixative began to boil. The vacuum was then released slightly, and the flask was shaken to sink the larvae, which were left in these conditions for from 6 to 12 hours.

(b) Embedding and sectioning.

Larvae were embedded in paraffin wax or ceresin wax, with a melting point of 58°C. Three changes of one hour each were given in a vacuum embedding oven. Ceresin wax was found to produce the best sections, which were cut on a Reichert rotary microtome, mostly at 5μ.

(c) Staining.

Heidenhain's iron haematoxylin was used according to Pantin (1946). Slides were kept in iron alum mordant for at least 12 hours, and stained for a similar time. The Giemsa-colophonium method of Shortt and Cooper (1948) was used in other histological studies. Feulgen's reaction for D.N.A. was carried out using de Thomasi's method as described by Pearse (1960). The use of Susa fixative necessitated hydrolysis for 18 minutes in N.HCl, at 60°C. The results of this reaction on the parasites were very indistinct, but the insect nuclei reacted strongly, proving that the technique was in order. Positive

reactions in the parasites were confirmed using unhydrolysed control sections.

The periodic acid-Schiff technique (P.A.S.) was used in the manner described by Pearse (1960). This reaction was particularly useful in the detection of light infections of L.tribolii.

Mercury bromophenol blue was used to stain proteins according to Pearse (1960). This general protein stain was found to be rapid and efficient in the present circumstances.

Sudan black was used for determining fats. A saturated solution of the dye in 70% ethanol was prepared, and infected larvae were crushed under a cover slip in a drop of the solution. The temporary preparations obtained were satisfactory for the observation of the fat content of the parasites.

5.   Examination of Material.

Microscopic examinations were made using a X20 binocular dissecting microscope. Sections were examined using a Reichert Zetopan binocular microscope with magnification up to X800. This microscope could be fitted with a negative phase contrast (anoptral contrast) condenser and objective lenses, which were particularly useful in the examination of fresh smears.

All measurements were made using an eyepiece scale, and are recorded either as units on this scale, or converted to metric units by calibration with a stage micrometer slide.

Drawings were made on graph paper, using an eyepiece graticule.

All other techniques used in the work presented in this thesis are described under the relevant headings in the Results section.

RESULTS

PART 1 LYMPHOTROPHA TRIBOLII

A. THE PARASITE

1. Description of the Stages and Life-cycle of Lymphotropha tribolii

   a) Spore hatching (Figs. 1, 2)

The use of negative phase contrast greatly facilitated the observation of the emergence of sporozoites. Larvae aged about ten days were fed on pure spore powder, and were observed in whole squashes in 1% saline.

Preparations made only $3\frac{1}{2}$ hours after exposure to infection showed hatching sporozoites, and these were very numerous after 24 hours. Empty spores in the anterior half of the mid-gut indicated the zone of hatching.

The sporozoites were not distinguishable within the spores until at least one had emerged, but the emergence of the last two or three could be observed in detail. The sporozoites moved within the spores by bending slowly until one end was inserted in the already open micropyle. These movements seemed to be exploratory, and the sporozoites were apparently free to move all around the spore. A small clear projection was first pushed through the micropyle; this soon became opaque, and was followed by the rest of the sporozoite, which glided out quite rapidly. The whole process of emergence sometimes took as little as five seconds, but occasionally, a sporozoite would come partly, or even completely out, only to withdraw within the spore, and emerge later.

Fig. 1.

20μ
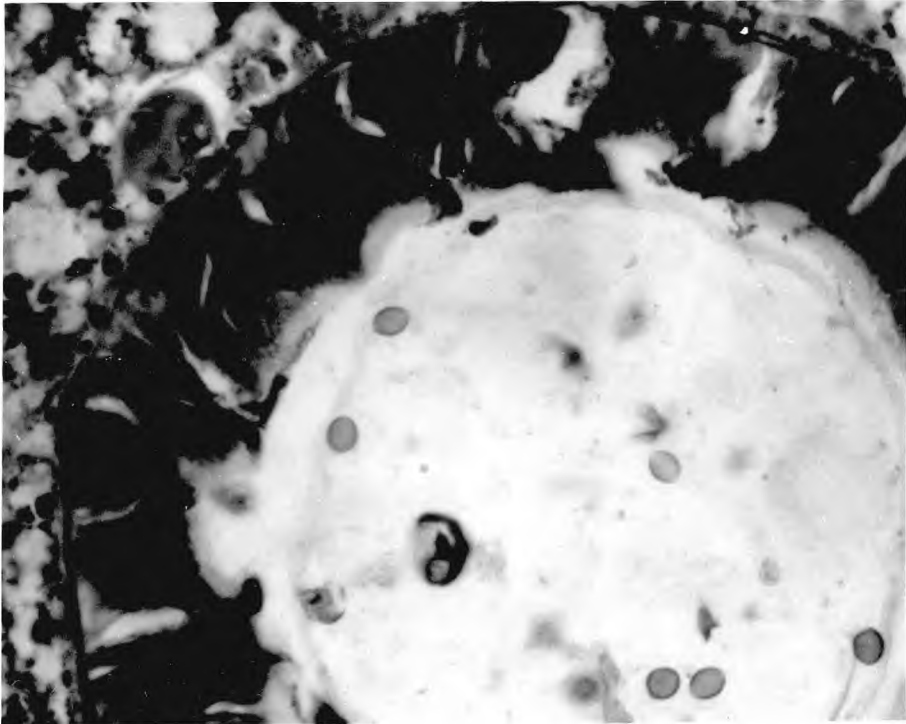
T.castaneum, t.s. mid-gut of young larva, with empty
spores of L.tribolii. Heidenhain's haematoxylin.

Once emerged, the sporozoite usually remained attached to the spore for a few moments, bending and rotating slowly about its fixed end, before breaking away and floating off.

No bending was observed during emergence, which seemed to be controlled by reversible peristaltic contractions. All the movements of the sporozoite except the actual emergence were very slow.

There was no observable difference between the ends of the sporozoite except in function. Both ends of the spore were used for emergence, and it was common to see one sporozoite coming out at each end simultaneously.

b) Description of the sporozoite (Figs. 3, 4, 29, 30).

Free sporozoites were never found in fixed preparations; the description is based on observations of free sporozoites in fresh smears, and on those within spores, in fixed and stained sections.

The sporozoites of the neogregarines show considerable uniformity, and those of L.tribolii are no exception. They are vermicular, tapering towards both ends, and measure 8μ in length by 1μ in central diameter. The nucleus occupies the entire cross section towards one end, and is slightly longer than broad. It stains darkly with Heidenhain's haematoxylin, and is coloured dark red with Giemsa's stain, while intranuclear granules are faintly positive to the Feulgen reaction. The cytoplasm, which is opaque in the fresh smears, but without visible granular inclusions, stains brightly with Heidenhain's haematoxylin, and more heavily, blue with Giemsa's stain. The cytoplasm and nucleus stain heavily with mercury-bromphenol blue, but no part of the sporozoite stains with P.A.S. or with Sudan black. The cytoplasm is therefore proteinaceous with no visible inclusions of polysaccharide or lipid material.

Explanation of Figs. 2-6.

L.tribolii, asexual stages, Fresh smears, Negative phase.

2. .Sporozoites emerging.

3. Young sporozoite.

4. Elongate sporozoite.

5. Sporozoite broadening.

6. Trophozoite with vacuolate cytoplasm and longitudinal
    striae.

2

3

4

5

6

5 μ
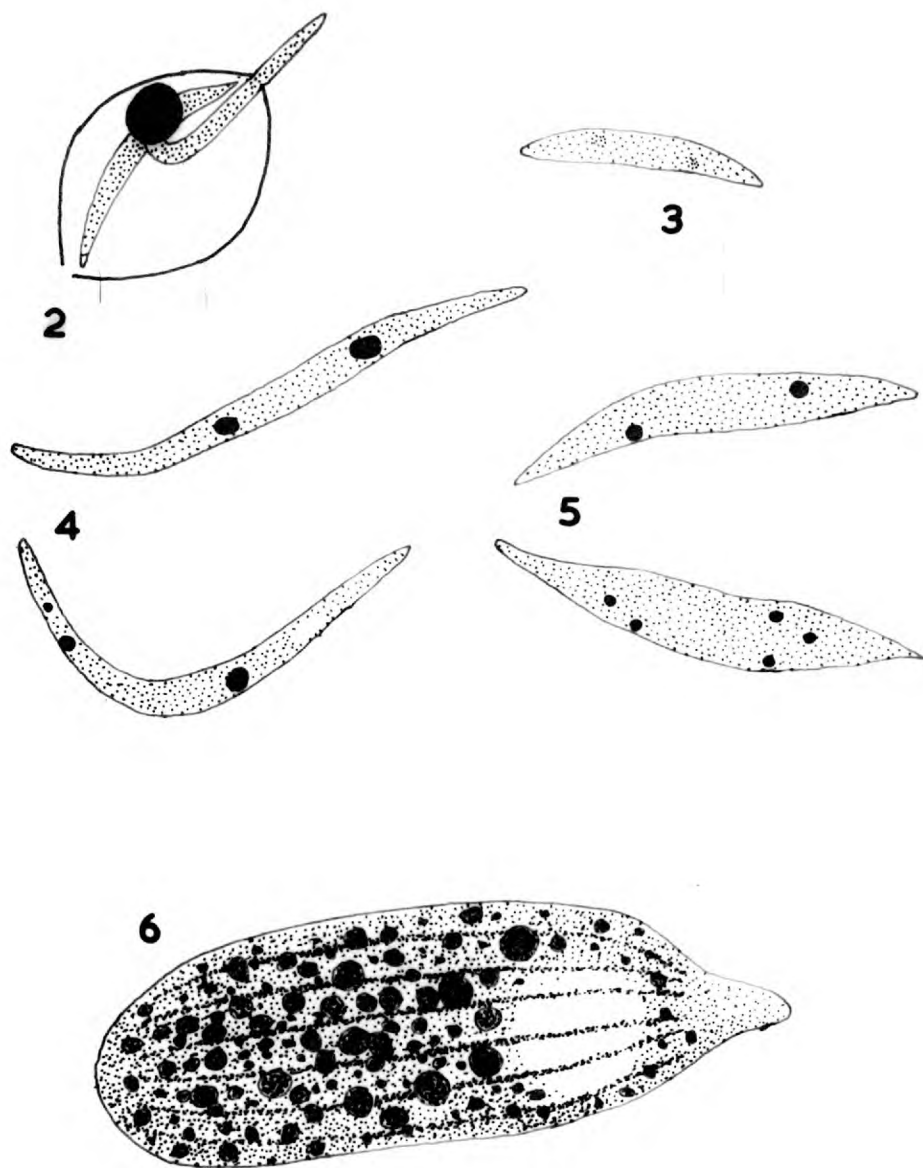
c) <u>Development of the trophozoite</u> (Figs. 5-13)

Penetration of the gut by the sporozoite was not observed, and no evidence was found that the Malpighian tubules are used in the route to the haemocoele, as suggested by Canning (1964) in the case of <u>Mattesia trogodermae</u> in <u>Trogoderma granaria</u>.   On reaching the haemocoele the sporozoite first elongates to about 15μ, without change in shape, and a few granules develop in the cytoplasm.   This elongation is later accompanied by lateral growth until the fully developed trophozoite measures up to 30μ by 15μ and is ovoid or spindle shaped.   The developmental stages of the trophozoite were only seen in unstained fresh smears.   One end of the fully developed trophozoite is more rounded than the other, which has a small clear projection or pseudomerite. The pseudomerite is usually visible in fresh preparations, but was rendered indistinguishable by fixation.   The cytoplasm of the trophozoite is almost entirely composed of granular and globular inclusions of varying refractivity and size, and stains very weakly with the histological stains used.   Mercury-bromphenol blue stains a fine proteinaceous network filling the interstices between the granules and globules. Proteins are therefore present in small quantities only, and restricted to these areas.   The majority of the granules stain intensely with   - P.A.S. and brown with Lugol's iodine, but not with Best's carmine, indicating their paraglycogen content.   The remaining inclusions in the cytoplasm are large randomly dispersed globules of lipid material which stain with Sudan Black.   All the trophozoites seen contained large quantities of paraglycogen, but the lipid globules were only present in the larger specimens.

Fig. 7.
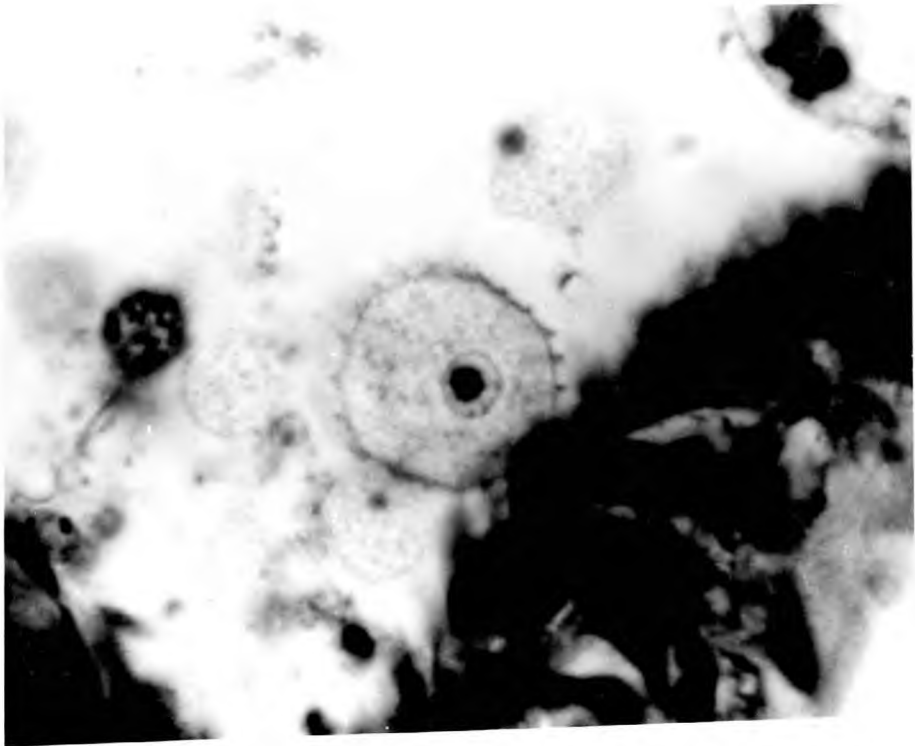
L.tribolii trophozoite showing striae in cross section.
Heidenhain's haematoxylin.

The surface of the trophozoite is marked by a number of long-itudinal striae. These are seen in fresh smears as faint, finely granular lines, and are visible in sections as equally faint lines of differential refractivity in the surface cytoplasm. None of the methods of preparation used stained the striae differentially.

The single nucleus is laterally central, and usually situated towards one end. It is large, 5μ diameter, with a clearly marked membrane on which a few small granules may be seen. The endosome occupies about half of the nuclear diameter, and may be spherical or connected by thin strands to the granules on the membrane. The endosome, granules and membrane stain heavily with Heidenhain's haematoxylin, and purple with Giemsa's stain. Mercury-bromphenol blue indicates proteins in the same areas. Feulgen's nuclear stain gave no visible reaction in the trophozoite nucleus.

Although no movement could be observed in the trophozoites, they become distributed throughout the haemolymph, and may even be found developing in the legs of the host. This distribution is probably effected by passive transportation in the haemolymph rather than by active migration of the parasites (see Fig.23).

d) Schizogony (Figs.14-17).

During the development of an infection, there is a considerable increase in the number of parasites, which cannot be accounted for by hyperinfection as the stages are well synchronised. However, schizogony was only rarely observed, which suggests that the division stages are passed through very rapidly. Schizonts are roughly rounded in shape,

Explanation of Figs. 8-16.

L.tribolii, asexual stages.

8. Trophozoite, Heidenhain's haematoxylin.

9. Trophozoite showing striae, side view, Heidenhain's haematoxylin.

10. Trophozoite showing striae, end view, Heidenhain's haematoxylin.

11. Trophozoite.   P.A.S. shows distribution of paraglycogen.

12. Trophozoite.   Fresh smear, Sudan black shows distribution of lipids.

13. Trophozoite.   Mercury bromophenol blue shows distribution of protein.

14. Schizont.   Fresh smear.   Negative phase.

15, 16. Schizonts.   Heidenhain's haematoxylin.

10μ
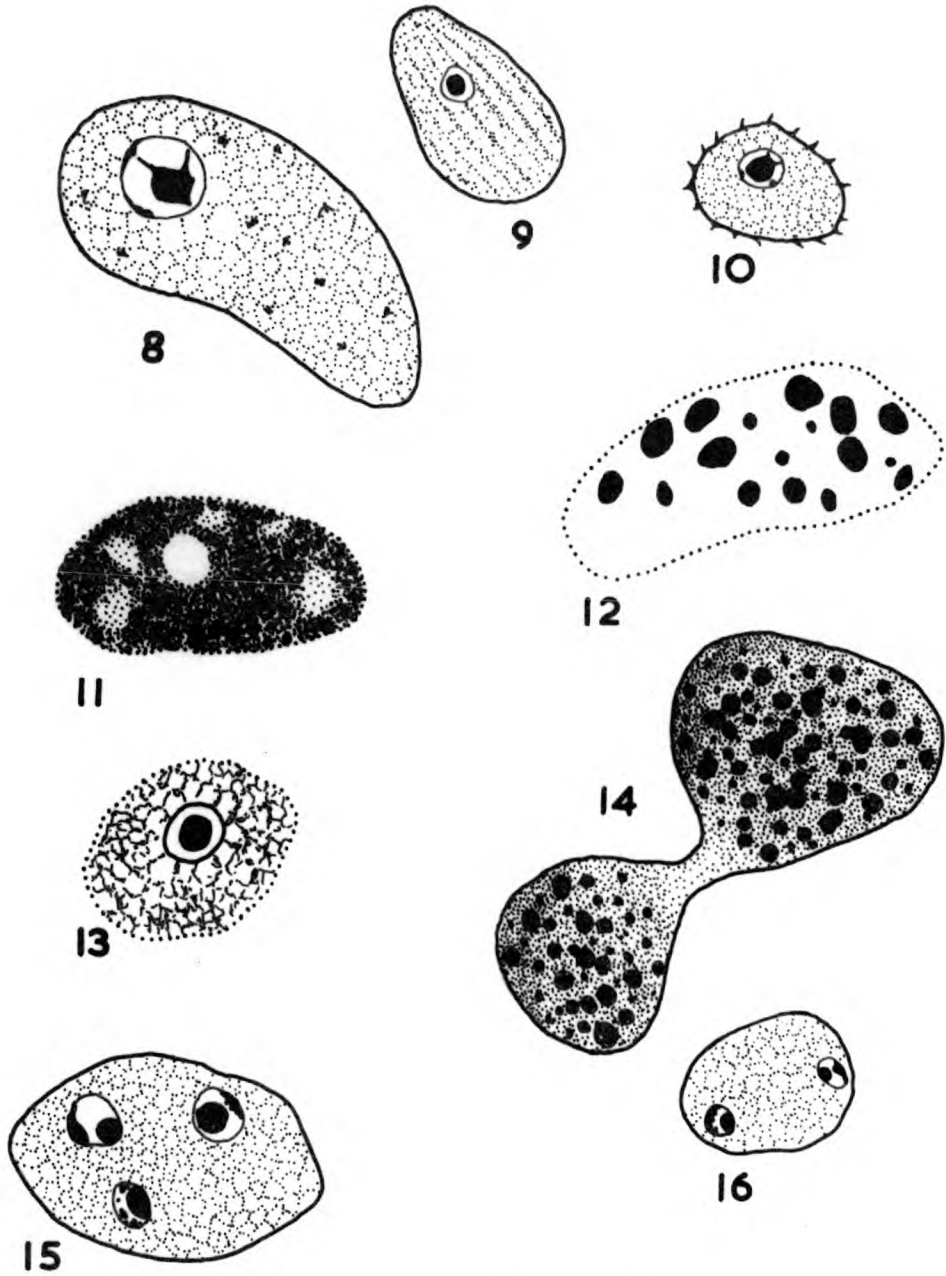
Fig. 17
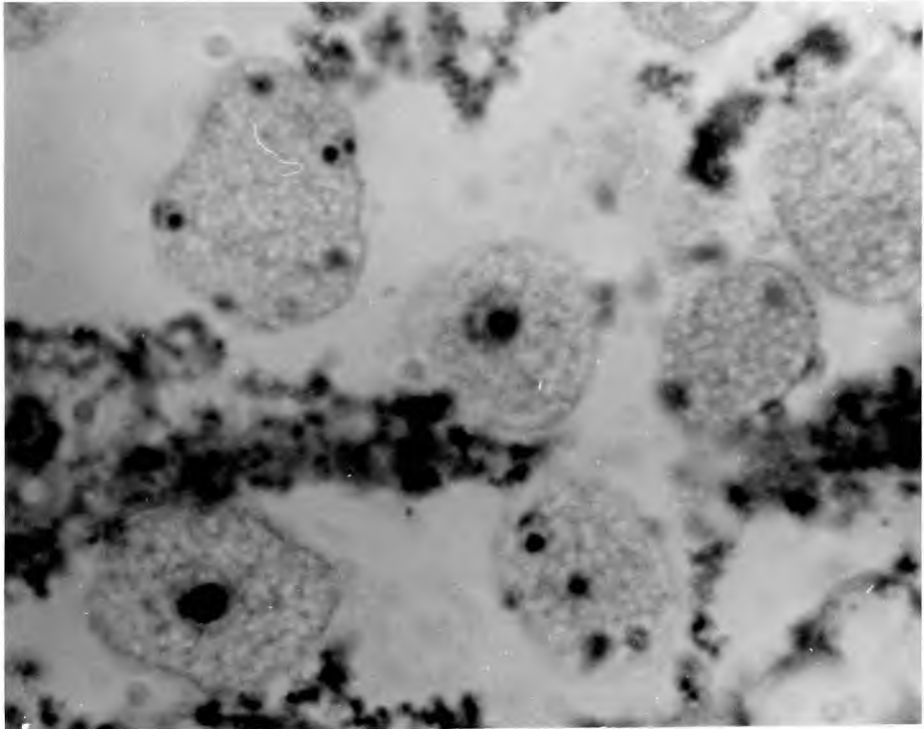
L.tribolii trophozoites and schizonts.   Heidenhain's
haematoxylin.

and contain from two to eight nuclei.   As early schizonts were not seen in fresh preparations, the fate of the pseudomerite could not be ascertained.   The size of schizonts varies greatly, from 10μ by 5μ to 30μ by 15μ, and their nuclei measure 2-3μ in diameter.   The cytoplasm of schizonts appears similar to that of trophozoites, as do the nuclei apart from their smaller size.   No schizonts were distinguished in Feulgen stained preparations, and so the reaction of their nuclei is not known.

There was no evidence of more than one schizogonic cycle.

The rupture of the schizonts was not observed, but merozoites, which are spherical, measuring 4μ to 6μ in diameter were frequently seen in aggregations, indicating a multiple fission of the parent plasmodium.

e) Gametogony (Figs.18-21, 25, 26).

Gametocytes, which presumably develop from merozoites, closely resemble large trophozoites, but have no striae or pseudomerite.   They associate in pairs while still uninucleate, and becoming flattened along the junction between them, together form a spherical body measuring about 20μ in diameter.   The gametocyst wall, which is very thin forms shortly after the association, when nuclear division commences.   The nucleus of each gametocyte divides into 16 daughter nuclei which become peripherally distributed.   These gametic nuclei, which measure 2μ in diameter commonly contain three granules of chromatin material which stain darkly with Heidenhain's haematoxylin, and red with Feulgen's reaction.

The cytoplasm separates round the gametic nuclei, giving rise to gametes of about 4μ diameter.   There is no apparent difference

L.tribolii, sexual stages.

18.   Associated uninucleate gametocytes.   Heidenhain's haematoxylin.

19.   4-nucleate gametocyst.   Heidenhain's haematoxylin.

20.   32-nucleate gametocyst.   Heidenhain's haematoxylin.

21.a,b,c.   Serial sections of a gametocyst with gametes and residual gametic nuclei.   Heidenhain's haematoxylin.

22.a,b,c. Serial sections of a gametocyst with 13 zygotes. Heidenhain's haematoxylin.

23.   Gametocyst with young spores.   Sudan black shows distribution of lipids.

24.   Gametocyst with young spores.   P.A.S. shows distribution of polysaccharides.

25.   Gametocyst with gametic nuclei. Feulgen's reaction shows distribution of D.N.A.

18

19

20

a

b

21

c

a

b

22

c

23

24

25

10 µ

Fig. 26.

10μ
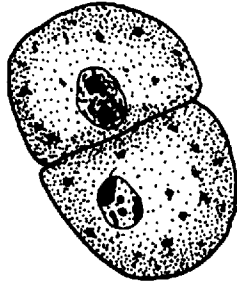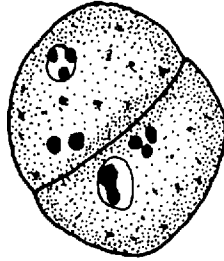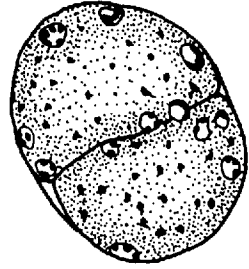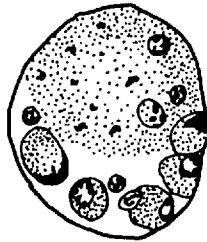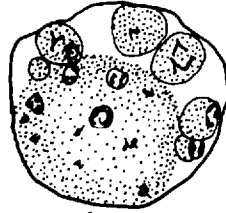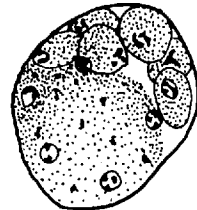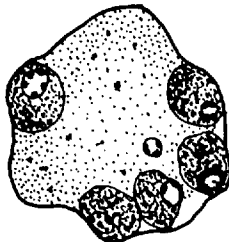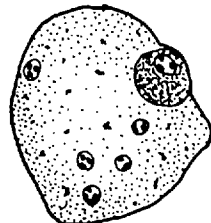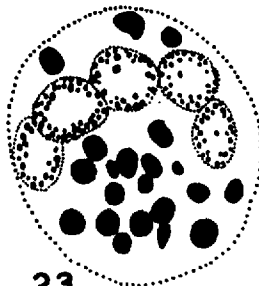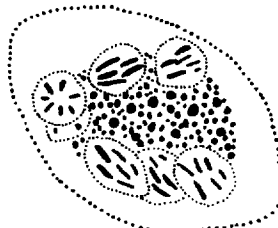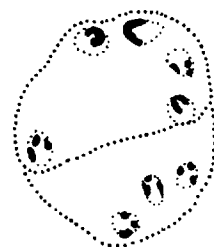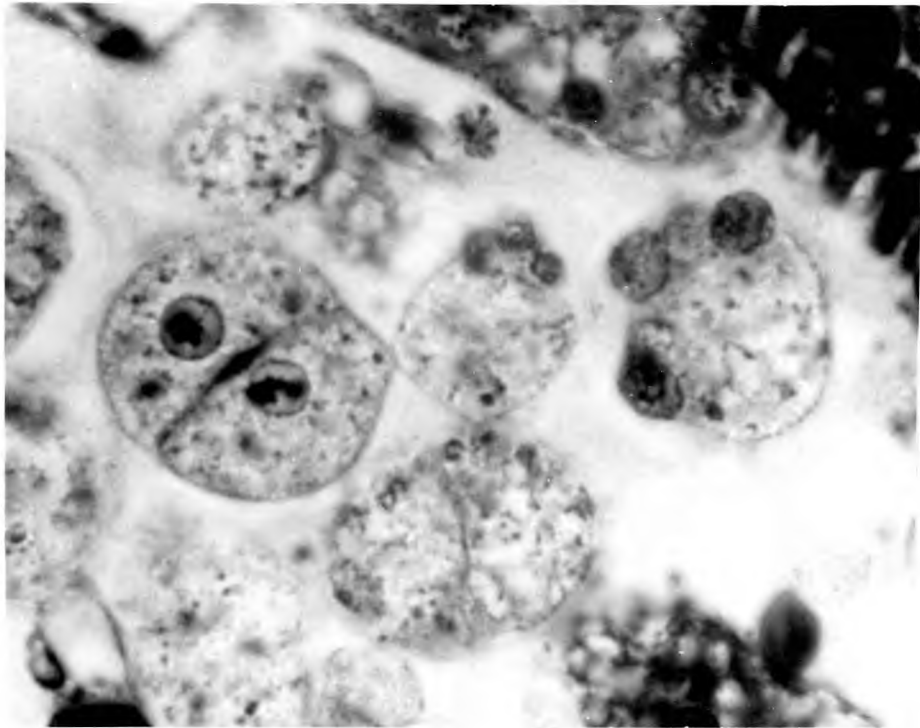
L.tribolii, sexual stages; binucleate gametocyst,
multinucleate gametocyst, gametocyst with gametes, gametocyst
with zygotes. Heidenhain's haematoxylin.

between the gametocytes of a pair, nor between the gametes they produce. Usually a number of nuclei in the gametocyte do not form gametes, and remain in a mass of undifferentiated residual cytoplasm.

A proportion of gametocysts are arrested in their development by being encapsulated by the host's haemocytes. This phenomenon is discussed at length in a later chapter.
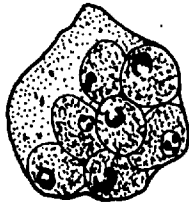
f) <u>Sporogony</u> (Figs. 23, 24, 26-30, 32).

Gametes fuse quite soon after their development; gametocysts containing gametes are infrequent. Zygotes are sometimes bunched towards one end of the gametocyst, and sometimes bunched at both ends, suggesting that fusion of gametes from opposite gametocytes is not invariable. The zygotes are initially spherical, becoming ovoid before the formation of the spore wall, and measure from 5μ diameter to about 6μ by 4μ in sections. Their nucleus, measuring 3μ diameter contains darkly staining granules and filaments which are faintly Feulgen positive, and represent the chromatin material from the two gametic nuclei.

Throughout their development, gametocytes contain large quantities of paraglycogen and lipid material. As the development of zygotes proceeds, however, the paraglycogen granules become smaller and rather more scattered than in the young gametocyte. The young zygote contains a few small granules of paraglycogen, and a large number of small fat globules which are concentrated around the periphery.

The spore wall forms before any division of the zygotic nucleus occurs, hindering further observations within the spore. The spore, or oocyst, is lemon shaped with slight protuberances at each end.

Explanation of Figs. 27-31.

L. tribolii.

27. Young spore in fresh smear. Negative phase.

28. Mature spore in fresh smear. Negative phase.

29. Mature spores, longitudinal section. Giemsa colophonium.

30. Mature spore, transverse section. Giemsa colophonium.

31. Gametocyst encapsulated by host's haemocytes. Giemsa-colophonium.

27 28

29 30

5μ

10μ
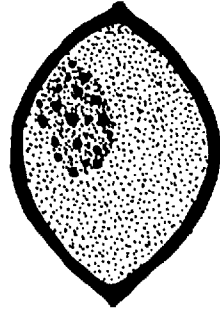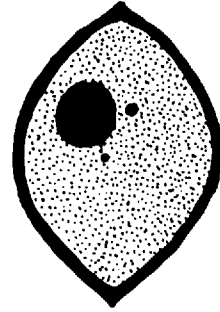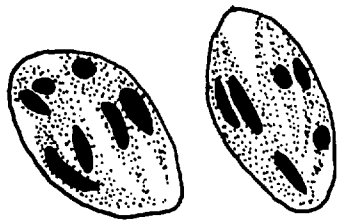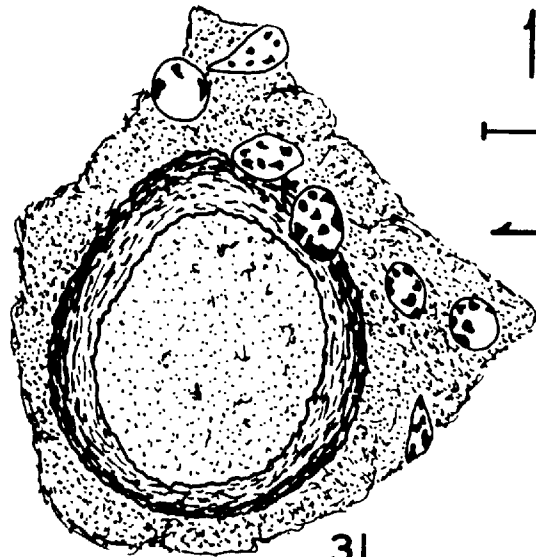
31
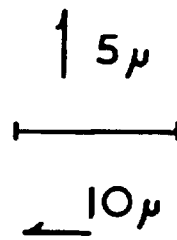
The poles are not, however, distinctly plugged as in some other neogregarines, but are capped, and surrounded by slight thickenings in the wall.   The cap does not stain differentially with any of the methods used, and could be demonstrated with certainty only by its absence from spores in the mid-gut of newly infected host larvae.   During the development of the spores, the residual cytoplasm of the gametocyst disappears.

The spores, which measure 8-9μ by 5-6μ (fresh) are still contained within the gametocyst on the death of the host, but are freed by slight pressure.   In order to count the spores in each gametocyst, the tissue of moribund or dead larvae was softened in 10 per cent KOH for three hours, then very gently squashed beneath a cover-slip.   The rupture of the host body released large numbers of intact gametocysts in which the spores could be counted.   It may be seen from the histogram, Fig.35, that while the theoretical number of spores (half the number of gametic nuclei) per gametocyst is 16, this number is rarely reached, and the most usual number is 9 or 10.   This is thought to be due to the failure of some gametic nuclei to form gametes, and possibly the failure of some gametes to fuse.

Nuclear division within the spore was not observed, but finally, eight sporozoites are formed.   The sporozoites lie longitudinally within the spore : four with their nuclei towards each end.

In fresh preparations, the sporozoites are not distinguishable within the spore.   The young spore is seen to contain a large number of scattered granules and globules, which congregate first around the long

Fig. 32.
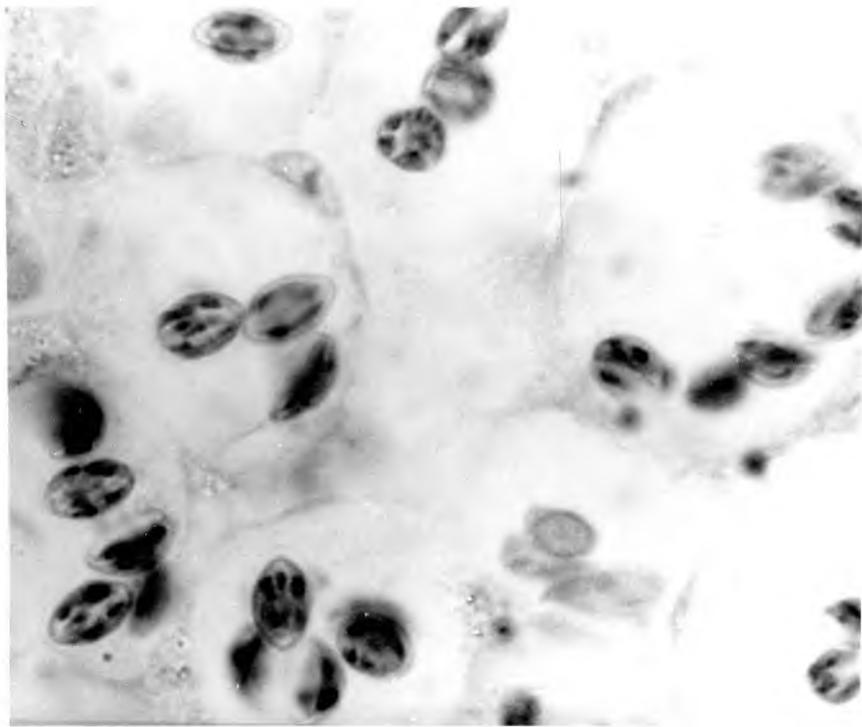
L.tribolii, mature spores.   Giemsa colophonium.

Fig. 33.

50µ

T.castaneum oblique section, posterior mid-gut region.
P.A.S. shows distribution of L.tribolii trophozoites
and young gametocysts.

axis, then asymmetrically towards one end. Finally these inclusions agglomerate into one to three large bodies which remain at maturity, but disappear on hatching.

g) Mode of infection

Dead larvae are packed with infective spores, and are readily eaten by healthy larvae (Fig.37) which thereby ingest spores. Spores may also be ingested fortuitously when larvae eat flour in which dead larvae have disintegrated. Dead larvae smell strongly of yeast which may have an attractive influence on live larvae and enhance the chances of continued infection.

The gametocyst wall breaks down during the process of ingestion releasing spores into the lumen of the gut (Fig.1). In the mid-gut of the new host, the polar caps are removed from the spore, either by the host's digestive enzymes, or from within, as the result of some precipitative stimulus. The sporozoites are thus released, and a new infection begins.

h) Duration of the stages

Under the conditions maintained in this study, sporozoites hatched within $3\frac{1}{2}$ hours of ingestion. Trophozoites took about 6 days to develop, and multiplied by schizogony on days 7 and 8. The first associating gametocytes were seen after 9 days, and zygotes were first present on day 10. Spores were mature from day 12.

Parasites were rare in the first six days after exposure to infective material, and during that time the infection may be termed pre-patent. From days seven to twelve, the development of stages is rapid,

and within an individual larva, well synchronised, even when larvae are exposed to constant hyper-infection. Larvae rarely die in less than twelve days, but commonly do so in fifteen to twenty days.

2. **The Viability and Maintenance of Virulence of Spores of L.tribolii.**

In order to estimate accurately the viability of spores, it is desirable to have a medium in which they can be hatched _in vitro_, so that the emerging sporozoites may be counted.

Spores of both _L.tribolii_ and _F.tribolii_ were subjected to a number of investigations which were designed to help develop such a medium.   As hatching depends on the digestion of the spore plugs, the chemical nature of these bodies is of great importance.

In a histochemical analysis of the spore walls and end plugs, fixed spores in smears were tested with P.A.S. and mercury bromophenol blue and unfixed suspensions of spores were tested with Millon's reagent, Alcian blue, Toluidene blue and Lugol's Iodine.   P.A.S. stained the spore plugs of _F.tribolii_ heavily, as did mercury bromophenòl blue.   The latter stain also coloured the polar region of the spore wall, leaving a barrel shaped region round the central third of the spore unstained.   Neither of these stains coloured _L.tribolii_ spores. Millon's solution, which is a violent reagent suited to the demonstration of proteins in larger masses of tissue destroyed the spores; Alcian blue, which colours acid mucopolysaccharides gave no reaction; Toluidene blue which is useful for distinguishing between proteins by their metachromatic properties gave no reaction, and nor did Lugol's Iodine which would have demonstrated the presence of starch or glycogen (including paraglycogen).   The negative results of histochemical tests such as those used cannot be regarded as conclusive, owing to the possible presence of protective layers surrounding the spore walls, and preventing the penetration of the stains.

These tests failed to indicate the nature of the spore cap of
L.tribolii, but demonstrated that the spore plugs of F.tribolii probably
contain a neutral muco-protein.

The osmotic pressure of any hatching medium would have to be
within the range suitable for the viability of sporozoites.   In order
to estimate this, six hours after being fed on a mixture of L.tribolii
and F.tribolii spore powder, when sporozoites would normally be hatch-
ing out in large numbers, young T.castaneum larvae were crushed in saline
of different concentrations.   In 0.1% saline, the sporozoites rapidly
swelled out and became immobile; in 10% saline, they shrank and died.
In 1% or 2% saline, however, hatching occurred normally.   The hatching
of sporozoites in 2% saline shows their tolerance of conditions hostile
to many parasites.   The final medium would therefore have to have an
uncritical osmotic pressure equivalent to that of about 1% or 2%
saline.

Sporozoites hatching in the variable medium of an insect's
gut would be expected to have moderately high tolerances of osmotic
pressure and pH.   It was thought pertinent, however, to attempt to
estimate the pH of the mid-gut of T.castaneum.

To this end, young larvae were allowed to feed on flour which
had been mixed with indicator powders.   Neutral red and Bromo-thymol
blue were the only indicators which the larvae could be induced to eat.

After an hour the larvae were dissected and the colour of their
gut contents noted.   Neutral red was dark crimson in the mid gut, and
Bromo-thymol blue was yellow with a tinge of green.   Comparison with

B.D.H. capillator tubes showed that an acid pH of between 6.0 and 6.5
was indicated by these tests.

Having determined the osmotic pressure and pH suitable for
hatching, and having gained some indication of the chemical nature of
the spore plugs, a number of easily available enzymes which were
thought likely to be suitable were tried.   The enzyme media prepared
were not all of the same pH as the insect gut; it was thought prefer-
able at this stage to use the enzymes close to their optimum pH in
order to observe effects on the spore plug.

The following media were prepared:

1. Human saliva, cleared by centrifugation, containing
    the starch-splitting enzyme, ptyalin.
2. Trypsin, 0.25% in pH 7.1 'PO$_4$ buffer $^m/5$
3. Trypsin, 0.025% in pH 7.5 'PO$_4$ buffer $^m/5$
4. Trypsin, 0.25% plus Diastase, 1% in pH 7.5 'PO$_4$ buffer $^m/5$
5. Trypsin, 0.25% plus Bile salts, 1% in pH 7.5 'PO$_4$ buffer $^m/5$
6. Papane, 1% in pH 5.7 'PO$_4$ buffer $^m/5$
7. Lysozyme, 1% in pH 6.3 'PO$_4$ buffer $^m/5$

Trypsin and Papane are protein splitting enzymes with a wide range of
activity.   The Trypsin plus Bile salts medium was found by Doran and
Farr (1962) to allow hatching of sporozoites of the coccidian
Eimeria acervulina.   Diastase is a polysaccharide-splitting enzyme, and
Lysozyme is a broad-spectrum enzyme which breaks down mucco-polysac-
charides.

No spore hatching, or degeneration of the spore plugs or caps
were observed when spores were incubated in these media for up to 12 hours.

As no success was obtained in developing an artificial medium, it was decided to attempt to hatch spores in a medium of crushed insect gut. Guts of T.castaneum larvae, and also of the larger, taxonomically and ecologically related beetle, Tenebrio molitor were dissected out and placed on microscope slides. They were then sprinkled with spore powder and crushed under a cover-slip. The slide was put in a petri with moist filter paper, and incubated at 30°C for up to 12 hours. Repeated observations during this period failed to show any spore hatching.

About 100 T.castaneum larvae were homogenised in 10 ml of 1% saline, and the soup was centrifuged. A drop of the resulting, almost clear liquid was sprinkled with spore powder, and incubated as above. This final effort also failed to induce hatching.

The above tests show that while the emergence of the sporozoite is not controlled by fine limits of osmotic pressure, the caps or plugs of the different spores require a fairly specific stimulus or digestive medium to be dissolved. It was therefore decided that the only feasible method in the present circumstances for estimating the viability of spores was by extrapolation from a bio-assay of their maintenance of virulence.

Estimation of maintenance of virulence of L.tribolii spores by bio-assay.

A fresh medium of spores in flour was prepared, containing about 2×10^5 spores per gm., which was expected to kill almost 100% of larvae raised in it. After an initial assay to prove this, the flour was divided into five parts, each to be stored at different temper-atures. The temperatures were chosen to represent those under which

infective material in flour might be expected to be stored in practice; 15°C, Room temperature (= 20°C approx.), 25°C and 30°C.

The virulence of the spores in these five media was assayed after three months and after nine months by determining the number of adults surviving from larvae reared in the medium.

This assay method showed in general how long infective material might be kept without serious loss of potency.

Assay method

1 gm. of flour was placed in each of ten two inch by one inch tubes, and 12 new laid eggs of T.castaneum were added.   After incubation for five weeks at 28°C, the number of surviving adults was counted.

Control mortality was measured in larvae reared in noninfective flour, and was taken into account using Abbott's formula.

The results of this experiment are summarised in Table 1 below.

Table 1   Percentage survival of T.castaneum to adult stage when reared in L.tribolii infective flour stored at different temperatures.

Storage Time	Fresh Spores		Storage Temp. °C	3 months		9 months	
	Obs.	Corr.		Obs.	Corr.	Obs.	Corr.
Uninfected	69	100		59	100	52	100
Infected	3.3	4.8	15	1.6	2.8	–	–
			R.T.	4.3	7.0	8.3	16
			25	–	–	9.2	18
			30	2.5	4.2	21.8	41

Obs : Observed percentage survival

Corr : Results corrected by Abbott's formula for control mortality.

R.T. : Room temperature (=20 ± 5°C approx).

Note: The flour stored at 15°C for nine months became mouldy and
was not assayed. The 25°C, three month assay was not made.

The preliminary assay showed the 95.2% mortality (4.8%
survival) occurred in the fresh medium. After storage for three
months, there was no noticeable change in this figure at any temperature.
After nine months, a marked decrease in mortality occurred; flour
stored at room temperature was the most potent, giving 84% mortality.
After storage at 30°C, only 59% mortality occurred.

No statistical analysis of the results would be valid, as
different larvae were used in the separate tests, and their suscept-
ibility cannot be assumed to have been identical.

The results indicate that although L.tribolii spores may be
stored for a considerable length of time, they lose much of their
potency if stored for nine months at 30°C.

Extension of this experiment to a wider range of temperatures,
and consideration of the effect of humidity, as well as further replic-
ation are desirable in order to draw accurate quantitative conclusions,
but were prevented because of the long periods for which the exper-
iment had to be set up.

3.   The Host Range of L.tribolii in Species of Tribolium.

50 eggs of each of the five available Tribolium species were collected by the method described above for T.castaneum, and placed in two by one inch tubes containing 3 gms. of flour with about 10^6 spores of L.tribolii per gm.   Control tubes contained 50 eggs of each of the species, in sterile flour.

The pure flour medium is not very satisfactory for the development of T.destructor or T.anaphae, and only 8 and 16 of these species respectively survived to maturity in the control cultures. Of the other species, 22 T.madens,   27 T.castaneum and 36 T.confusum adults emerged.   In the infected cultures, no adults developed in any save that of T.confusum, where 22 survived.   Examination of these adults showed no sign of infection.

The dead larvae were removed from the remaining cultures, and examined.   All were infected with L.tribolii, and had died from the disease.

The low survival rate in control cultures was not due to L.tribolii, but probably to cannibalism in the crowded conditions. The use of large numbers of eggs, which encouraged cannibalism, would have allowed any low rate of infection to become apparent.

Thus, all readily available species of Tribolium are susceptible to the disease, except T.confusum, which is highly resistant. The resistance of this species to Lymphotropha contrasts with its sensitivity to Nosema, but compares with its resistance to Adelina.

B. PATHOLOGY OF L.TRIBOLII IN T.CASTANEUM

1. Macroscopic Effects of Infection

Larvae which become infected show no external symptoms until shortly before they die, or until encapsulated parasites become visible through the integument.

About ten days after infection, some of those larvae which have received a massive dose show slight darkening of the posterior abdominal segments. The rest of the body is slightly less opaque than in healthy larvae of the same age, owing to the degeneration of the fat body. At death, after twelve or more days, the larvae are straight, laterally shrunken, rigid, and black in colour. Dead larvae (Fig.37) dry out, become dorso-ventrally flattened, shrunken and dark brown in colour. In cultures with old infections, such larvae are commonly found to have been partially eaten. The only stage at which heavily infected larvae can be separated with certainty from healthy ones, or from those which have died from other causes, is when they are moribund or just dead, and immobile, shrunken and blackened.

Larvae which have received a sub-lethal dose of spores develop normally, but more slowly than healthy ones. Pupation may be delayed for as much as a month. In these larvae, when the fat body becomes opaque and white just before pupation, the capsules surrounding the parasites become clearly visible externally as brown spots below the integument. This symptom does not become noticeable until at least 12 days after infection.

Many larvae were found to be intermediate between those described above, and some showed no externally visible signs of a light infection.

Larvae which survive an infection may pupate. Some of the pupae are speckled with capsules, while others show no external signs of infection. Pupae which die do not blacken, they shrivel dorso-ventrally on drying out, and retain their live colour, which depends on the state reached by the developing adult inside. Apart from the capsules visible in some specimens, infected pupae while still alive are indistinguishable externally from healthy ones.

A large proportion of adults which die as the result of disease do so shortly after emergence. Such specimens are often grossly deformed in the development of their wings and elytra (Fig.38). Capsules may be seen in large numbers through the translucent cuticle of moribund teneral adults. Those which die prematurely after emerging as perfect insects show no differences from adults which die from other causes.

Fig. 34.

20μ
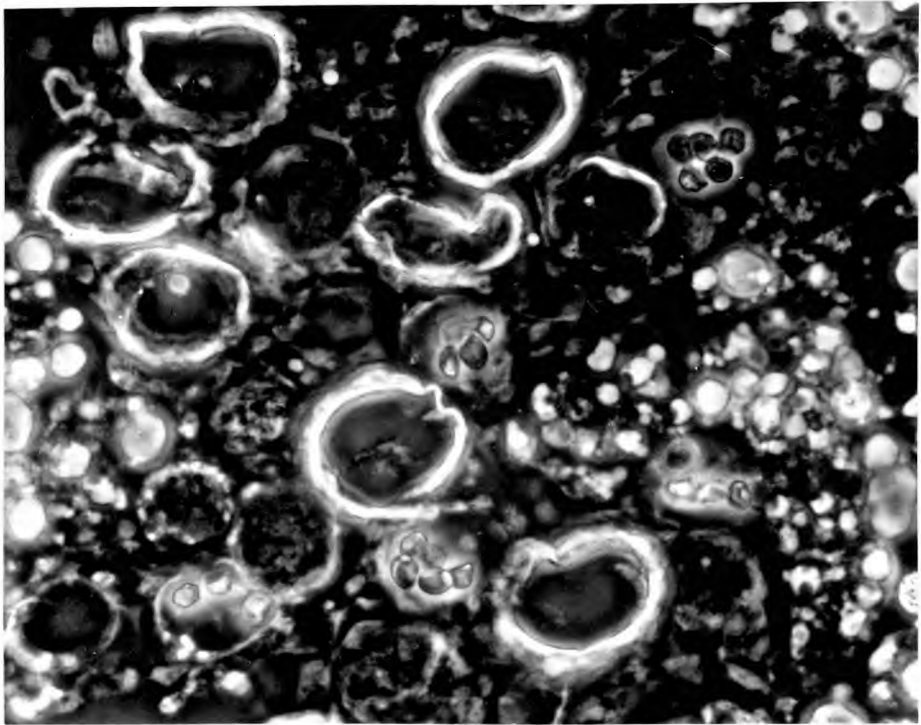
L.tribolii in T.castaneum moribund pupa.   Unstained, negative
phase, shows encapsulated gametocysts, but gametocysts with
mature spores unaffected.

L. TRIBOLII:
SPORES PER
GAMETOCYST

Fig. 35.
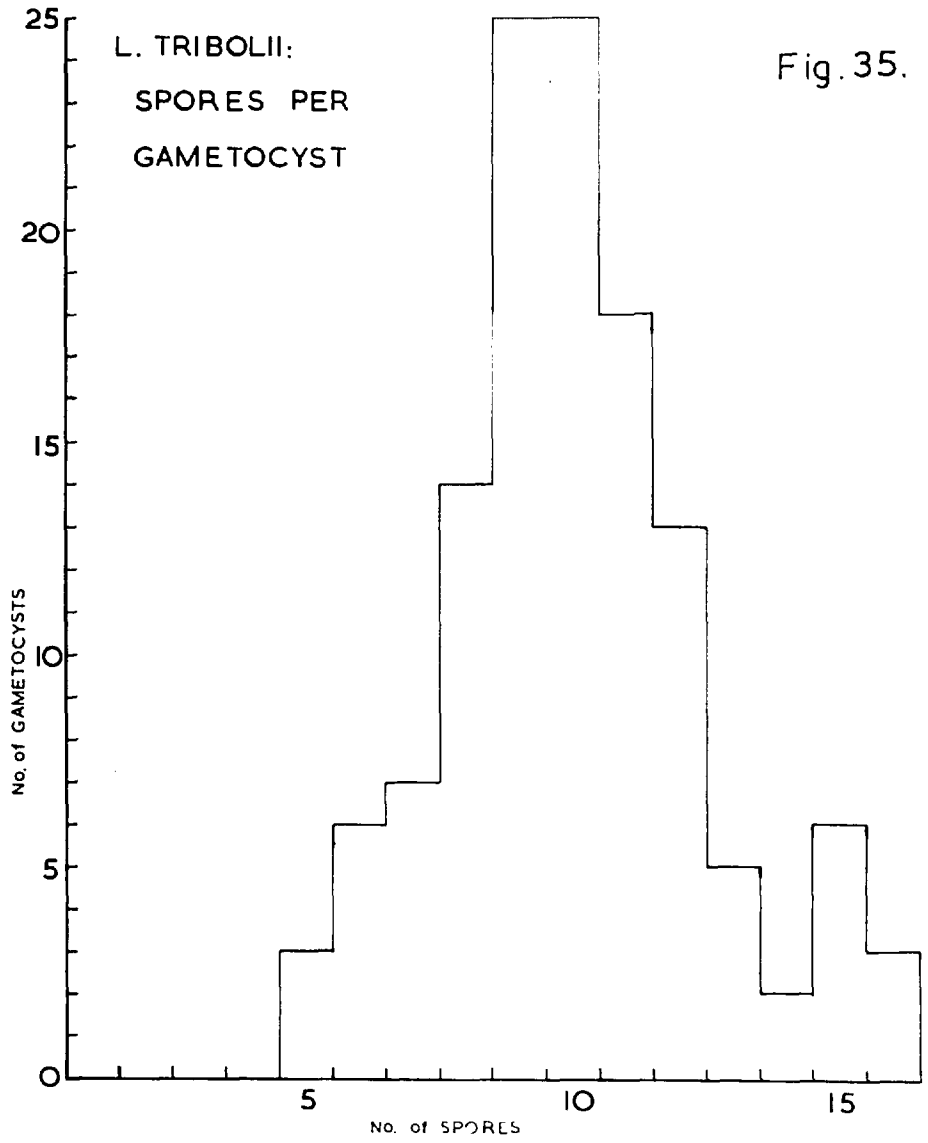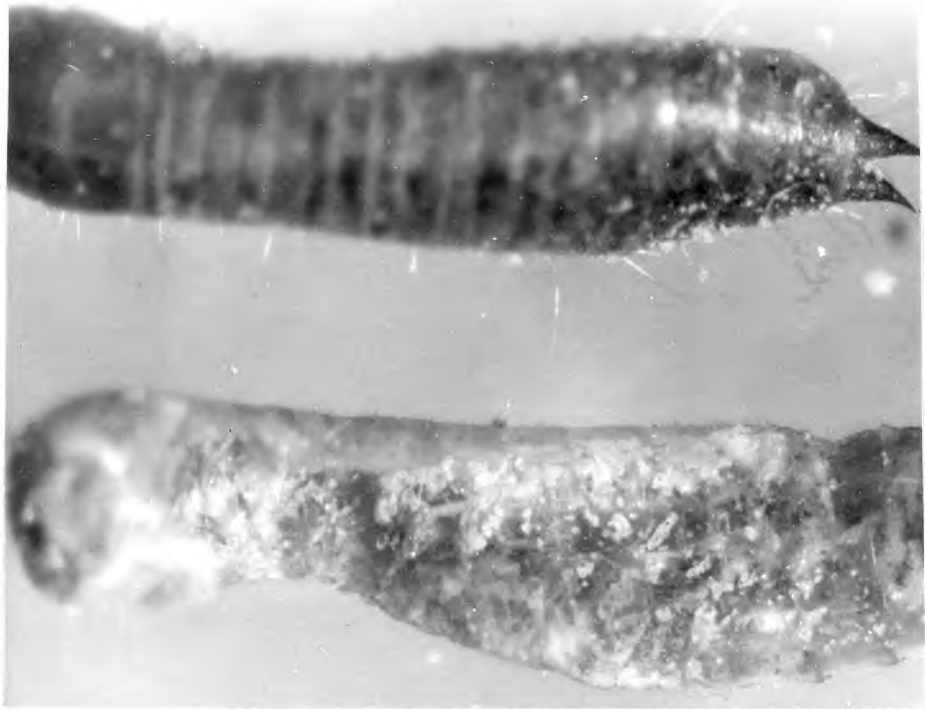
1 mm.

Fig.37.   T.castaneum larvae dead from L.tribolii infection.

Fig 38.

1mm.

T.castaneum adult, dead shortly after emergence with deformed elytra due to L.tribolii infection.

2. Histopathology of Infection

a) Encapsulation of parasites by the host

A conspicuous feature of L.tribolii infection is the host reaction, encapsulation, which it provokes. Encapsulated parasites were found in larvae, pupae and adults which had been exposed as young larvae to moderate or light infections. The capsules were clearly visible on external examination of larvae, pupae and teneral adults, appearing as brown spheres, often in groups, mostly just below the cuticle. Those pupae which died or would have been expected to give rise to deformed adults contained particularly large numbers of capsules, and a series of such pupae was examined histologically. (Figs. 31, 34)

The capsules consist of two layers, though there is no clear division between them. The outer layer is composed of haemocytes which have agggegated round the parasite, forming a thick coat. The inner haemocytes in this layer are flattened and appear spindle shaped in sections. As haemocytes are very rare as free cells in healthy larvae, it was not possible to compare those in the capsule with normal ones.

The inner layer of the capsule, which is about 5 μ thick is of fibrous texture, and light brown in colour. This layer, which sometimes only partially surrounds the parasite has very much the same appearance as insect cuticle. Cell walls and nuclei are not distinguishable in the inner layer of the capsule. It is difficult to say whether this layer is composed of compacted dead haemocytes, or is secreted by the surrounding layer of living cells. In sections, there appears to be a gradation between the outer and inner layers, but in

fresh preparations, the haemocytes are washed off, and the parasite is surrounded by the brown inner layer, which then appears to have been secreted.

The inner layer of the capsule stains very heavily with Heidenhain's haematoxylin, and bright blue-green with Giemsa's stain. The haemocytes of the outer layer also stain heavily with Heidenhain's haematoxylin, and both their cytoplasm and nuclei stain red with Giemsa's stain. The cell boundaries and nuclei are therefore difficult to distinguish. The outer haemocytes stain strongly with P.A.S. which has a fainter reaction in the inner layer. The entire capsule stains deeply with mercury bromophenol blue, and while the outer layer is destroyed by boiling with KOH, the inner layer remains intact. After treatment with KOH, the inner layer does not stain with iodine. These tests suggest that the inner layer is composed of a protein polysaccharide complex of high inertia, which does not contain chitin. Proteins and polysaccharides are also present in large quantities in the cytoplasm of the surrounding haemocytes.

A remarkable feature of encapsulation is that it only occurs round developing gametocysts of the parasite, which degenerate, their nuclei becoming indistinguishable, until the capsule finally contains only amorphous matter. Trophozoites, and gametocysts containing mature spores are not affected.

Capsules are found from about 12 days after infection, and may form in any part of the host's haemocoele. They are mostly, however, close to the remnants of the fat body, where most of the

parasites occur.   The brown inner layers of adjacent capsules may
coalesce, and aggregations of five or more may be formed.

Encapsulation of parasites was observed in all species of
Tribolium which became infected, but was particularly prominent in
T.anaphae.

The importance of encapsulation as a defense mechanism against
heavy parasitemia cannot be great as it rarely occurs in heavy infections,
and then only a very small proportion of the parasites are affected.
In light infections, however, a large proportion of the parasites may
become encapsulated, and effective control of the disease is probably
obtained.

b) Effects on host organs

Besides the phenomenon of encapsulation described above, the
only histopathological effects of the disease which were noticed occurred
in the fat body.   The fat body of healthy T.castaneum larvae (Fig. 39)
almost fills the haemocoele from an early age.   In the young larva it
comprises a small group of cells with well defined margins, containing
a few small granules and globules.   As the larva grows, the cells swell
with increased volumes of reserve vacuoles, and their cell walls become
indistinguishable.   The most conspicuous of the vacuoles stain heavily
with Heidenhain's haematoxylin, obscuring the nuclei of the cells.
They also stain with mercury bromophenol blue, and faintly with P.A.S.
and may thus be interpreted as containing a mucopolysaccharide of high
protein content.   These are probably the "albuminoid spheres" commonly
found in large numbers in insect fat body.   In young larvae aged up to

10 µ
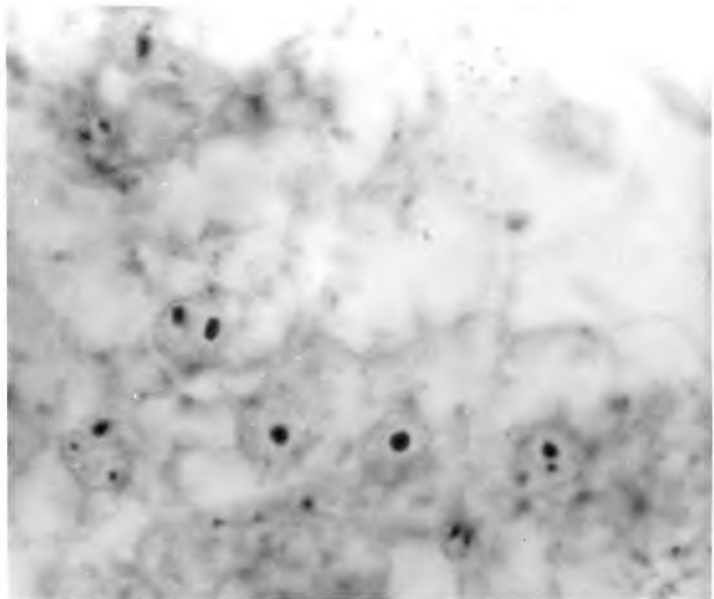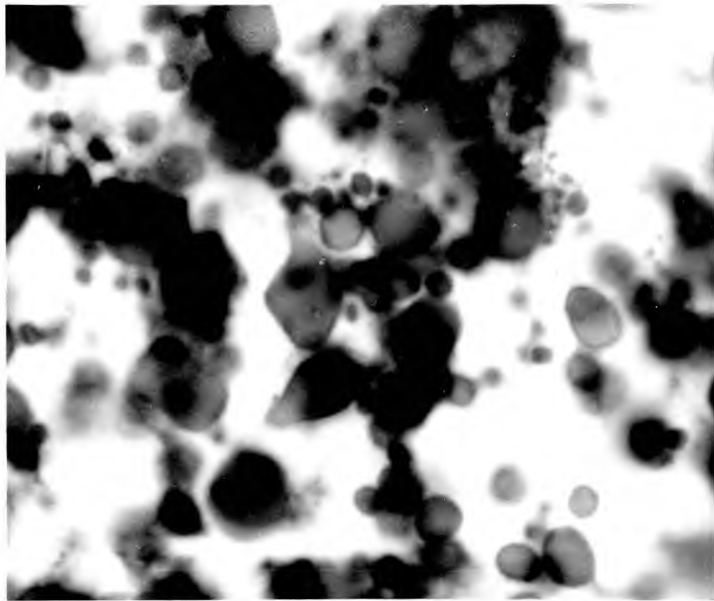
T.castaneum. Section of healthy larval fat body.
Heidenhain's haematoxylin shows albuminoid spheres. Empty
spaces are lipid vacuoles.
Fig. 40 T.castaneum. Section of infected larval fat body.
Heidenhain's haematoxylin.

6 days, the albuminoid spheres are small or absent, but they develop rapidly so that by 12 days the fat body is largely composed of them. They are variable in size, from 2 to 9 μ in diameter, mostly about 6 μ in old larvae.

Globules of lipid substance develop from an early age, and also increase in size and number as the larva grows. The lipid globules may best be observed as clear areas of the fat body in fixed preparations. In squashes in Sudan Black in 70% alcohol, they stain heavily, but cannot be measured, as on release from the cells they agglomerate. Fresh smears in saline of larvae aged over 15 days are almost obscured by the numerous lipid droplets which float up from the specimen to lie below the cover slip.

The remaining granules in the healthy fat body are of two kinds; one is proteinaceous, staining heavily with mercury bromophenol blue, and the other a polysaccharide staining heavily with P.A.S. These granules are present in the early fat body, and though they increase in number, do not grow to more than 3 μ in diameter. They are therefore obscured by the albuminoid spheres and oil droplets in older larvae.

The nuclei of the fat body are uniform and constant in structure throughout the larval development. They are spherical, 5-6 μ in diameter, and contain from 2 to 6 darkly staining granules.

The nuclei are not affected noticeably by infection; the effect of L.tribolii on the fat body may be interpreted mainly as slowing down its normal development, arresting it, and causing degeneration

in heavy infections. (Figs. 39, 40)

The albuminoid spheres are most affected. These fail to develop in infected larvae, or do so to a limited extent, and are almost entirely wanting in moribund specimens. The lipid globules are affected more slowly, persisting in small numbers in quite heavily infected larvae. At death, however, there is very little lipid material in the fat body. The other proteinaceous and polysaccharide granules are decreased in number, but a few may still be present at death.

While the fat body of healthy larvae occupies almost the entire haemocoele, the lack of development of storage vacuoles in infected larvae causes it to remain of constant size as the larva grows, and thus to occupy only a small proportion of the body cavity. The space thus made available is occupied by parasites.

The effects of infection on the fat body seem to be reversible; in larvae in which there are a large number of encapsulated parasites, the albuminoid spheres and lipid globules may develop, and in surviving pupae the fat body has a normal appearance.

3. The Succeptibility of Larvae at Different Ages to Infection

Eggs of T.castaneum were collected at ten day intervals, and allowed to begin development. When the third lot were beginning to hatch, batches of 20 larvae were selected from each age group, and were exposed to "massive", "heavy" and "light" infections. 20 further larvae of each age were reared in sterile flour.

The infective media were prepared as follows:

1. "Massive" dose : 10% powdered dead larvae in flour
   $= 10^7$ spores per gm. approx.

2. "Heavy" dose : one part of the above mixture + 20 parts of flour
   $= 5 \times 10^5$ spores per gm. approx.

3. "Light" dose : one part of number 2 above + 50 parts of flour
   $= 10^4$ spores per gm. approx.

The 20 larvae of each age to be exposed to each infective dose were put in two inch by one inch tubes with two gms. of the requisite medium. The surviving adults were counted as they emerged. Table 2 shows the results obtained.

The oldest larvae pupated within a few days of exposure. The other larvae, however, were exposed for long enough for the infection to develop before pupation.

Table 2   Numbers of _T.castancum_ (/20) reaching the adult state

after exposure at different ages to different doses of

L.tribolii.

Age	Dose			
	Control	Light	Heavy	Massive
4 days	20	10	7	0
14 days	20	20	20	0
24 days	20	20	20	20

A clear decrease in susceptibility with age is shown by these results.   Taking the youngest larvae as having the maximum suscepti- bility (100%) 24 day old larvae may be said to be 100% resistant to light, heavy and massive doses.   14 day old larvae are 100% resistant to light and heavy doses, but 100% susceptible to a massive dose.

Examination of the adults showed no active stages of the disease.   Some of those surviving light and heavy doses from 4 day larvae, and surviving a heavy dose from 14 day larvae did, however, contain either capsules or gametocysts.

It was not felt necessary to repeat this experiment, as many independent observations on age resistance supported the findings. Larvae aged 15 days or more from egg laying were very difficult to infect. Adults could not be infected even by feeding them on pure spore powder, nor did they contain actively developing stages remaining from larval infection.

4. The Éffect of Standard Serial Doses of <u>L.tribolii</u> on the Mortality

of <u>T.castaneum</u> Larvae.

A series of flour media was prepared containing fresh

<u>L.tribolii</u> spores as follows: 10^7, 10^6, 10^5, 10^4, 10^3 10^2, 0 spores

per gram.   The media were prepared by the serial dilution of a 10^7

spores per gm. medium.   Care was taken in each dilution to ensure a

homogeneous mixture of spores in flour; small quantitits of the sterile

flour were added at a time to the infective flour, and at each addition

the mixture was stirred and shaken.   A control mixture of powdered

dye (Bromophenol blue) was prepared in the same way.   No heterogeneity

could be detected when this control mixture was examined under a dis-

secting microscope.

Five eggs of <u>T.castaneum</u> were placed in each of five two inch

by one inch tubes for each concentration of spores, and one gram of the

appropriate flour was added.   25 individuals were thus allowed to

develop, exposed to each infective level.

In the two repetitions of the experiment, the 10^7 spores per

gm. dose was not used, and ten eggs were placed in each tube.

After four to seven weeks, the surviving adults in these

cultures were counted as they emerged.   Survivors were counted as those

which showed no sign of infection on emergence.

Control mortality was measured similarly, in larvae reared

in sterile flour.   A surprisingly high control mortality was recorded,

and this was thought to be due to the infertility of some eggs.   Abbott's

formula was used to correct for control mortality.

Table 3   Numbers of T.castaneum surviving to the adult state when reared in flour containing serial concentrations of L.tribolii spores.

No. of eggs	Dose: spores per gm. 10^7	10^6	10^5	10^4	10^3	10^2	0
25	0	0	1	7	18	21	20
50	–	–	15	32	38	38	33
50	–	1	13	37	30	36	34
Total survivors	0	1	29	76	86	95	87
% (observed)	0	0.8	23.2	60.8	68.8	76.0	69.6
% (corrected)	0	1.1	33.3	87.3	98.9	100	100

Table 3 shows that there is a relationship between the dose to which larvae are exposed and their mortality.   There is no "all or none" response to the disease, as might be expected if the organism multiplied enormously within the host.   Exposure to flour containing 1,000 spores per gram causes very little mortality attributable to the disease, but 10^6 spores per gram cause almost 100% mortality.

The variation between the results of the assays shows that a finer series of doses would not be useful.   However, there are not enough results intermediate between 20% and 80% mortality for the median lethal dose to be calculated with accuracy.   Estimating the LD50 obtained from the graph (Fig.41) of percentage mortality against log dosage, gives a figure of $10^{4.8}$ (= 90,310) spores per gram.   In view of the variation inherent in this type of experiment, this figure can only be regarded as accurate within an order of magnitude.

Fig. 41.

T. CASTANEUM:
LARVAL MORTALITY IN
INFECTIVE FLOUR
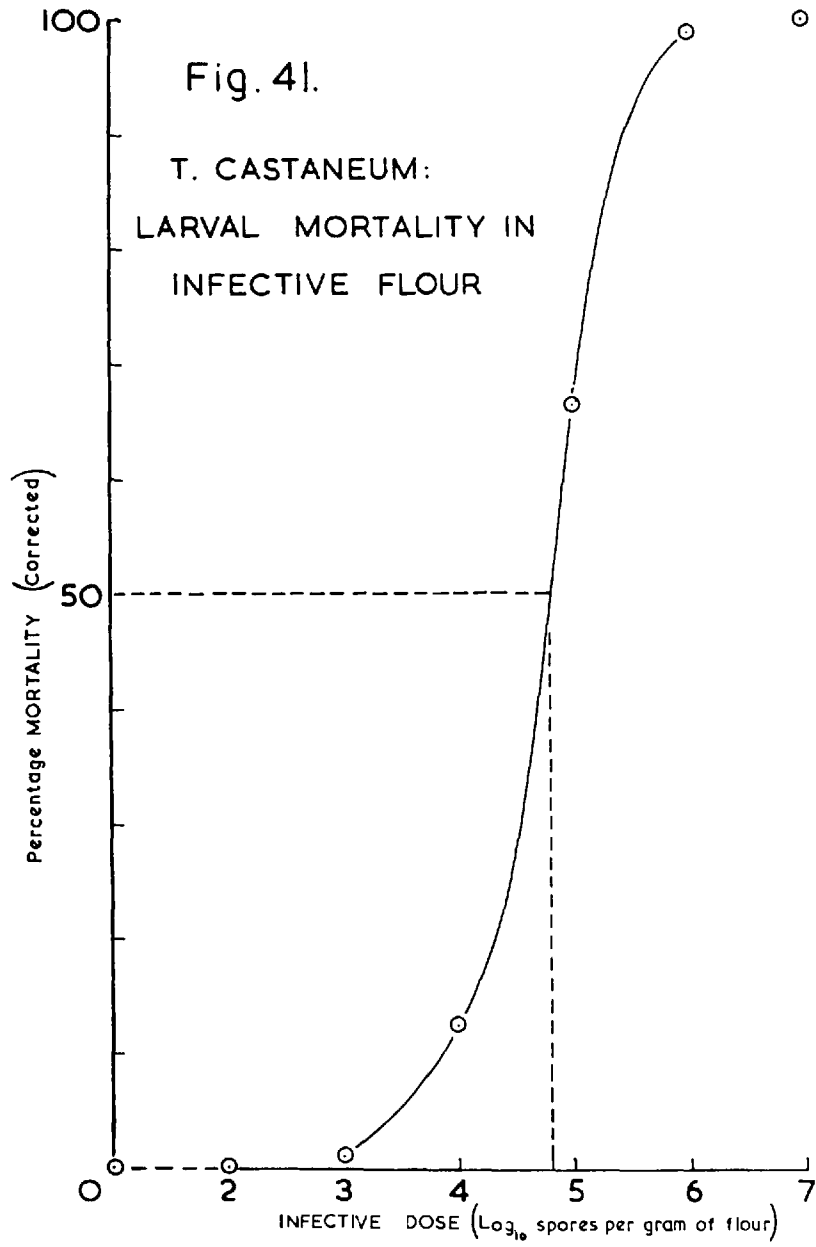
5.   The Effect of Disease on Larval Gain in Weight.

In the experiments on the susceptibility of larvae to D.D.T. (p. 90), and on oxygen consumption (p. 84), larvae infected 10 and 11 days from egg laying, and uninfected larvae were weighed.   The infected larvae were expected to die within 15 days, and uninfected ones to pupate within the same time.   The weights recorded are presented here.

Larvae were cleaned of adhering flour particles by allowing them to crawl on paper and blowing off unwanted matter.   In order to avoid inaccuracies due to water loss or starvation, larvae were weighed within one hour of removal from the culture.

Larvae were not weighed individually, but in groups of 20 or more.   The average weight of individual larvae of each age was calculated and recorded.

Dry weights were measured similarly after the larvae had been kept in an oven at 110°C overnight.

Tables 4 and 5 show the weights recorded at each age and state of infection.

Table 4   Weights of uninfected larvae and larvae infected at 10 days from egg laying (from D.D.T. experiments).

Age, days from egg laying.	Average live wt. mgms.		Average dry wt. mgms.	
	Infected	Uninfected	Infected	Uninfected
10	0.07	0.06	0.03	0.03
13	0.15	0.11	0.05	0.04
16	0.29	0.33	0.10	0.09
19	0.39	0.74	0.12	0.26
22	0.49	1.35	0.15	0.54
25		1.47		0.60

Table 5   Weights of uninfected larvae, and larvae infected 11 days

from egg laying (from QO_2 experiments).

Age, days from egg laying.	Average live wt. mgms.		Average dry wt. mgms.	
	Infected	Uninfected	Infected	Uninfected
10	0.04	0.05	0.02	
12	0.07	0.08	0.02	0.03
14	0.09	0.11	0.03	0.03
16	0.13	0.18	0.04	0.06
18	0.19	0.21	0.06	0.08
20	0.26	0.55	0.08	0.17
22	0.46	1.18	0.13	0.41
24	0.56	1.64	0.12	0.60
26	0.54	2.14	0.16	0.87

Particularly in the middle of their development, larvae showed great variation in size.   Groups of 20 or more were thought adequate to give a fair average.

It will be noticed from the figures, which are represented graphically in fig. 42, that infected and uninfected larvae cultured simultaneously show a distinct relationship with each other which is not so clear when infected larvae are compared with uninfected ones from the other group.   Slight differences in the consistancy of the flour, and slight variations in the temperature of the incubator are thought to be responsible for this.   Only cultures reared simultaneously in identical media can be critically compared.

The results demonstrate that the disease had no measurable effect on the rate of larval growth until at least six days after exposure to infection.   After eight days, however, when the uninfected larvae were growing rapidly, the rate of increase in weight of infected larvae

decreased.   Infected larvae continued to grow at a greatly reduced

rate until they died.   Examination of the dry weight figures showed

no difference in pattern from live weight readings.

Fig. 42.

LARVAL WEIGHTS

O   UNINFECTED A.

●   UNINFECTED B.

△   INFECTED A. 10 DAYS

▽   INFECTED B 11 DAYS
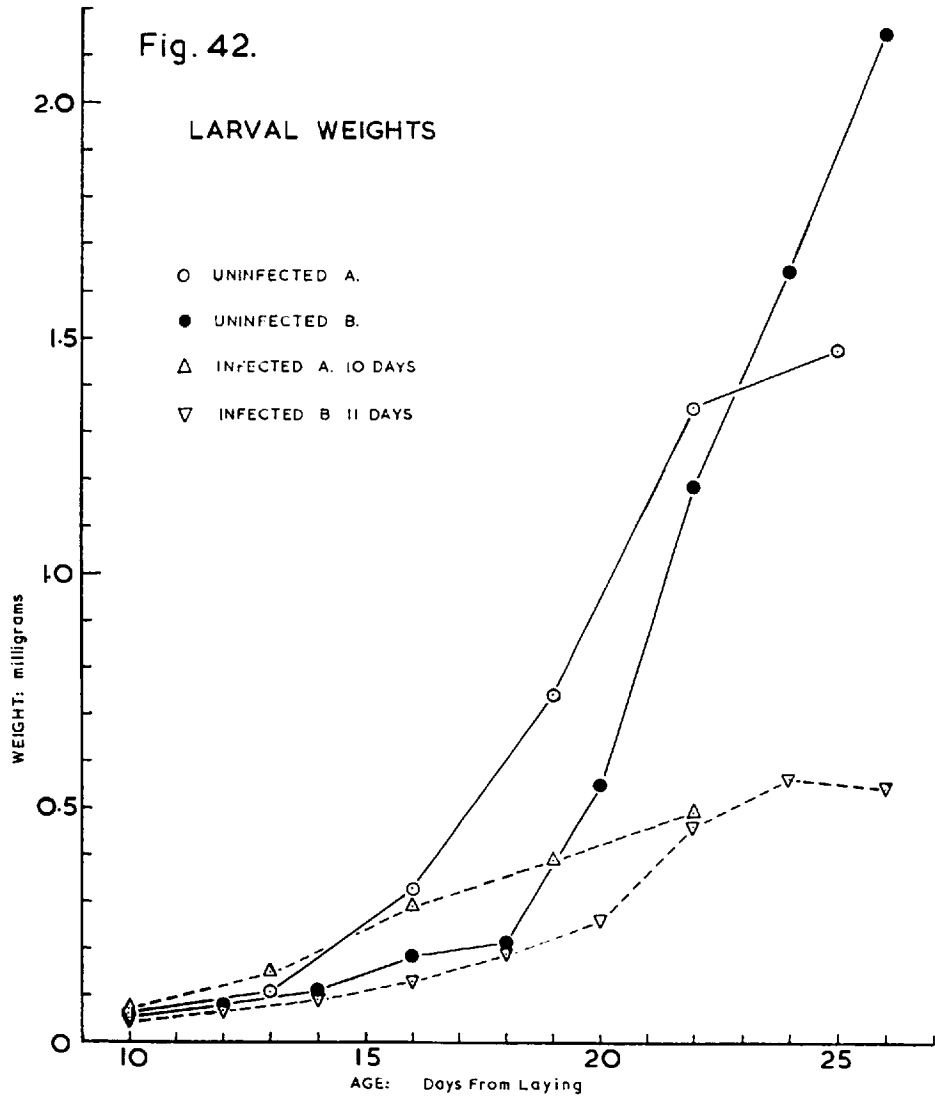
6.   The Size at Death of Larvae Exposed to Different Doses of

L.tribolii.

In order fully to estimate the effects of a control measure

on an insect pest, it is important to know how much development of the

affected insects is allowed before death.

An estimate of the size of T.castaneum larvae which had died

following exposure from birth, to different infective doses of L.tribolii,

was therefore made.   The most convenient measurement was that of head

capsule width, which is independent of the shrinkage which occurs during

desiccation.   The measurements were made using a X20 binocular microscope

and an eyepiece scale, with units equal to 1/14 mm.   Larvae from two

of the dosage/mortality experiments (p.71) were used, and the values

obtained are shown in Table 6 below.

Table 6   The head capsule widths of T.castaneum larvae which

have died following exposure to different doses of

L.tribolii.

Dose spores/gm.	No. measured	Head capsule width in eyepiece units	
		Mean	Range
$0-10^3$	None dead	–	–
10^4	7	7.76	6.0-9.0
10^5	40	6.88	4.0-10.0
10^6	72	5.78	3.0-9.0

The table shows that while the maximum size reached by larvae from any

dosage level is constant, the minimum and mean sizes both decrease with

increasing dose from 6.0 (min.) and 7.76 (mean) at 10^4 spores per gm. to 3.0 (min.) and 5.78 (mean) at 10^6 spores per gm. For comparison, newly emerged larvae have a mean head capsule width of about 2 units, and those about to pupate, 10 units.

This result, which would be expected from a disease relying partly on cumulative infection to cause death (i.e. where mortality and the time taken to cause death are related to dosage) shows that even when death eventually occurs, larvae exposed to a lighter dose develop further, and therefore cause more damage than larvae which die from a heavier dose.

7.   The Effect of L.tribolii Infection on the Activity of T.castaneum

Larvae.

Activity in insects may be measured by a variety of methods, the results of each being open to different types of interpretation. The prime purpose of the present investigation was to determine whether or not, in the experiments below,on D.D.T. susceptibility, differences in activity between larvae of different ages and states of infection might be responsible for different mortalities on exposure to dry films of insecticide.   Inactive larvae might show deceptively low mortalities, and hyperactive larvae would be apparently hypersensitive to the insecticide.

It was therefore necessary to use a method which would be repeatable for larvae of all sizes.   The distance moved per unit time is dependent upon size as well as activity, and is therefore not suitable without considerable adjustment of each reading.   It was decided after investigating the merits of a number of alternatives to count the number of larvae out of a batch of ten which moved in a ten second period.   This method had the advantage that conditions could be made identical with those of the D.D.T. experiments, and readings could be repeated over a long time.

Larvae were infected 10 days from egg laying, and at each age and state of infection, four batches of ten infected and healthy larvae were placed on filter papers, and enclosed in nickel-plated brass rings one inch in diameter.   The rings were covered with a plastic petri dish in order to eliminate disturbance caused by air movements.   The

observations were carried out at a temperature of $20 \pm 1^\circ C$.

It was found that the number of larvae moving in a 10 second period was usually more than zero, and almost always less than 10. This time was therefore regarded as suitable for repeated comparative readings. Ten larvae were used in each observation as this was the number used in the D.D.T. experiments, for ease of calculation of the results, and as ten was the greatest number of larvae which could be observed accurately at one time.

Twenty readings were taken from each of the eight batches of larvae (4 infected and 4 control), the readings being taken in four groups of five, the groups separated by intervals of 1 hour.

Table 7 shows the total score for each group of larvae observed i.e. the sum of the numbers out of ten larvae moving in twenty ten second intervals, spread in groups of five over four hours.

The percentage activity is expressed as the average number moving in any group in any ten second period.

Table 7   The activity of infected and uninfected T.castaneum larvae of different ages.

Culture	Group	Days from egg laying							
		10	12	14	16	18	20	22	24
Control	1	81	47	64	105	54	44	80	38
	2	74	54	58	62	53	51	62	53
	3	72	45	71	45	76	60	43	62
	4	63	71	71	69	44	65	63	101
	Total	290	217	264	281	227	220	248	254
	Mean %	36	27	33	35	28	27.5	31	32

82.

Table 7   Continued

Culture	Group	Days from egg laying							
		10	12	14	16	18	20	22	24
Infected day 10.	1		48	68	31	62	43	87	35
	2		54	49	44	74	59	89	55
	3		78	69	76	43	71	49	69
	4		46	63	62	52	68	51	80
	Total		226	249	213	231	241	276	239
	Mean %		28	31	27	29	30	36	30

Maximum score = 200

Maximum total = 800

mean % = total/8 to nearest whole number.

The table shows that there may be considerable difference between the activity of groups of larvae from identical cultures, under identical conditions.

In all cases the mean percentage activity is between 27% and 36%. The activity of individual batches is between 15.5% and 52.5%. No pattern or trend is discernable in these results either in the figures for any one culture, or between the cultures.

It may be concluded that the disease has little if any effect on the activity of larvae at least until very shortly before the time of death. Differences in activity may therefore be excluded as affecting the results of the insecticide experiments.

An interesting observation on the effect of the disease on the behaviour of larvae may be reported here.   It was noticed that small numbers of infected larvae would crawl up on the filter paper in culture jars.   These larvae were invariably heavily infected and close to death.   Uninfected larvae only left the culture medium in cases of very heavy overcrowding.

9. The Effect of Disease on the Rate of Larval Oxygen Uptake.

The respiration of T.castaneum larvae was measured using a Warburg apparatus with flasks of about 2.5 ml. volume. Larvae were placed in the flasks, and the flasks were immersed in a water bath at 30.3°C. This temperature was chosen because it is suitable for the larvae, and very convenient in the calculation of the flask constant. The CO_2 in each flask was absorbed by 0.02 ml of 20% KOH in the centre well. A small roll of filter paper stood in the well increased the absorbtive surface area. For each reading, three flasks contained larvae, and two were used as thermo-barometers.

The number of larvae in the flasks had to be varied according to their size. 20 of the smallest larvae used gave a good deflection on the manometer in one hour, and 5 older larvae gave a full scale deflection in about 40 minutes. As the average oxygen consumption of a group of larvae was being measured, groups of less than five were not used as individual variation would then lead to great inaccuracy.

20 minutes were allowed for the equilibration of the flasks with the water bath. Manometer readings were then taken every 10 minutes, for one hour, and after correcting for thermobarometer changes, the readings were plotted on graph paper. The graphs of corrected manometer readings against time were of almost constant shape for larvae of all ages, and showed that oxygen consumption decreased considerably during the hour. It was not the intention to investigate the causes of this non-linearity, which may have been due for instance to the accomodation of the larvae to their new surroundings or to the absence

of food. Therefore, the closest fitting straight line was drawn by eye through the points. This line usually crossed the plotted curve near the 35 minute mark. The point at which it crossed the 60 minute mark was taken as the estimated total manometer reading for one hour's respiration, and further calculations were based on this figure.

Multiplication by the relevant flask constant gave the hourly oxygen consumption for each group of larvae, from which, using the dry weights measured in larvae from the same culture, the oxygen quotient could be found (Oxygen quotient = QO_2 = μl O_2 consumed/mgm. dry weight per hour).

Table 8 shows a sample set of data, and the treatment of the figures. The theory of this type of experiment and the formulae involved are given by Umbreit et.al (1957).

The oxygen quotient was measured throughout the development of four cultures of larvae:

       1. Control, uninfected a.

       2. Infected 15 days from egg laying.

       3. Control, uninfected b.

       4. Infected 11 days from egg laying.

Cultures 1 and 2 were from a single batch of eggs, as were cultures 3 and 4. Only about 60% of larvae in culture 2 became infected, so the results obtained were rejected. Table 9, below, shows the three estimates of the QO_2 of larvae of each age and state of infection from the remaining 3 cultures.

Table 8 The measurement of the oxygen consumption of _T.castaneum_ larvae aged 12 days, infected 11 days from egg laying.

Flask No.		3		6	9		10	12		
Man. No.		1		2	3		4	5		
Conts. KOH+		20 larvae		(T-b)	20 larvae		(T-b)	20 larvae		
Flask Vol.; µl		2698			2384			3093		
Cont. Vol.; µl		21			21			21		
Diff. = V_g; µl		2677			2363			3072		
Flask const. = K		0.241			0.213			0.276		
	Mins.	obs.	corr.		obs.	corr.		obs.	corr.	(T-b) mean
	0	0	0	0	1	0	0	1	0	0
Man.	10	-2	7.5	6	-1	7.5	5	-1	7.5	5.5
readings	20	-11	15.5	4	-10	15.5	5	-8	13.5	4.5
mm.	30	-14	20.5	6	-15	22.5	7	-11	18.5	6.5
	40	-18	24.5	6	-21	28.5	7	-16	23.5	6.5
	50	-22	30.5	8	-29	38.5	9	-20	29.5	8.5
	60	-31	35.5	4	-37	42.5	5	-28	33.5	4.5
Est'd total		38			45			36		
xK (= µl O_2)		9.158			9.585			9.936		
Dry wt. mgms.		0.372			0.372			0.372		
$Q\ O_2$		24.62			25.74			26.71		

90.

Table 9 CO_2 of T.castaneum larvae of different ages and states

of infection.

Days from egg laying	Culture 1 Control a				Culture 3 Control b				Culture 4 Infected 11 days from laying			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
10	–	–	–		23	19	19	20	23	26	26	25
12	–	–	–		27	21	20	23	25	26	27	26
14	29	30	–	29	31	28	26	28	28	27	24	26
16	33	33	45	37	22	31	20	24	41	48	41	43
18	36	23	24	28	35	31	22	30	30	33	29	31
20	22	24	18	21	21	25	22	23	18	15	20	18
22	18	22	23	21	24	18	20	21	14	19	14	16
24	11	10	3※	11	21	18	14	18	10	12	12	11

※   This reading was made out of interest, using larvae which had
entered the inactive state which occurs just prior to pupation,
and is not relevant to the present discussion.

Figures are shown to the nearest whole number; means are taken

from original readings which were calculated to one decimal place.

The table shows that there is considerable variation between

readings made at a given time on larvae from the same culture.   This

is particularly true of the 16 and 18 day readings, and is probably

largely due to the fact that the larvae in any small sample vary consider-

ably in weight, and that the larvae weighed were necessarily not the ones

used in the Warburg flasks.   About day 16, a great increase occurs in

the rate of weight gain, and small variations in this time lead to large

variations in weight for the following few days.

The mean values are plotted graphically in fig.43 , from which it can be seen that the readings from the two control cultures do show some correlation, and indicate that a rise in QO_2 occurs about the middle of larval development, which is followed by a drop from about 30 to about 15 in the last 6 days. Prepupal larvae have a comparatively low oxygen quotient of about 3.

The infected larvae showed a sudden increase in QO_2 on day 16 (5 days after infection), but the figure on this day of 43 is not greatly different from that of 37 for one of the control cultures at the same time. In view of the great variation between readings, the difference between control and uninfected larvae cannot be taken as significant. As the infection takes a grip on the larvae, their oxygen quotient falls. In the present case, the fall is almost coincident with that observed in uninfected larvae. The causes, however, are thought to be different; healthy larvae are nearing the end of their development, and are about to enter the inactive pupal state, while infected larvae are about to die. In both cases a slowing down of the metabolism, and consequent fall in oxygen consumption is indicated.

It must be emphasised that it was not possible to differentiate between the respiration of the parasite and that of the host; the measurements concern the oxygen consumption of the host parasite system as a whole.

Fig.43.

T. CASTANEUM:
LARVAL OXYGEN QUOTIENT

OXYGEN QUOTIENT

O   CONTROL A.

●   CONTROL B.

△   INFECTED, DAY 11

AGE: Days From Laying
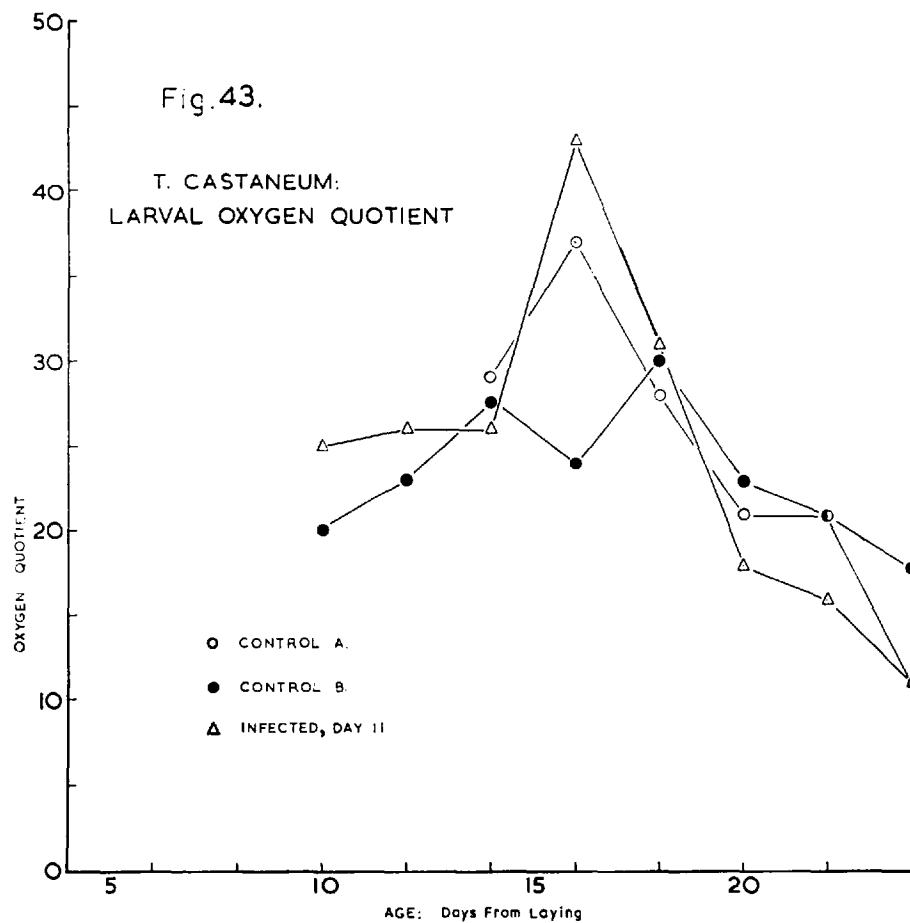
9. The Susceptibility to D.D.T. of Healthy and Infected T.castaneum Larvae.

Susceptibility to D.D.T. of T.castaneum was estimated by allowing the larvae to crawl on filter paper impregnated with D.D.T., and counting the number dead after 24 hours. Measurements of the median lethal dose (LD_{50}) were made throughout the larval life of uninfected insects, and throughout the development of the disease in infected ones.

The deposits of D.D.T. were prepared by pipetting solutions of the requisite concentration of D.D.T. in acetone onto 9cm. filter-papers, and evapourating the acetone in a draught of warm air. The filter-paper was suspended on the heads of pins to prevent the spread of the solution beyond its borders.

A series of D.D.T. solutions was prepared by serially diluting a 10% solution by a factor of three. Preliminary tests showed that deposits from 1 ml. of solution did not give adequate dosage response curves, so intermediate deposits were also prepared with 1.73 ($\sqrt{3}$) ml. of each solution. Thus, a logarithmic series of deposits was obtained each differing from the next by a factor of $\sqrt{3}$. This series was found to give a dosage response regression suitable for the calculation of the L.D.$_{50}$ in larvae of all ages.

The concentrations of D.D.T. solutions used, and the deposits obtained are shown in Table 10 below.

Table 10   The range of D.D.T. deposits used in experiments on the

susceptibility of larvae.

Dose Number	% D.D.T. in acetone	Volume applied in ml.	gms. of D.D.T. per paper	log. 10^5 x gms. of D.D.T.
1	10.0	1	0.100	4.0000
2	3.33	1.73	0.0576	3.7604
3		1	0.0333	3.5224
4	1.11	1.73	0.0192	3.2833
5		1	0.0111	3.0453
6	0.370	1.73	0.00640	2.8062
7		1	0.00370	2.5682
8	0.123	1.73	0.00213	2.3284
9		1	0.00123	2.0899
10	0.0413	1.73	0.000714	1.8537
11		1	0.000413	1.6160
12	0.0137	1.73	0.000237	1.3747
13		1	0.000137	1.1367
14	0.00457	1.73	0.0000791	0.8982
15		1	0.0000457	0.6599
16	0.00152	1.73	0.0000263	0.4200
17		1	0.0000152	0.1818

Larvae from four cultures were tested; two uninfected cultures, one exposed to infection at birth, and one infected 10 days from egg laying (= 6 days from birth, approximately).

On each filter-paper including a control paper without D.D.T.,
three batches of ten larvae were allowed to crawl for 24 hours.   Each
batch was enclosed in a nickel plated brass ring of one inch diameter.
After this period of exposure, the dead and "seriously affected" larvae
were counted.   "Seriously affected" larvae were defined as those which
showed convulsive twitches in the body, or which were incapable of
righting themselves in 30 seconds after being turned on their backs.
The proportions of dead and seriously affected larvae from the 3
replicate rings were pooled, and termed "mortality".

A series of observations were made at intervals during the
development of the larvae but when larvae reached the inactive state
just prior to pupation, and when control mortality exceeded 50%, the
observations were stopped.

All the experiments were carried out at a temperature of 20°C.

Owing to the high control mortality in infected groups, the
effective numbers of larvae in a given test were variable.   The results
are not therefore suitable for statistical analysis.

In Tables 11-14 the observed mortalities are recorded for each
of the tests, as well as the calculated L.D.$_{50}$.

The L.D.$_{50}$ was calculated as follows : first, observed
mortalities were corrected for control mortality using Abbott's formula.
The corrected results were transformed to angles, which were expected
to convert the S shaped regression to a straight line.   The slope of
this straight line, and its mean point were calculated; the L.D.$_{50}$
value was determined by substitution.   The formulae used are explained
in Snedecor (1956), and were confirmed as being suitable by Hague and

Murdie (personal communication). The L.D.$_{50}$ is expressed as log 10^5 x median lethal D.D.T. deposit in gms. per filter-paper.

Table 11   Numbers (/30) of T.castaneum larvae of different ages and states of infection dying when exposed to different doses.

(1) Control : Uninfected A.

Days from egg laying.	Control No D.D.T.	Dose number									LD$_{50}$
		15	14	13	12	11	10	9	8	7	
10	1	10	8	23	23	29	30	–	–	–	0.99
13	0	4	3	17	21	28	30	–	–	–	1.15
16	0	3	1	13	29	26	30	–	–	–	1.24
19	0	–	0	3	6	22	21	28	–	–	1.58
22	0	–	1	0	5	19	18	21	–	–	1.71
25	0	–	–	–	2	12	22	25	21	29	1.80

Table 12   Numbers (/30) of T.castaneum larvae at different ages and states of infection dying when exposed to different doses.

(2) Infected at 10 days.

Days from egg laying.	Control No D.D.T.	Dose number									LD$_{50}$
		15	14	13	12	11	10	9	8	7	
10	1	5	6	26	27	30	30	–	–	–	1.00
13	0	0	5	18	27	26	29	–	–	–	1.12
16	0	1	4	10	17	19	25	–	–	–	1.41
19	2	–	5	8	13	27	29	27	–	–	1.38
22	12	–	15	25	27	29	30	–	–	–	1.09
25	–	Over 50% control mortality						–	–	–	

Table 13   Numbers (/30) of T.castaneum larvae of different ages and
states of infection dying when exposed to different doses.

(3)   Control : Uninfected B.

Days from egg laying.	Control No D.D.T.	Dose number											LD$_{50}$
		16	15	14	13	12	11	10	9	8	7	6	5	
11	0	4	6	11	12	25	30	–	–	–	–	–	–	0.93
16	0	–	–	2	15	22	26	30	–	–	–	–	–	1.23
20	0	–	–	–	6	12	22	27	25	28	28	–	–	1.45
24	0	–	–	–	–	–	5	14	13	23	16	22	22	2.20

Table 14   Numbers (/30) of T.castaneum larvae of different ages and
states of infection dying when exposed to different doses.

(4)   Infected at Birth.

Days from egg laying.	Control No D.D.T.	Dose number								LD$_{50}$
		16	15	14	13	12	11	10	9	
6	0	2	8	5	17	30	–	–	–	0.93
12	5	4	4	4	12	25	27	30	–	1.23
14	6	7	7	4	15	20	21	30	–	1.45
17	14	–	11	10	17	20	21	29	30	2.20

Tables 11 and 13 show the results obtained using healthy larvae.
Only those doses which were expected to give mortalities between 0 and
100% were used.   The susceptibility of larvae as measured by this method

can be seen to decrease with age.   This is certainly due in part to their increased weight; the effect of activity is shown to be negligible by the previous experiment (p.80).

The susceptibility of larvae infected from birth (Table 14) was measured at irregular intervals, but does not differ considerably from that of control larvae, even when almost 50% of the larvae were moribund from disease.

Larvae infected 10 days from egg laying (Table 12) were more convenient for estimation owing to their greater size.   A slight increase in L.D.$_{50}$ (decrease in susceptibility) relative to uninfected larvae is recorded six days from exposure to infection, after which, susceptibility increases until death occurs after 9 to 15 days.

During this period of increased susceptibility, the larvae do not stop growing altogether (see section on rate of gain in weight p.74), so assuming that the quantity of D.D.T. absorbed during the 24 hours exposure is equal in infected and uninfected larvae, this increased susceptibility may be regarded as genuine, and not due to a decrease in the weight of infected larvae.

The results of the D.D.T. experiments are summarized graphically in Fig.44 .   These experiments show that the disease has little effect upon the susceptibility of larvae to D.D.T., at least until the larvae are moribund.

The L.D.$_{50}$ of larvae which have been infected for 12 days and almost 50% of which are moribund from disease is only about 4 times less than that of healthy larvae of the same age, and only very slightly less than that of healthy larvae of the same weight.

Fig. 44.

SUSCEPTIBILITY TO D.D.T.

○ UNINFECTED A.

● UNINFECTED B.

△ INFECTED 10 DAYS FROM LAYING

▽ INFECTED FROM HATCHING

L.D.$_{50}$ (log 10X gms. DDT per Filter paper)

AGE   From Egg Laying (Days)

96.
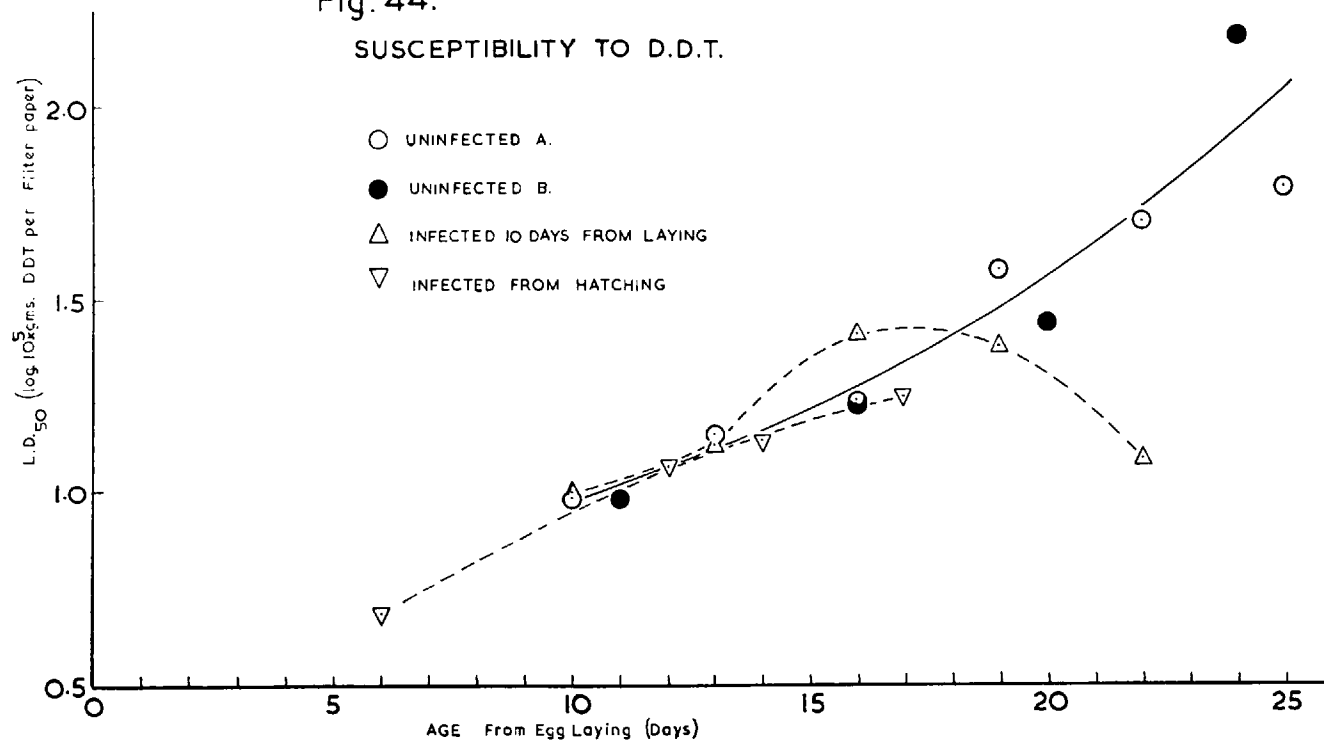
10.   The Effect of Disease on Surviving Adults.

In the following sets of data, the size, sex ratio, oviposition rate and longevity of surviving T.castaneum adults are discussed.   The size and sex ratio figures are supplementary data obtained from the dosage/mortality experiments; the oviposition rate and longevity were measured in adults surviving from cultures in which over 80% of larvae had died.   Survivors are defined as adults which are alive and normal in appearance on emergence from the pupa.

a)   The size of survivors

Estimates of the sizes of survivors were made by measuring the length of their elytra from the base of the scutellum to the tip. Measurements were made in the same way as those of the head capsule width of dead larvae, and are shown in table 15 below.

Table 15   The elytra length of T.castaneum adults surviving
                exposure to different doses of L.tribolii.

Dose spores per gm. survived.	No. measured	Elytra length(eyepiece units)	
		Mean	Range
0	32	34.89	32.0 - 37.5
10^2	38	34.86	31.5 - 38.0
10^3	38	34.54	31.0 - 37.0
10^4	32	34.44	31.5 - 37.0
10^5	12	33.96	25.0 - 36.5
10^6	No survivors	-	-

There is a distinct trend shown by these figures, indicating that healthy adults (average elytra length 34.89 units) are slightly

larger than those which have survived a heavy dose (average 33.96 units). The difference between the extreme means however is well within the range about either one, and is so small that no significance can be attached to the results.

It seems therefore that while disease in the larval state may affect the size of surviving adults, the difference is very small.

b) Sex ratio of survivors.

The sexes of the adults emerging from one of the dosage/mortality studies were determined after preservation for one week in 70% alcohol. This treatment made the hair lined pit on the fore femur of males more conspicuous. Table 16 shows the results obtained.

Table 16   The sexes of surviving T.castaneum adults.

Dose spores per gm. survived.	Males	Females
0	17	16
10^2	11	27
10^3	24	13
10^4	16	16
10^5	6	5
10^6	No survivors	
Total	74	77

The sex ratio of healthy T.castaneum is variable, and usually slightly biased in favour of females (Howe, 1956). No difference in the rates of survival of the two sexes can be detected here.

Many other counts were made, mainly on surviving pupae; as there was never any suggestion of differential mortality between the sexes it was not felt necessary to repeat the above observations.

c) Longevity of survivors

From a culture in which all the larvae had been infected, pupae were removed and separated into sexes. On hatching, the adults were placed in 2 × 1 in. tubes with about three gms. of flour. Deformed adults, which died within a few days of hatching were not used.

The tubes containing adults were examined at intervals of about five days, and dead beetles were recovered. The flour was changed every 15 days. The experiment was repeated once, and the longevity of adults from two uninfected cultures was measured as control.

The results of this experiment are shown in table 17 below, with the data converted for clarity to exact five day intervals.

The results show that there is no constant difference between the mortality of males and females. Adults from uninfected cultures had a high rate of survival for the first 50 days. Those from infected cultures, however, had a distinctly lower survival rate for the first 25-30 days, during which time about 25% of adults died. After 30 days, the mortality rate was approximately equal in surviving and uninfected adults over the period investigated.

Surviving adults may thus be separated into two distinct categories; those which are affected and have a reduced life span (about 25%), and those which are apparently unaffected with respect to their longevity (about 75%).

The data in table 17, converted to percentages are shown graphically in fig.45.

Table 17   The longevity of T.castaneum adults.

State	Sex		Days from hatching											
			0	5	10	15	20	25	30	35	40	45	50	100
Infected	Males	1	73	70	62	59	57	54	52	51	51	51	51	50
		2	22	22	22	17	17	17	15	15	15	15	15	-
	Females	1	77	75	69	65	58	56	54	53	53	52	52	51
		2	14	14	14	14	14	14	13	13	13	13	13	-
Uninfected	Males	1	76	73	69	69	69	69	69	69	68	67	67	63
		2	23	23	23	23	23	23	23	23	23	23	23	-
	Females	1	66	66	63	63	63	63	63	63	63	63	63	63
		2	26	26	26	26	26	26	26	26	26	26	26	-
Total Infected			186	181	167	155	146	141	134	132	132	131	131	
Total Uninfected			191	188	181	181	181	181	181	181	181	181	181	

Fig. 45.

LONGEVITY OF T. CASTANEUM ADULTS

O CONTROLS

△ SURVIVORS

BEETLES LIVING Percent

AGE: Days From Emergence

101.
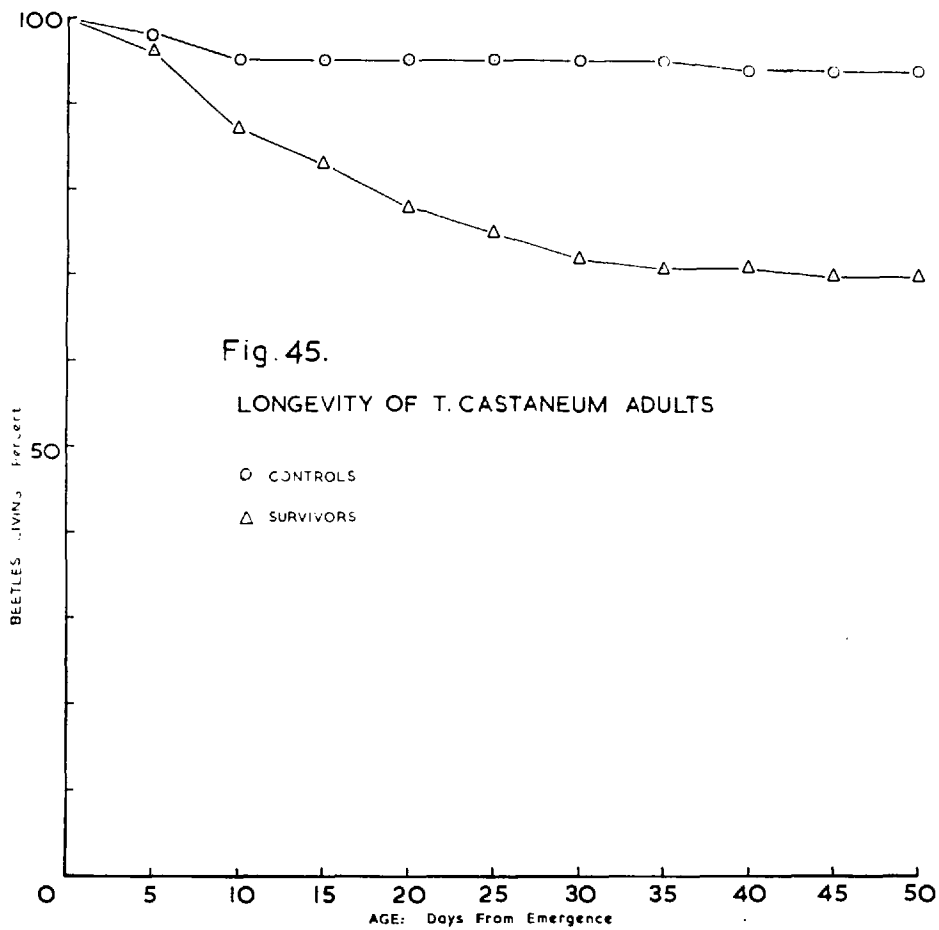
d) <u>Oviposition rate of survivors.</u>

In order fully to estimate the effect of a disease on an insect population, not only the mortality must be measured but also the reproductive potential of those individuals which survive or avoid infection.

Pupae from a culture in which about 90% of larvae had died were extracted and separated into sexes. Adults were collected on the day of emergence, paired, and incubated in 2 × 1 in. tubes containing about three gms. of flour. One pair was kept in each tube.

Every two to four days, the adults were removed and the flour was sieved to separate eggs. The eggs were counted, and the adults and flour replaced. The flour was changed every ten days.

As adults emerged over a number of days, and it was more practicable to make many counts every three days rather than a few every day, the intervals between counts were irregular. For clarity of presentation the results are tabulated as average egg counts for three day intervals.

A series of beetles from uninfected cultures were similarly treated and the numbers of eggs laid by the two groups were compared (tables 18 and 19, graph, fig. 46).

Table 18 shows that healthy females lay no eggs in the first three days after emergence, then lay at an increasing rate of up to 18 eggs per day (usually about 10) for the whole length of the observed period.

Table 18.   Eggs laid by T. castaneum females.

A.   Control.   Adults from healthy cultures.

| Pair No.	Days from emergence														Total
	3	6	9	12	15	18	21	24	27	30	33	36	39	42	
1	0	6	19	26	27	33	38	30	29	30	28	49	46	38	399
2	0	2	13	28	34	41	46	42	43	40	34	53	53	43	477
3	0	0	13	21	27	37	27	31	27	24	26	46	48	45	372
4	0	0	5	11	17	26	26	25	21	23	24	41	39	34	292
5	0	7	18	18	21	34	37	33	36	29	27	42	48	46	396
6	0	18	21	29	31	32	28	27	27	25	24	43	47	44	386
7	0	12	36	45	44	45	42	36	46	51	42	51	52	49	551
8	0	1	6	9	23	31	26	21	25	26	24	40	39	28	299
9	0	9	16	14	19	38	42	38	33	31	27	36	40	39	382
10	0	9	12	21	30	41	35	29	30	31	28	48	40	40	394
11	0	4	26	25	29	44	36	27	29	32	29	47	49	45	422
Total:	0	58	185	247	302	402	383	339	346	342	313	496	501	456	4370

Table 19.   Eggs laid by <u>T</u>. <u>castaneum</u> females

B.   Survivors

| | Days from emergence | | | | | | | | | | | | | | |
Pair No.	3	6	9	12	15	18	21	24	27	30	33	36	39	42	Total
1	0	13	27	36	38	41	37	32	38	40	29	28	36	33	428
2	0	11	27	36	40	47	47	41	41	38	36	35	35	30	464
3	0	9	28	44	48	49	26	29	38	44	32	31	42	31	451
4	0	0	5	19	29	36	34	26	28	34	31	32	36	28	338
5	0	4	15	31	35	30	21	20	28	29	23	21	19	35	311
6	0	0	0	0	0	0	X female died								0
7	0	9	22	35	41	48	40	30	32	32	33	36	41	29	428
8	0	0	X female died.												0
9	0	13	29	44	45	37	32	21	31	32	34	37	40	32	427
10	0	0	X female died												0
11	0	5	3	1	1	4	X female died								14
12	0	14	20	19	20	27	22	22	28	44	38	34	39	37	364
13	0	X female died													0
14	0	5	11	15	15	13	26	27	33	37	33	30	26	23	294
Total:	0	83	187	280	312	332	285	248	297	330	289	284	314	268	3519

As in the longevity experiment, the survivors may be clearly separated into two groups. The females of pairs 6, 8, 10, 11 and 13 all died within 21 days of emergence having laid no or very few eggs. The remaining 9 females lived for the whole period of the experiment, laying an average of 388 eggs each. This number is very similar to the average of 397 eggs laid by healthy females.

The graph, fig.46 shows the oviposition rates of healthy and surviving female adults. The two lines for surviving adults are based on average egg counts taken from the number of living survivors, and from the original number of survivors. It will be seen that there is no meaningful difference between the oviposition rates of living survivors and healthy beetles, but that when the total population surviving at emergence is considered a decreased oviposition rate is observed.

Fig. 46.

T. CASTANEUM:
OVIPOSITION RATE

○ CONTROL

△ SURVIVORS LIVING

▽ SURVIVORS TOTAL
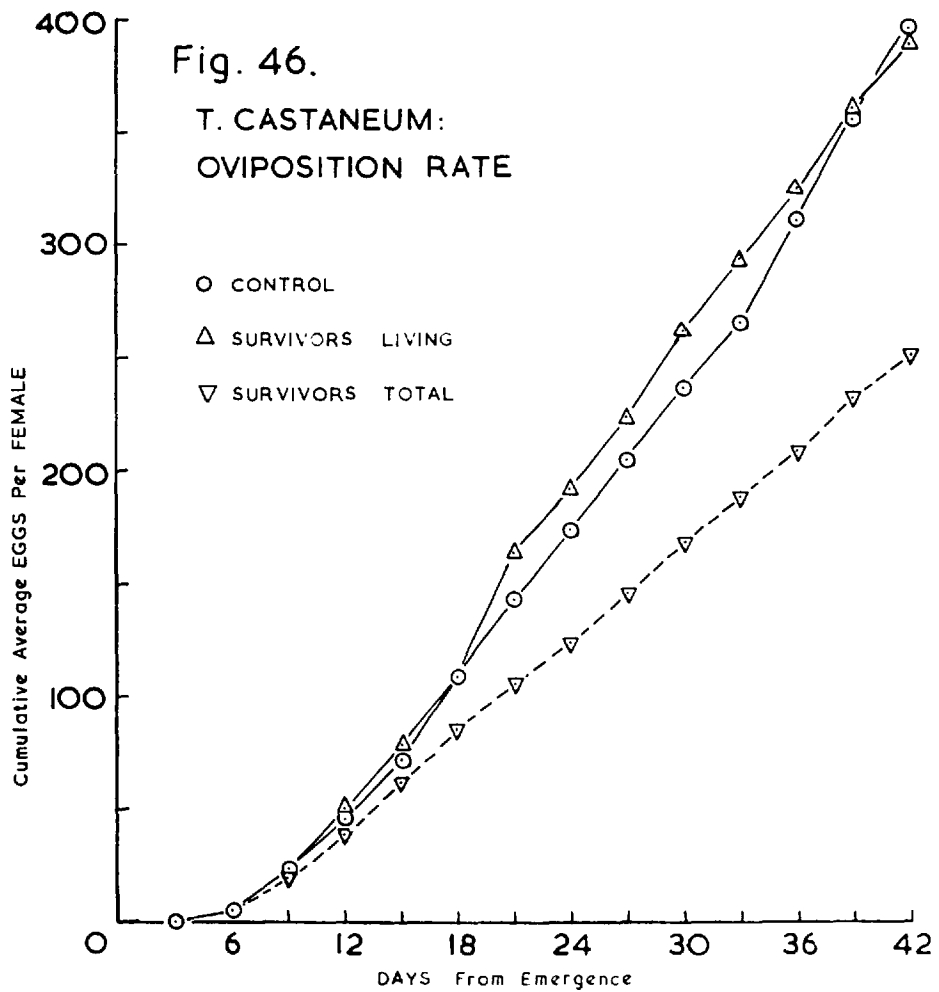
PART 2   FARINOCYSTIS TRIBOLII

1. Description of the Stages and Life-cycle of <u>Farinocystis tribolii</u>
   <u>Weiser</u>.

   All parasites resembling <u>F.tribolii</u> Weiser (1953) which were
examined during this work were identical as far as could be ascertained,
and their life cycles agreed closely with that described for
<u>Triboliocystis garnhami</u> Dissanaike (1955).   As it was felt possible
that the two descriptions might have arisen from different interpretations
of the life cycle of a single species, and in view of the slight
discrepancies between Dissanaike's observations and my own, it was felt
that a brief re-description of the life cycle, based on new observations
was called for.

   a)   <u>Description of the trophozoite</u>

      The sporozoite hatches in the mid-gut of the host, and
migrates by an unknown route to the fat body.   The development of the
trophozoite in this species differs considerably from that of
<u>Lymphotropha</u>, as within 24 hours of exposure of larvae to infection,
the trophozoite becomes an intracellular micronuclear schizont.

   b)   <u>Schizogony</u>

   There are two distinct cycles of schizogony in this species.
The micronuclear schizonts (Figs.47, 54) develop within the fat body and
grow up to 40 × 30 μ.   They are usually rounded, but may be flattened
and elongated.   The fully developed plasmodium contains up to 200
nuclei, each measuring 1-2 μ in diameter, and having a conspicuous
endosome.   The endosome is spherical, and varies considerably in size,

Explanation of Figs. 47-53.

F.tribolii.   Giemsa-colophonium.

47.   Section of 200 nucleate micronuclear schizont.

48.   Meroz oite.

49.a,b. Section of a 13 nucleate macronuclear schizont.

50.   Associating uninucleate gametocytes.

51.   Binucleate gametocytes in association.

52.   4-nucleate gametocytes in association.
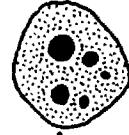
53.   Gametocyst with early gamete formation.
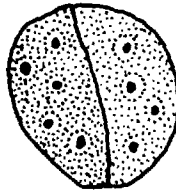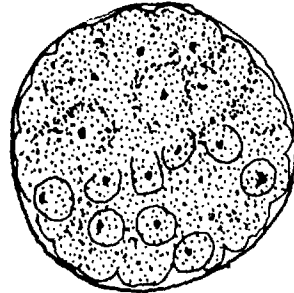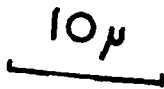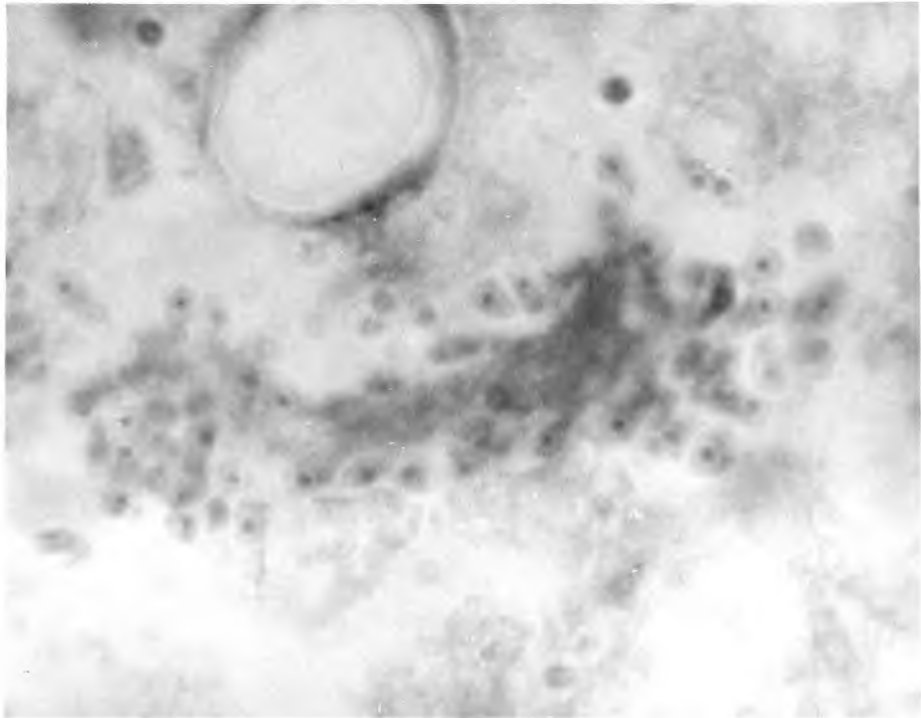
47

48

a

49

b

50   51   52

10μ

53

Fig. 54.

10μ
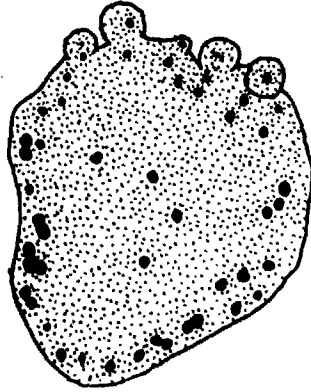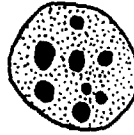
E.tribolii, the rupture of a micronuclear schizont.
Giemsa colophonium.

occupying almost the entire nuclear diameter in the undivided plasmodium, but becoming much smaller during the separation of merozoites. The majority of the nuclei are distributed peripherally in the plasmodium, but a few are scattered throughout. In section, the terminal part of a large plasmodium could easily be taken for the whole organism with nuclei distributed throughout the cytoplasm.

Micronuclear schizonts, and further stages of this species have apparently homogeneous cytoplasm which stains quite heavily with mercury bromophenol blue, and contains no granules of P.A.S. – positive material. The densely proteinaceous nature of the cytoplasm, without paraglycogen inclusions is unusual among gregarines, and contrasts sharply with Lymphotropha.

Merozoites are produced by budding from the surfaces, or by the rupture en masse of the plasmodium (Figs. 48 , 54). They are spherical, measuring 4 μ in diameter, or elongate, 3 × 5 μ, and may presumably develop into micro- or macronuclear schizonts.

Macronuclear schizonts (Figs. 49 , 55) are first seen three days after infection. They are more uniform in shape than the micro-nuclear schizonts, being almost spherical, 6 μ diam., to 20 × 15 μ, and containing up to 40 nuclei. The nuclei measure 3 μ in diameter, and have conspicuous endosomes occupying about $\frac{1}{2}$ of the diameter. The rupture of the macronuclear plasmodium was not observed, but gives rise to uninucleate merozoites, 4 μ in diameter, which, without further growth, become ovoid in shape, and associate as gametocytes.

Intermediate schizonts with a few large nuclei and many small ones, similar to those described by Dissanaike were also present.

Fig. 55.
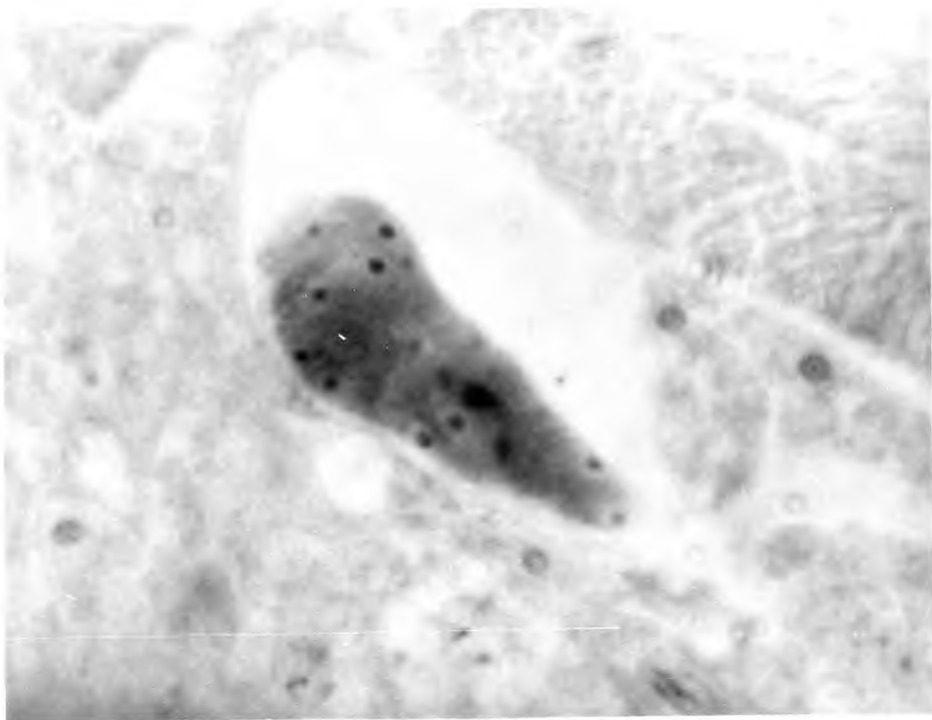
10μ

Macronuclear schizont.   Giemsa colophonium.

c) <u>Gametogeny</u> (Figs. 50-53, 56, 57)

Shortly after association of the gametocytes, their nuclei divide. Nuclear division is accompanied by considerable growth of the cells, so that the diameter of the pair increases from 5 μ to 15 μ. Occasionally one gametocyte surrounds the other as a mantle, but usually both maintain the same hemi-spherical shape.

The gametocyst, which is very thin, is laid down about the time of the last nuclear division of the gametocytes, when growth of the pair is complete. The cyst wall stains distinctly with P.A.S., indicating the presence of polysaccharide (Fig. 66)

Eight-nuclear gametocytes in association were commonly seen, but others with smaller, more numerous nuclei, which were difficult to count were also common. Eventually, each gametocyte produced 16 gametes. Thus, the mature gametocyst contains 32 gametes which can readily be counted in well serialised sections. This is twice the number implied, but not specifically stated by Dissanaike, and equals the number of "sporonts" observed by Weiser. The gametes are spherical and measure 3 μ in diameter.

Feulgen's nuclear stain was applied to the gametic and other stages, but while it gave a good reaction in the insect tissues, no reaction was visible in the parasites. This is not taken to imply an absence of D.N.A. in the nuclei, but that perhaps the small quantities present were not adequately preserved.

The fusion of the gametocytic cytoplasm before the separation of gametes was reported both by Weiser and Dissanaike. This phenomenon is difficult to confirm with certainty, though no membrane was visible

Fig.56.

10μ
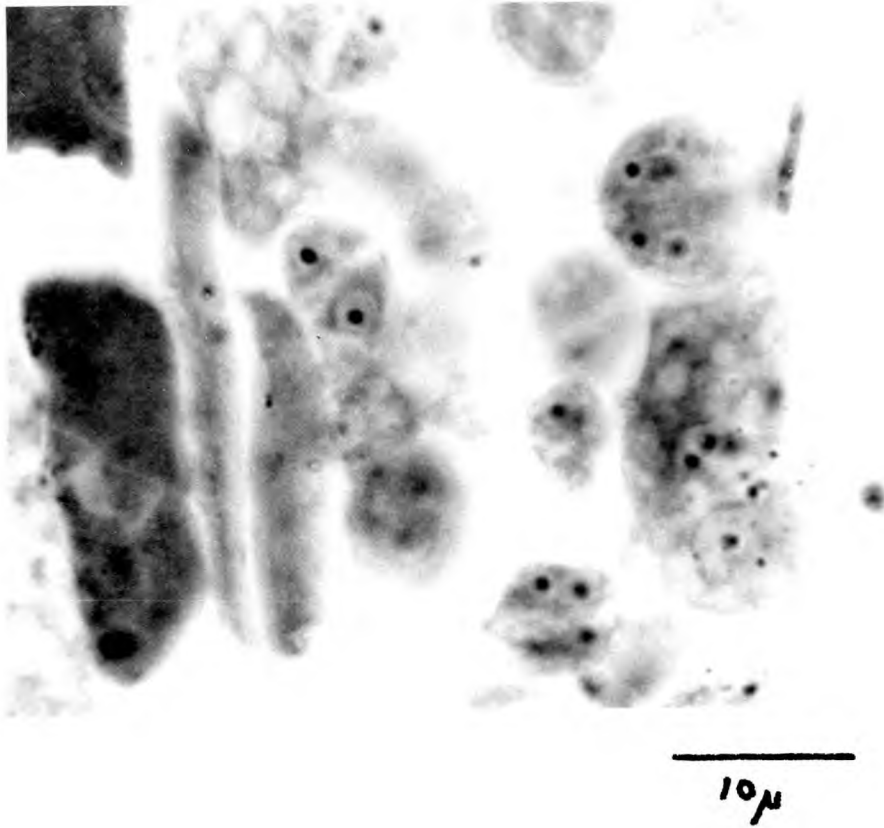
F.tribolii.   Uninucleate and plurinucleate gametocytes in
association.   Giemsa colophonium.

114.
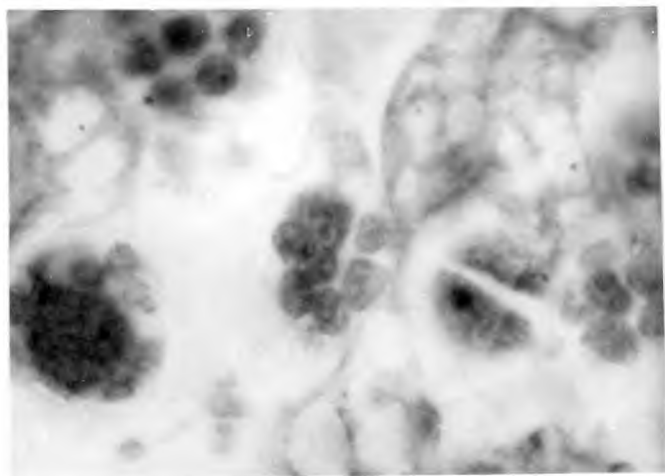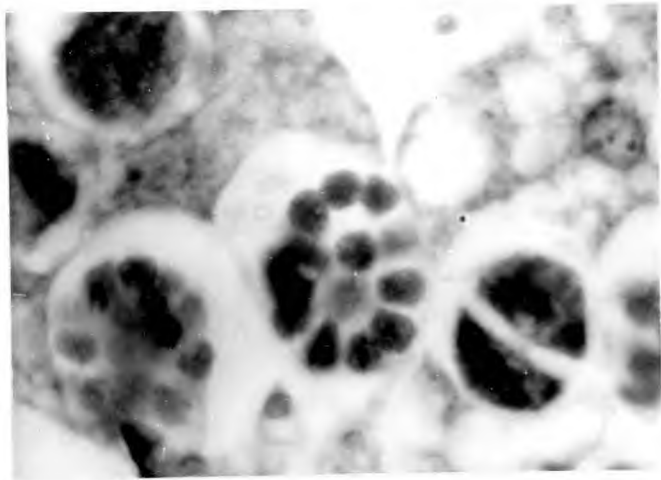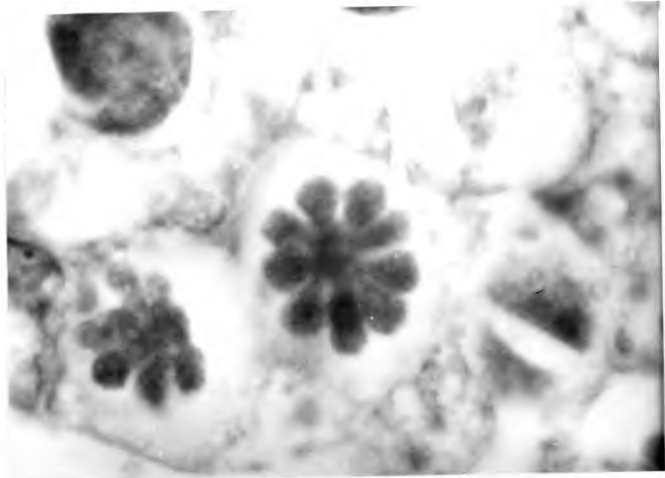
Fig.57 F.tribolii.   Three sections of a gametocyst
with 32 gametes.

between the gametocytes at this time, and the gametes were not divided into two groups after differentiation.

    d)   Sporogony   (Figs. 58-63, 64, 65).

Gametes, presumably from opposite gametocytes fuse to form up to 16 zygotes. This number is rarely reached, however, and the number of spores produced is usually eight or nine, as observed by Dissanaike. Zygotes are initially spherical, measuring 5 μ diameter. Their nucleus is large, 3 μ diam., and before nuclear division commences, the zygote becomes ovoid, and grows considerably to 9 μ by 4 μ.

The formation of the oocyst wall prevented observation of the division of the zygotic nucleus. The spore is elongated, lemon-shaped, measuring 13 μ × 7 μ, with prominent thickenings at the poles, which are distinctly plugged, not merely capped as in Lymphotropha.

In fresh preparations, the sporozoites are not visible within the spore. The only internal structures visible under these conditions are small vacuoles at either end, just within the spore plug. Sections of mature spores show eight sporozoites lying longitudinally, with four pointing each way. The sporozoites at this stage contain elongated granules of polysaccharide as indicated by the P.A.S. reaction.

The gametocyst often breaks during the development of the zygotes; in light infections, however, the cyst may remain intact until ingestion by a new host.

Explanation of Figs. 58-63.

F.tribolii. Sporogony.

58. a,b,c.   Three serial sections.of a gametocyst with
developing zygotes.   Giemsa colophonium.

59. Young spores. Giemsa colophonium.

60. Mature spore.   P.A.S. shows polysaccharide at poles and
in sporozoites.

61. Mature spore.   Fresh smear.   Negative phase.

62. Empty spore.   Mercury-bromophenol blue shows
protein in walls.

63. Mature spore (entire) Mercury-bromophenol blue shows
protein at poles.
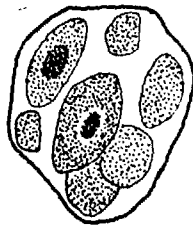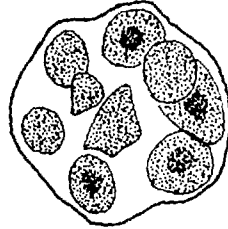
a   58   b   c
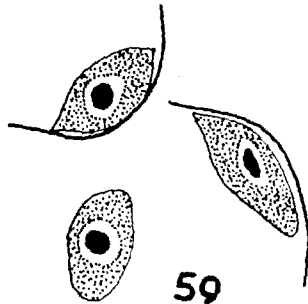
59

60
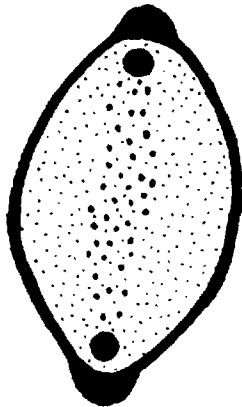
61   62   63
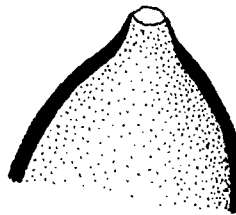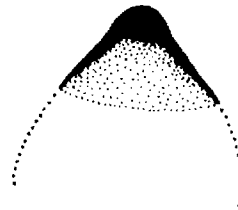
58, 59 : 10μ

60-63 : 5μ

Fig. 64.
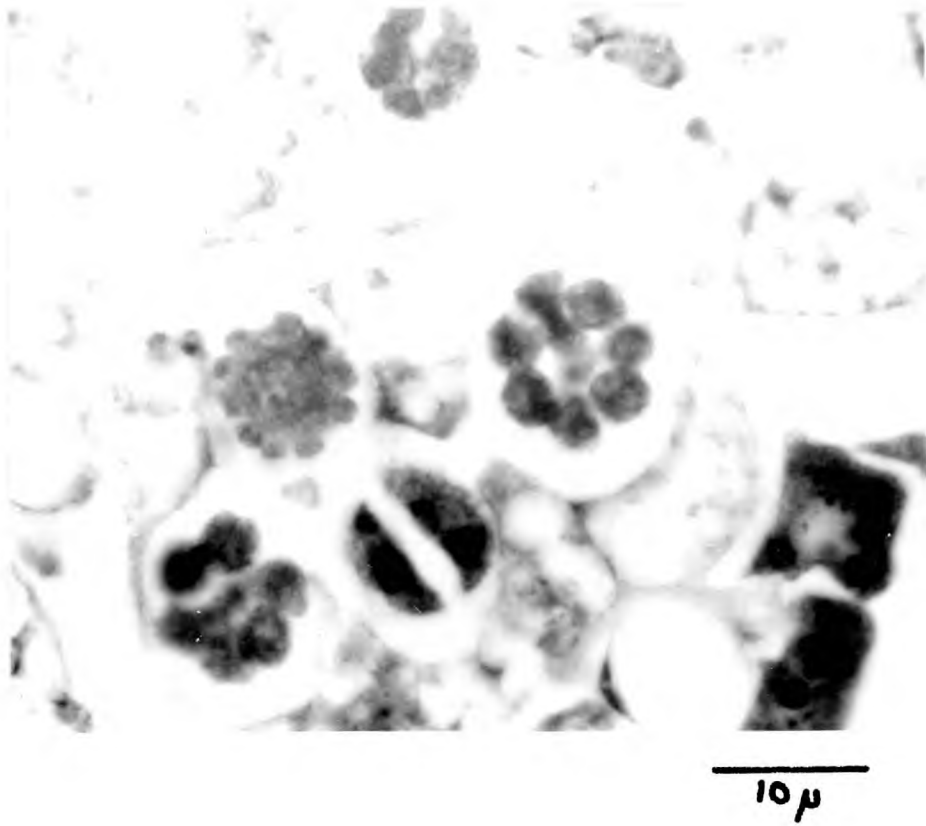
F.tribolii, zygotes.   Note also separating gametes.   Giemsa
         Colophonium.

Fig. 65.

20μ

F.tribolii developing spores.   Giemsa colophonium.

Fig. 66.
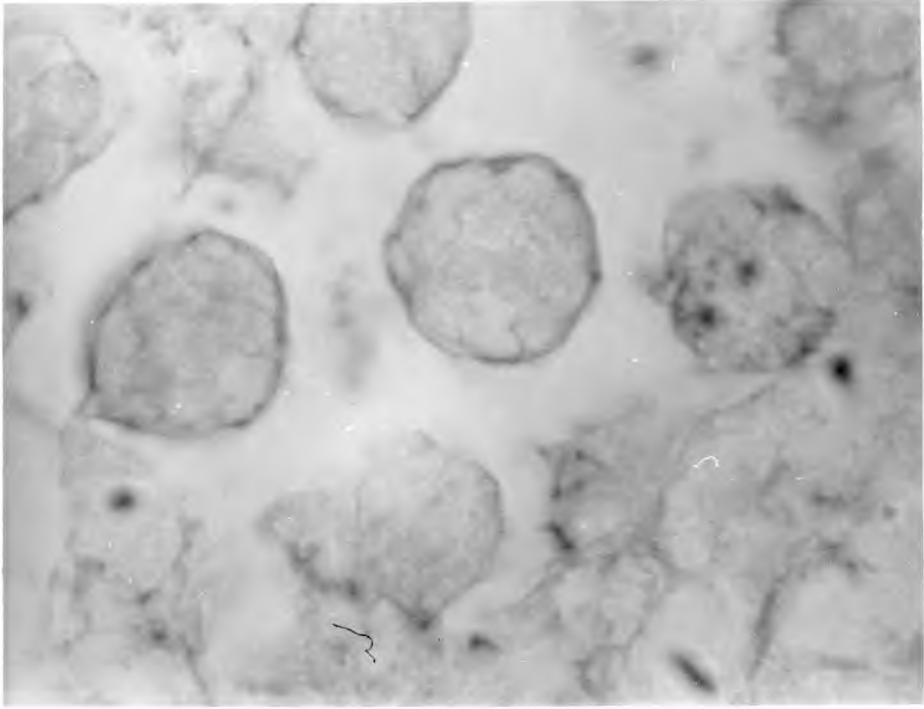
10μ

F.tribolii.   P.A.S. shows polysaccharide in gametocyst wall.

2. Notes on Pathology

The mode of infection of F.tribolii seems to be the same as that of L.tribolii. The progress of the infection is, however much more rapid, schizonts being produced within 24 hours, gametocysts within 4 days and mature spores within 7 days. Death commonly occurs after 10 days.

Larvae which are suffering, or which have died from this disease are in no way distinguishable macroscopically from those infected with L.tribolii, except by the absence of capsules.

Survivors from F.tribolii infections were not noticed; as adults may become infected, survival would be difficult to confirm.

Encapsulation did not occur in any of the F.tribolii infections examined. As the parasites are intracellular during their early development, only later stages could be encapsulated, and by the time these develop, the larvae are probably too weak to produce the large numbers of haemocytes required.

In one experiment where 3×10^4 L.tribolii spores per gm., of flour were mixed with those of F.tribolii in the ratio of 40:1, 100% mortality occurred in larvae exposed to the mixture, and the ratio of L.tribolii to F.tribolii spores in the resulting dead larvae was about 1:3. F.tribolii may thus rapidly supersede L.tribolii in mixed infections. This is probably accounted for by the rapid multiplication of F.tribolii within the host, and by the death of the larvae before many L.tribolii spores have matured.

It was however, more difficult to produce infections using old F.tribolii spores, than using L.tribolii spores of the same age.

As in the case of L.tribolii infections, the fat body is the only host organ noticeably affected. This degenerates as in the former case, and the albuminoid spheres and lipid globules fail to develop. Infected cells degenerate, and the lobes of the fat body break up. Reversal of the process was not observed in this case.

DISCUSSION

In any biological investigation, it is important that the taxonomic positions of the organisms studied should first be established. Data were obtained in the present work which permit a re-evaluation of the taxonomy of _Farinocystis tribolii_ and _Triboliocystis garnhami_.

Dissanaike (1955) described the life cycle of _T.garnhami_ in larvae of _T.castaneum_, beginning with trophozoites entering the fat body and becoming schizonts. The plasmodia of these micronuclear schizonts measured 7-25 μ in diameter, and the largest contained over 100 nuclei. Merozoites, released mainly by multiple fission, developed into macronuclear schizonts measuring 5 μ diameter to 15 by 12 μ, with up to 12 nuclei measuring 2 μ diam. Intermediate schizonts with a few large nuclei and many small ones were also seen.

The macronuclear merozoites, which were budded off from the surface of the parent plasmodium, were uninucleate, and associated as gametocytes in this condition. After association, the gametocytes grew, and their nuclei divided. A thin gametocyst was laid down about the stage when each gametocyte contained 8 nuclei. Occasionally one gametocyte would enclose the other in a cup-shaped mantle. The details of gametogony were not described, but eventually eight zygotes were formed, each of which developed into a spore (oocyst). Thus about eight (occasionally three to ten) spores were formed in each gametocyst.

The morphology and development of the organism described in the present work (as _Farinocystis tribolii_) conform closely to

Dissanaike's description, and examination of some of his original

slides convinced me that only one species was concerned. Micro-

nuclear schizonts were sometimes larger (up to 40 × 30 μ), and contained

up to 200 nuclei. Similarly, macronuclear schizonts measured up to

20 × 15 μ, and contained up to 40 nuclei. Gametogony was observed

in more detail in the present work. Nuclear division proceeded in

each gametocyte to a 16-nucleate stage, the gametic nuclei becoming

very small, and difficult to distinguish against the dense surrounding

cytoplasm. However, arising from the two gametocytes, 32 gametes were

regularly counted. The early zygotes usually numbered less than the

expected 16, and during their considerable growth, a number of these

degenerated. Thus only about 8 spores were formed in the mature game-

tocyst (occasionally upto 12).

It now remains to be shown that these parasites do not differ

significantly from <u>Farinocystis tribolii</u> described by Weiser (1953a).

Weiser's description does not include division of the macro-

nuclear schizonts into merozoites; rather, they were thought directly

to become gametocytes, and were thus plurinucleate on association.

This view was modified slightly when he stated (1953b) that macronuclear

schizonts divided into uninucleate merozoites, whose nuclei divided

usually to an 8-nuclear stage before they associated as "gametes".

A further contrast between Weiser's description and those outlined

above is his statement that 32 sporonts were formed in each gametocyst,

after which the wall broke down, and each developed into a spore.

However a photograph published in a later work (Weiser 1963) clearly

shows entire gametocysts, each containing about eight spores.

From the present additional observations it appears probable that Weiser's observations on the last stages of schizogony and on the early association of gametocytes were incomplete. Also his "sporonts" were presumably gametes, and owing to the breakdown of the gametocysts in some of his material he was unable to count the number of spores. The gametocysts commonly disintegrated in my own infections, and only remained intact when the density of parasites was quite low.

One further aspect of Weiser's description remains to be accounted for; this is his observation that gametocytes invariably associated in the asymmetrical manner described as occasional by Dissanaike and also observed in many of my own specimens. Such variability in the association of gametocytes is surprising, but the frequency of one or other type is probably a matter of strain variation, and the occurrence of the asymmetrical type of association in all three sets of observations strongly reinforces the argument that only one species is concerned.

In a personal communication, Weiser said, regarding his opinion that Farinocystis and Triboliocystis are identical, that this was supported by the structure of the spores, types of gametes, and all other facts including the host and locality. The cysts in Farinocystis had not been described, as in some materials they autolysed before the maturity of the spores. The type of gametes and number of nuclei forming spores were so specific for Farinocystis that if ever T.garnhami were to survive any earnest revision it would only be another species of the same genus.

The conclusions derived from the present work are in agreement with those of Weiser. It is believed that the present work clarifies the sequence of development at the end of macronuclear schizogony, and in the early stages of sporogony.

By way of summary, fig. 67 shows diagrammatically, and in simplified form, the life cycle of the organism, _Farinocystis tribolii_ Weiser, with which _Triboliocystis garnhami_ is thought to be synonymous. The figure shows how, in the light of new observations, the two original descriptions have been amplified and combined.

The life cycle of _L.tribolii_ contrasts sharply with that of _F.tribolii_, particularly in the development of large extracellular trophozoites, the single type and low rate of schizogony, and the association of large gametocytes which do not grow after pairing. The presence in the former of longitudinal striae in the trophozoites, and the abundant accumulation of paraglycogen are additional contrasting features. Some of these differing characteristics are no doubt associated with the extracellular habitat of the one, and the intra-cellular habitat of the other, and it is not clear which factors are important in the phylogenetic classification within the group.

Grassé's (1953) classification is the one into which both species fit most naturally. Grassé divided the order Neogregarina into five families, leaving four genera as _incertae sedis_.

The family Ophryocystidae Léger and Duboscq comprises a single genus, _Ophryocystis_ Schneider with nine species inhabiting the lumen of the Malpighian tubules of various beetles. The schizonts

Explanation of Fig.67

Summary of the life cycle of F.tribolii combining the observations of Weiser and Dissanaike with the present work.

a. Mature spore

b. Sporozoite

c. Micronuclear schizont with merozoites

d. Macronuclear schizont (Weiser's gametocytes)

e. Macronuclear merozoites

f. Associating gametocytes

g. Gametocytes, 4 nuclear

h. Gametocyst with 32 nuclei

i. Gametocyst with 32 gametes (Weiser's sporonts)

j. Gametocyst with developing zygotes

k. Young spore

Fig. 67

Weiser ⎯ ⎯
Dissanaike ⎯ · ⎯
Ashford ⎯ ⎯
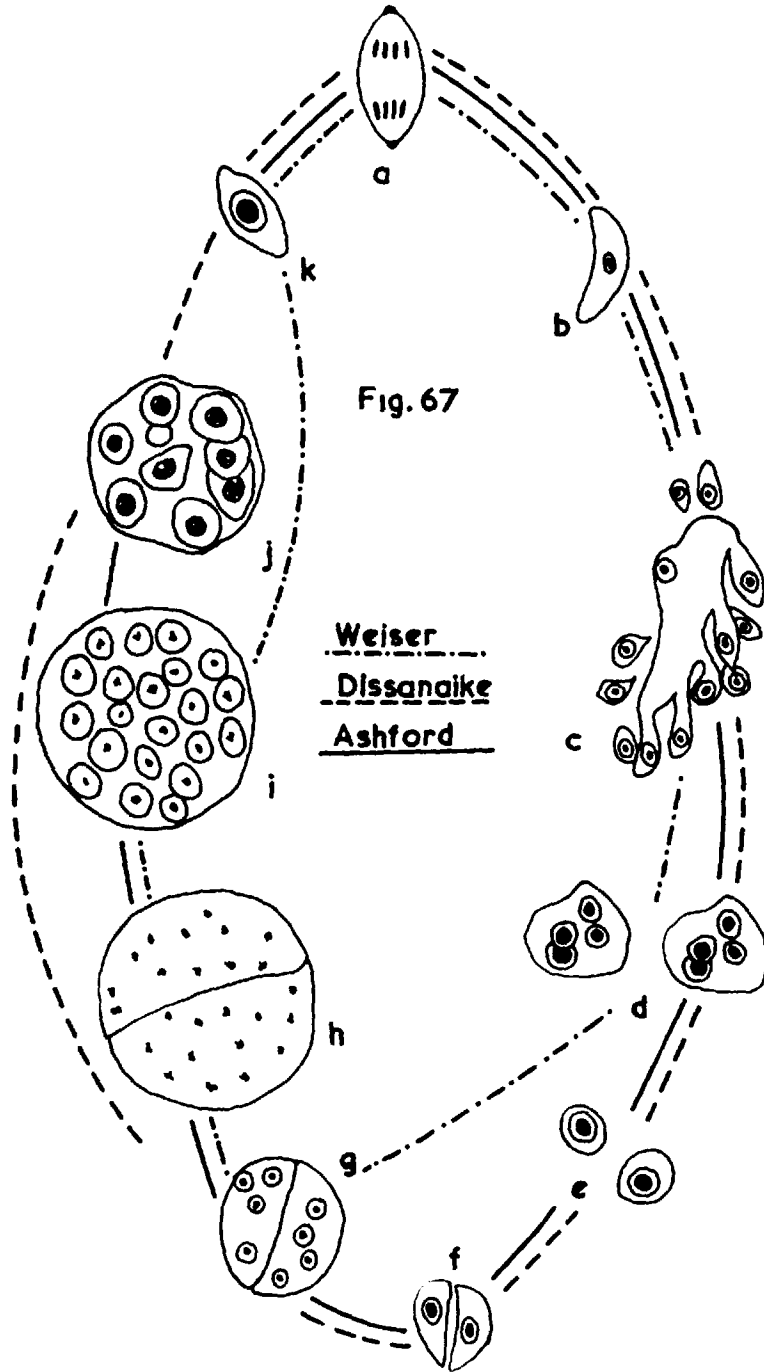
in this family are attached to the walls of the tubule by branching
processes which give them a mycetoid appearance. The family is
further characterised by the production of a single spore in each
gametocyst.

The Schizocystidae Léger and Duboscq are extracellular parasites
in the digestive tract of Malpighian tubules of Diptera and Hemiptera.
The family as originally erected included only the genus Schizocystis
Léger. The subsequent inclusion of Machadoella Reichenow (1935)
necessitated some extension of the familial diagnosis.

The species of the genus Schizocystis are remarkable among the
Neogregarina in that they exhibit anisogamy. The trophozoites are
large, vermiform, and develop directly into schizonts which are
attached to the gut walls of the host by means of a complex terminal
apparatus. When Reichenow (1935) erected the genus Machadoella, he
proposed that it should include a species of Schizocystis, S.spinigeri
Machado, as well as M.triatomae Reichenow. Machadoella differed from
Schizocystis in many ways, but also had vermiform trophozoites with
longitudinal striae and with an indistinct terminal apparatus.
Schizogony in Machadoella occurred by the irregular multiple fission
of a multinucleate plasmodium, in contrast to Schizocystis, where
division is more regular. MAchado (1913) had thought to observe
the association of binucleate gametocytes in S(=M) spinigeri, but
Reichenow refuted this, thinking that the binucleate bodies were schizonts,
whereas the gametocytes were uninucleate on association. He also
pointed out the difficulty in differentiating between nuclei and cyto-
plasmic inclusions of volutin. Mayer & Pifano (1949) in their partial

description of an unidentified species of _Machadoella_ also observed
the association of binucleate gametocytes, and were able to distinguish
these from schizonts by their different cytoplasmic reaction to
Giemsa's stain.

The organism described in the present work as _Lymphotropha_
tribolii appears to be closely related to _Machadoella_. The tropho-
zoites while not vermiform, are large, and have longitudinal striae.
The schizonts are very similar in appearance, as are the stages in
gametogony and sporogony, and the spores. The differences in the
shape of the trophozoite, the indistinctness of the striae in
Lymphotropha, the probable association of binucleate gametocytes in
Machadoella and the difference in habitat (_Machadoella_ inhabits the
Malpighian tubules of Triatomid bugs, and _Lymphotropha_, the haemocoele
of beetles) warrant the generic status of _Lymphotropha_ at least until
further related species are found, and the bases for the taxonomy of
the group are more clearly established.

It is interesting to note in this context that Bucher (1965)
recently found a neogregarine in the fat body of the grasshopper
Melanoplus bivattatus Say. Only the spores were seen for certain,
but these bore a very close resemblance to those of _Lymphotropha_,
and varied similarly in their number per gametocyst. Bucher tentatively
identified his specimens as being close to _Lipotropha_.

The members of the family Caulleryellidae Keilin live extra-
cellularly in the intestine of Diptera, and have flask-shaped trophozoites,
regularly bunched merozoites, and spores which differ considerably from

the species at present under consideration.

Grassé (1953) erected the family Syncystidae to include Syncystis mirabilis Schneider, which has spores furnished with spines at the poles, indicating its possible relationship with the Acanthosporinae among the Eugregarina.

The family Lipotrophidae Grassé includes three genera whose members, in the same way as Farinocystis, parasitise the fat body of insects intracellularly. Members of the genus Lipotropha Keilin have a single schizogonic cycle, and gematocytes which grow before, not after association. In the genus Mattesia, there are two cycles of schizogony as in Farinocystis, and the gametocytes associate before being fully grown, but only two spores are produced in each gametocyst. Although typically the spores do not have distinct plugs, Canning (1964) has shown that M.trogodermae has spores which closely resemble those of F.tribolii. The species of the genus Lipocystis Grell have two schizogonic cycles somewhat similar to those of Farinocystis, but between 100 and 200 thin walled spores are produced in each gametocyst, which ruptures shortly after the fusion of gametes.

Farinocystis must therefore retain its generic status, and occupy an intermediate position between Mattesia, with two spores per gametocyst and Lipocystis with many. The occurrence of two cycles of schizogony indicates relationships between Farinocystis and both of these genera; the spore structure resembles that of Mattesia, and the breakdown of the gametocyst indicates ties with Lipocystis.

Weiser's (1955) classification of the Schizogregarina divides the group (a sub-order in this case) into two families. The

Ophryocystidae, characterised by two distinguishable types of
schizogony are divided into two sub-families, Ophryocystinae, with
uninucleate associating gametocytes, and Machadoellinae with pluri-
nucleate gametocytes. The latter sub-family contains both Machadoella
and Farinocystis. Quite apart from the fact that the presence of a
second schizogony in Machadoella is in question (see above) and that
the present work shows Farinocystis to have uninucleate gametocytes,
the two genera differ greatly in their habitat, and in the structure
of their trophozoites. The fortuitous discovery of Lymphotropha,
which is clearly related to Machadoella, but not to Farinocystis
supports the argument that these two genera are not closely allied.

Weiser bases the sequence of his genera largely upon the
number of spores produced in each gametocyst. The present work has
shown this number to be variable in both of the species studied, and
not directly related to the number of gametic nuclei produced, which
is perhaps more constant.

There is a strong correlation between the habitat of neogre-
garines and their morphology, which is emphasised in Grassé's system.
Whether or not this is also correlated with phylogeny is open to question,
as is even the homophylogeny of the Neogregarina as a whole (Grassé, 1953).
The facility with which the two genera dealt with in the present work
fit into Grassé's system speaks at least for its practical convenience.

The present discovery of a new genus of neogregarine in such
a well-known beetle as Tribolium castaneum suggests that only a small
proportion of the living members of the order are known. Neogregarines
are usually pathogenic parasites which cause no externally visible

symptoms in live insects. They are therefore rare in healthy
populations, and are most likely to be discovered in laboratory cultures
where conditions are suitable for the development of epizootics.
The discovery of neogregarines in wild insect populations is an arduous
task, depending largely on fortune, but considerably more will have to be
known before any definitive classification of the group will be acceptable.

Detailed studies on the pathology of neogregarine diseases have
not yet been attempted. In the present work, a broad investigation
was undertaken into the relationships between the parasite, <u>L.tribolii</u>
and its host, <u>T.castaneum</u>, in order to find the gross effects of one
upon the other, and the elucidate the fields in which more detailed
investigation might be rewarding.

The disease caused by <u>L.tribolii</u> reached its fullest develop-
ment when larvae aged up to about 12 days from egg laying (8-9 days
from hatching) were exposed to infective doses of 10^6 spores per gram
or more. The effects of this full expression of the disease will
first be considered, then effects of smaller doses will be discussed.

During the incubation period of about six days following
exposure to infection, there were very few parasites to be found in the
haemocoele of the host, and no pathological effects could be detected.
During the following ten days, the parasites multiplied by schizogony
and increased in size. The fat bodies of infected larvae ceased to lay
down fat globules and albuminoid spheres, and the rate of gain in weight
decreased relative to that of healthy larvae.

The parasites accumulate large quantities of paraglycogen which must be synthesised from carbohydrate substances in the blood. The nature of the food of _Tribolium_ larvae is such that in order to obtain adequate protein for development, large excesses of carbohydrate (starch) must be ingested. It seems probable therefore that the massive accumulation of paraglycogen by the parasite has little harmful effect on the host.

While large volumes of carbohydrate are not stored by the insect, lipid and proteinaceous materials are. Infection with _L.tribolii_ has been shown to disrupt the storage of both these types of substance. The storage of relatively large quantities of lipid material by the parasites probably prevents their accumulation in the cells of the fat body. Proteins, however, are not present in large volumes in the parasite, and the lack of development, and degeneration of the albuminoid spheres of the host fat body requires more elaborate explanation. Perhaps the parasites in some way disrupt the normal processes of protein synthesis within the host, causing material which would normally be stored, to be required immediately.

While it was not possible to say at what stage feeding ceased, the reduction of the rate of larval weight gain may be explained by the parasites absorbing nutrients from the blood, thus preventing the accummulation of reserves in the fat body, and causing demands to be made on that organ, as happens when uninfected larvae are starved.

During this clinical period, when, in effect the larva is suffering from starvation, changes in its physiology are remarkably slight.

The oxygen quotient falls gradually as death approaches, and at no time differs significantly from that of healthy larvae, where a parallel reduction was observed as they completed their development and neared pupation.   This lack of change in oxygen consumption, which contrasts sharply with the findings of Sussman (1952) and Lysenko and Slam u (1961) in the case of fungal and bacterial diseases, is simultaneous with the decrease in growth rate, and is consistent with the absence of profound physiological changes.   The indication simply is, that the host's metabolism is slowed down by advancing parasitemia.

The susceptibility of healthy and diseased larvae to D.D.T. was measured by allowing them to crawl on insecticide-coated filter-paper for a standard time.   No significant change, not accountable by differences in weight, could be detected until shortly before the death by disease of infected larvae.   Shortly before death, these larvae did become slightly more susceptible than healthy larvae of the same weight.   Observations on the activity of healthy and infected larvae confirmed the validity of the D.D.T. experiments, and showed that the disease has no demonstrable effect on activity until within a few hours of death.

These experiments show that the disease caused by L.tribolii in T.castaneum probably has no marked effect on the nervous or muscular systems of the host until shortly before death.   The degeneration of the fat body has been shown to be reversible, so it is concluded that death is caused in part by the physical obstruction of the host's haemocoele, and in part by a form of starvation.

The observation that some larvae crawl up on filter-paper when heavily infected is of particular interest as it is the only indication of any change in their behaviour. It is well known that many insects crawl upwards before dying from disease (Steinhaus, 1949). Personal observations have regularly been made on the cockroaches Ectobius lapponicus Lin. and the cantharid beetles, Rhagonycha fulva Scop., clinging to the tips of grass stems after death by mycosis. In the present case, it has been shown that no increase in activity occurs in infected larvae, so the change in behaviour must be due to a change in orientation. It seems strange that insects suffering from the effects of starvation should leave the culture medium where ample food is available.

When larvae were exposed to doses of 10^4 or 10^5 spores per gm. nearly all became infected, but a varying proportion survived. In some cases the disease ran about the same course as above, death being delayed by a few days only. In other cases, death was delayed for up to two weeks. Some larvae pupated before dying, and others reached the adult state in a deformed condition. Of those which became externally normal adults, about 25% had greatly reduced longevtiy and female fecundity. The remaining 75% of the surviving adults lived apparently normal lives.

The size of insects at death, as the metamorphic stage, is only partly controlled by the dose to which they are exposed. Thus, while there is a minimum size at death, which is only produced by heavy doses, some larvae exposed to heavy doses develop almost the prepupal size before dying.

Survival and the postponement of death may be partly due to encapsulation. Capsules are formed round encysted gametocytes, isolating them and causing them to degenerate. It is improbable that encapsulation is the only factor important in survival, as the growing stages of the parasite; trophozoites, schizonts and merozoites are not affected. The fact that gametocysts isolated by encapsulation degenerate implies that some interchange still exists between the parasite and host, and by preventing this interchange the larva benefits.

The breakdown of the resistance of the parasite to encapsulation at the time of encystation is probably due to the change in the nature of the surface at this time, from a living cell wall to the dead cyst, and possibly also to the fact that the parasites are no longer motile. Why gametocysts with fully developed spores do not become encapsulated remains unexplained, except that usually the host is moribund at this stage.

Encapsulation has been suggested by Salt (1963b) to occur when parasites are in unnatural hosts. It is possible that the presence of Lymphotropha in Tribolium is the result of contact between the parasite and host under the abnormal conditions of laboratory culture. The resistance of T.confusum to infection might support the idea that L.tribolii is not completely suited to the other members of the genus. However, until the hypothetical natural host is discovered this is mere speculation.

Factors other than encapsulation must be operative in the survival of large larvae of T.castaneum and all larvae of T.confusum,

where sporozoites hatch, but infection does not succeed.   Phagocytosis

was not observed in L.tribolii infections, though both Dissanaike

and Weiser described it in F.tribolii.   It seems that phagocytosis

and encapsulation by insect haemocytes are closely related phenomena,

being the result of a surface stimulus on the same cells.   Only if the

parasite or foreign body is too large to be ingested by a single cell

is it encapsulated.

Some speculation on the effects of L.tribolii on T.castaneum

populations may be made from the dosage/mortality figures and from the

studies of the effects of disease on survivors.   An experimental study

along similar lines was conducted by Park (1948) who accumulated an

enormous amount of data on T.castaneum populations infected with

Adelina tribolii.   As this worker renewed the culture medium and

removed all dead insects every 30 days, he placed the coccidian at a

considerable disadvantage.   Nevertheless, 8 out of 40 of his

T.castaneum populations died out during the four year study period.

Under sterile conditions, T.castaneum usually eliminated T.confusum

from mixed populations, but the presence of the parasite reversed

this trend, and T.confusum survived at the expense of T.castaneum.

The present experiments would lead to the following possible

conclusions.   In the case of a very low rate of infection in a well

dispersed population with unlimited food, the disease would remain

enzootic, causing very few deaths, but perhaps slightly reducing the

average fecundity of females by greatly reducing the fecundity and

life span of some of them.

In any suitable natural medium under normal circumstances, a peak population of healthy T.castaneum would be reached within a few generations. The high rate of reproduction and low rate of dispersal would ensure this. With the build up of a high population density, the chances of dead individuals being eaten would increase. The disease would thus spread, and become epizootic.

In the case of a population suffering from an epizootic, the adults mostly have a normal life span, and lay the usual number of eggs. The eggs are viable in the normal proportions, but very few larvae develop to maturity. Of those that do, a proportion of the resulting adults have very low fecundity, and greatly reduced longevity. The remainder live and reproduce normally.

In the presence of many of their dead fellows, larvae of the third generation have small chance of survival. Thus, even when the original density of insects and rate of infection are low, epizootic conditions and the extinction of the population would be expected to occur within very few generations. The affected substrate would be considerably altered by the beetles which had developed on it, but would remain contaminated with viable spores for many months, preventing any further infestation by the beetles.

The work presented in this thesis has shown that T.castaneum is susceptible to a number of pathogenic protozoa. L.tribolii has been studied in some detail, and has been shown to be capable of

preventing increase in populations. However, as the insects are
unaffected by the parasite from the age of 15 days from egg laying,
economic application of spores of the protozoan would not be expected
to be profitable except as introducing an additional natural control
factor into a population. The benefit would only accrue over a
long term, and stored products pests usually require more rapidly
effective measures for their economic control.

LIST OF REFERENCES

ASHFORD, R.W. (1965) Lymphotropha triboli gen. nov., sp. nov., Neogregarinida, Schizocystidae, from the haemococle of Tribolium castaneum (Herbst.). J. Protozool., 12: 609-615.

BENZ, G. (1963) Physiopathology and histochemistry. In Steinhaus, E. A., Insect Pathology, Academic Press Inc., New York, Vol. 1: 299-331.

BHATIA, B. (1937) On Adelina tribolii, a coccidian parasite of Tribolium ferrugineum F. Parasitology, 29: 239-246.

BRONSKILL, J.F. (1960) The capsule and its relation to the embryogenesis of the ichneumonid parasitoid Mesoleius tenthredinis Morl., in the larch sawfly, Pristophora erichsonii (Htg.). Canad. J. Zool. 38: 769-775.

BUCHER, G.E. (1965) Schizogregarine infection in grasshoppers. J. Invert. Path. 8: 127-129.

BUCHER, G.E., and MORSE, P.M. (1963) Precision estimates of the median lethal dose of insect pathogens. J. Insect Path. 5: 289-308.

BUTSCHLI, O. (1885) Bemerkungen über einen dem Glycogen verwandten Körper in den Gregarinen. Z. Biol. 21: 603 -

CANNING, E.U. (1964) Observations on the life history of Mattesia trogodermae so. n., a schizogregarine parasite of the fat body of the khapra beetle, Trogoderma granarium Everts. J. Insect Path. 6: 305-317.

CONFAVREUX, M. (1961) A study of two schizogregarine parasites of Tribolium. Undergraduate thesis, Imperial College, London.

COTTON, R.T. (1956) Pests of Stored Grain and Grain Products, Revised ed., Burgess Publishing Co., Minneapolis, Minn., 306 pp.

DANIELS, M.I. (1938) A cytological study of the gregarine parasites of Tenebrio molitor using the ultra-centrifuge. Q. Jl microsc. Sci. 80: 293-320.

DISSANAIKE, A.S. (1955) A new schizogregarine, Triboliocystis garnhami n.g., n.sp., and a new microsporidian, Nosema buckleyi n.sp., from the fat body of the flour beetle, Tribolium castaneum. J. Protozool. 2: 150-156.

DOFLEIN, F. and REICHENOW, E. (1953) Lehrbuch der Protozoenkunde, Vol. 2; Spezielle Naturgeschichte der Protozoen, second half: Sporozoa und Ciliophora, Gustav Fischer, Jena, 6th., edition, pp. 777-1214.

DORAN D.J. and FARR, M.L. (1962) Excystation of the poultry coccidium Eimeria acervulina. J. Protozool. 9: 154-161.

FINLAYSON, L.H. (1950) Mortality of Laemophloeus (Coleoptera, Cucujidae) infected with Mattesia dispora Naville (Protozoa, Schizogregarinaria). Parasitology, 40: 261-264.

FISHER, F.M., and SANBORN, R.C. (1962) Observations on the susceptibility of some insects to Nosema (Microsporidia: Sporozoa). J. Parasit. 48: 926-932

FISHER, R.A. (1963) Bioassay of microbial pesticides. In Zweig, G., Pesticides, Plant Growth Regulators and Food Additives, Academic Press Inc., New York, Vol. 1: 425-442.

FOERSTER, H. (1938a) Gregarinen in Schlesischen Insekten. Z. ParasitKde 10: 157-209.

FOERSTER, H. (1938b) Beobachtungen über das Auftreten von Gregarinen in Insekten. Z. ParasitKde 10: 644-673.

GHORE, E. (1943) Untersuchungen über den plasmatischen Feinbau der Gregarinen mit besonderer Berücksichtigung der Sexualitätsverhältnisse. Arch. Protistenk. 96: 297-324.

GOOD, N.E. (1933) Biology of the flour beetles Tribolium confusum Duval and T. ferrugineum Fab. J. agric. Res. 46: 327-334.

GOOD, N.E. (1936) The Flour Beetles of the Genus Tribolium. Technical bulletin no. 498, United States Department of Agriculture, 57 pp.

GRASSE, P.P. (1953) Classe des Grégarinomorphes.
In Grassé, P.P. Traité de Zoologie, Masson,
Paris, Vol. 1, part 2: 550-689.

HALSTEAD, D.G.H. (1963) External sex differences in
stored products coleoptera. Bull. ent. Res.
54: 119-134.

HINTON, H.E. (1942) Secondary sexual characters of
Tribolium. Nature, Lond., 149: 500-501.

HINTON, H.E. (1945) A Monograph of the Beetles Associated
with Stored Products. The Trustees of the
British Museum, London, Vol. 1, 433 pp.

HONIGBERG, B.M., BALAMUTH, W., BOVEE, E.C., CORLISS, J.O.,
GOJDICS, M., HALL, R.P., KUDO, R.R., LEVINE, N.D.,
LOEBLIGH, A.R., WEISER, J., and WENRICH, D.H.
(1964) A revised classification of the phylum
Protozoa. J. Protozool. 11: 7-20.

HOWE, R.W. (1956) The effects of temperature and
humidity on the rate of development and
mortality of Tribolium castaneum (Herbst.)
(Col., Tenebrionidae). Ann. appl. Biol. 44:
356-368.

HOWE, R.W. (1960) The effects of temperature and
humidity on the rate of development and mortality
of Tribolium confusum Duval. Ann. appl. Biol. 48:
363-376.

HOWE, R.W. (1962) The effects of temperature and
humidity on the oviposition rate of Tribolium
castaneum (Herbst.). Bull. ent. Res. 53: 301-310.

HUGER, A. (1960) Untersuchungen zur Pathologie einer
mikrosporidiose von Agrotis segetum (Schiff.)
(Lepidopt., Noctuidae), verursacht durch
Nosema perezioides nov. spec.
Z. PflKrankh. PflPath. PflSchutz, 67: 65-77.

ISHII, S. (1914) On four polycystid gregarines from the
intestine of Tribolium ferrugineum F.
Annotnes zool. jap. 8: 435-441.

JAFRI, R.H. (1961) Synergistic action of radiation and
of Bacillus thuringiensis toxin on protozoan
diseases of insects. In Progress in Protozoology,
Abstracts of the First International Conference
on Protozology, Prague: 510-515,

JAFRI, R.H. (1964) Influences of pathogens on the life span of irradiated insects. Revue Path. vég. Ent. agric. Fr. 43: 37-51.

JENNINGS, J.B. (1962) Periodic acid - Schiff technique for the demonstration of extra and intra-cellular stages of eugragarine and coccidian protozoa. Nature, Lond. 192: 473-474.

JOYET-LAVERGNE, P. (1926a) Les colorations vitales des grégarines et les caractères de sexualisation du cytoplasme. C. r. hebd. Séanc. Acad. Sci., Paris, 182: 1295-1297.

JOYET-LAVERGNE, P. (1926b) Recherches sur le cytoplasme des sporozoaires. Archs Anat. microsc., 22:

KANE, J. (Undated) A method for culturing Tribolium castaneum from clean eggs. Internal circular, Pest Infestation Laboratories, Slough.

KOZLOFF, E.N. (1958) Development and dehiscence of gametocysts of the gregarine Pyxinia crystalligera Frenzel. J. Protozool. 5: 171-174.

KOZLOFF, E.N., and BROWN, H.E. (1963) Influence of enzymes on germination of sporocysts of the gregarine Pyxinia crystalligera Frenzel. J. Protozool. 10: 377-379.

LAIRD, M. (1959) Gregarines from laboratory clonies of flour beetles Tribolium castaneum (Herbst) and T. confusum Duval, at Montreal. Canad. J. Zool. 37: 378-381.

LEGER, L. (1900) Sur un nouvreau sporozoaire des larves de Diptères. C. r. hebd. Séanc. Acad. Sci., Paris, 131: 722-724.

LEGER, L., and DUBOSCQ, O. (1908) L'evolution schizogonique d'Aggregata (Eucoccidium) eberthi Labbé. Arch. Protistenk. 12: 44-108.

LEVINE, N.D. (1961) Problems in the systematics of the "Sporozoa". J. Protozool. 8: 442-451.

LEVINE, N.D. (Ed.) (1966) Discussion of the classification of the Protozoa at the Second International Conference on Protozoology, London, England. *J. Protozool.* **13**: 189-195.

LIPA, J.J., and STEINHAUS, E.A. (1962) Further reports on the identification of protozoa pathogenic for insects. *Acta. parasit. pol.* **10**: 165-175.

LYSENKO, O., and SLAMA, K. (1959) The relation between oxygen consumption and bacterial infection in sawflies. *J. Insect Pathol.* **1**: 184-188.

MACHADO, A. (1913) Sobre o ciclo evolutivo de *Schizocystis spinigeri* n. sp., gregarina do intestina de uma especie de *Spiniger*. *Mems Inst. Oswaldo Cruz*, **5**: 5-15.

MAYER, M., and PIFANO, F. (1949) Sobre una schizogregarine de *Eutriatoma maculata* de Venezuela. *Arches venez. Patol. trop. Parasit. med.* **1**: 333-340.

Mc LAUGHLIN, R.E. (1965) Some relationships between the Boll Weevil *Anthonomus grandis* Boheman and *Mattesia grandis* Mc Laughlin (Protozoa: Neogregarinida). *J. Invert. Path.* **7**: 464-473.

MUNRO, J.W. (1966) *Pests of Stored Products*, Hutchinson, London, 234 pp.

PANTIN, C.F.A. (1946) *Notes on Microscopical Technique for Zoologists*, University Press, Cambridge, 77 pp.

PARK, T. (1948) Experimental studies of interspecies competition. 1, Competition between populations of the flour beetles, *T. confusum* Duval and *T. castaneum* (Herbst). *Ecol. Monogr.* **18**: 265-308.

PEARSE, A.G.E. (1960) *Histochemistry, Theoretical and Applied*, J. and A. Churchill Ltd., London, 998 pp.

REICHENOW, E. (1935) *Machadoella triatomae* n. g., n. sp., eine Schizogregarine aus *Triatoma dimidiata*. *Arch. Protistenk.* **84**: 431-445.

ROSICKY, B. (1951) Nosematosis of _Otiorrhynchus ligustici_.
2, The influence of parasitism by _Nosema otiorrhynchi_ Weiser on the susceptibility of the beetles to insecticides. Věst. čsl. zool. Spol. _15_: 219-234.

SALT, G. (1963a) The defence reactions of insects to metazoan parasites. _Parasitology_ _53_: 527-642.

SALT, G. (1963b) Experimental studies in insect parasitism. XII, The reactions of six exopterygote insects to an alien parasite. J. Insect Physiol. _9_: 647-669.

SHORTT, H.E., and COOPER, W. (1948) Staining of microscopical sections containing protozoal parasites by a modification of Mc Namara's method. Trans. R. Soc. trop. Med. Hyg. _41_: 427-428.

SNEDECOR, G.W. (1956) _Statistical Methods Applied to Agriculture and Botany_. Iowa State University Press, Ames, Iowa, 5th. Ed., 534 pp.

STANLEY, J. (1961) Two techniques of use in the control of _Triboliocystis garnhami_. Canad. J. Zool. _39_: 121-122.

STANLEY, J. (1964) Washing the eggs of _Tribolium_ for gregarine control. Canad. J. Zool. _42_: 920.

STEINHAUS, E.A. (1947) _Insect Microbiology_. Comstock Publishing Company, Ithaca, New York, 763 pp.

STEINHAUS, E.A. (1949) _Principles of Insect pathology_. Mc Graw Hill Book Company, New York, 757 pp.

STEINHAUS, E.A. (1956a) Microbial control - the emergence of an idea. _Hilgardia_ _26_: 107-159.

STEINHAUS, E.A. (1956b) Potentialities for microbial control of insects. J. agric. Fd. Chem. _4_: 676-680.

STEINHAUS, E.A., and MARSH, G. (1962) Report of diagknoses of diseased insects 1951-1961. _Hilgardia_ _33_: 349-490.

STEPHENS, J.M. (1963) Immunity in insects. In Steinhaus, E.A., Insect Pathology, Academic Press Inc., New York, Vol. 1: 273-297.

SUMNER, R. (1936) Relation of gregarines to growth and longevity in the mealworm Tenebrio molitor L. Ann. ent. Soc. Am. 29: 645-648.

SUSSMAN, A.S. (1952) Studies of an insect mycosis. IV, The physiology of the host-parasite relationships of Platysamia cecropiae and Aspergillus flavus. Mycologia 44: 493-505.

THEODORIDES, J. (1955) Les eugrégarines du genre Gregarina parasites des coléoptéres ténébrionides. Annls. Parasit. hum. comp. 30: 5-21.

THOMPSON, H.M. (1960) A list and brief description of the Microsporidia infecting insects. J. Insect Path. 2: 346-385.

TUZET, O. (1931) Une grégarine parasite de Bythinia tentaculata L., Gonospora dubosequi n.g., n.sp. Archs Zool. exp. gén. 71: 16-20.

TYLER, P.S. (1962) On an infection of Tribolium spp. by the sporozoan Triboliocystis garnhami Dissonaike. J. Insect Path. 4: 270-272.

UMBREIT, W.W., BURRIS, R.H., and STAUFFER, J.F. (1957) Manometric Techniques. Burgess Publishing Company, Minneapolis, Minn., 338 pp.

VEBER, J., and JASIC, J. (1961) Microsporidia as a factor in reducing fecundity in insects. J. Insect Path. 3: 103-111.

VINCENT, M. (1922) On the life history of a new gregarine: Pyxinia anobii n. sp., intestinal parasite of Anobium paniceum L. (Coleoptera). Parasitology 14: 299-306.

WATSON, M.E. (1916) Studies on gregarines. Illinois biol. Monogr. 2: 1-258.

WEISER, J. (1953a) Schizogregariny z hmyzu škodícího zásobám mouky. Věst. čsl. Zool. Spol. 17: 199-212.

WEISER, J. (1953b) Parasites of the flour beetle, Tribolium castaneum. Proc. Soc. Protozool. 4: 21.

WEISER, J. (1955a) A new classification of the
Schizogregarina. J. protozool. 2: 6-12.

WEISER, J. (1955b) A new classification of the
Schizogregarina: a correction.
J. Protozool. 2: 88.

WEISER, J. (1962) Biological Insect Control.
In: Málek, I., Ed., Advances in Biological
Sciences Ceskoslovenská akademie Ved., English
edition: Tinková, S., Collets, London, pp. 123-133.

WEISER, J. (1963) Sporozoan infections. In Steinhaus, E.A.
Insect Pathology, Academic Press Inc., New York,
Vol. 2: 291-334.

WEST, A.F. (1960) The biology of a species of Nosema
(Sporozoa: Microsporidia), parasitic in the
flour beetle, Tribolium confusum.
J. Parasit. 46: 747-754.

Lymphotropha tribolii gen. nov., sp. nov., Neogregarinida, Schizocystidae, from the Haemocoele of Tribolium castaneum (Herbst)

R. W. ASHFORD

Imperial College Field Station, Silwood Park, Sunninghill, Berkshire, England

SYNOPSIS. *Lymphotropha tribolii* gen. nov., sp. nov. (Neogregarinida, Schizocystidae) is described from the haemocoele of *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae). The trophozoites are large, ovoid and uninucleate with distinct longitudinal striae. Schizogony occurs in one type of cycle only, each schizont giving rise to up to 8 merozoites. Gametocytes are uninucleate when they associate. Up to 16 oocysts each with 8 sporozoites are formed in each gametocyst. The oocysts are lemon-shaped, and sporozoites emerge from the poles.

The parasite causes considerable mortality in young larvae, though its pathogenicity is probably inferior to that of *Farinocystis tribolii* Weiser.

L ARVAE of the flour beetle, *Tribolium castaneum* (Herbst) in cultures at the Imperial College Field Station were found to be heavily infected with *Lymphotropha tribolii* gen. nov., sp. nov., a schizocystid neogregarine, which caused high mortality among young larvae. The life cycle of the parasite is here described, along with some discussion of its pathogenicity.

Five sporozoan diseases have been described from larvae of *T. castaneum*: *Adelina tribolii* Bhatia, 1937 (1); *Nosema whitei* Weiser, 1953(9); *Nosema bucklei* Dissanaike, 1955; *Farinocystis tribolii* Weiser, 1953; and *Triboliocystis garnhami* Dissanaike, 1955(3). Thompson, 1960(8) questioned whether the two *Nosema* species are in fact valid,[1] and Weiser, 1963(12) has stated his belief that the two schizogregarines, *F. tribolii* and *T. garnhami* are identical. It was nevertheless surprising to find yet another sporozoan disease affecting this beetle.

In the present paper, oocysts resembling those of *F. tribolii* and *T. garnhami*, cultured from material from Czechoslovakia and Gambia, are mentioned under the name *F. tribolii*.

---

[1] At least one species of microsporidian does occur in *Tribolium* spp. *Nosema whitei* Weiser was described from *T. castaneum* and *Tribolium confusum* (Duval) and *N. bucklei* Dissanaike, from *T. castaneum*. These descriptions are incomplete, and therefore invalid (Thompson, 1960)(8). West (1960)(13) described part of the life cycle of a *Nosema* species from *T. confusum*, but did not identify or name it.

MATERIALS AND METHODS

Cultures of *T. castaneum* were fed on wholemeal flour with a small quantity of yeast added and kept in an incubator at 28°C. Infected cultures were maintained by feeding young larvae on the ground-up bodies of others which had died of the disease.

Infected larvae were examined by the following methods: (a) Larvae were fixed in Heidenhain's Susa, embedded in 58°C paraffin wax and sectioned at 5 μ. Alcoholic Bouin, Carnoy and formalin proved unsatisfactory as fixatives. Sections were stained with Heidenhain's iron haematoxylin, Giemsa, and the periodic acid-Schiff technique. Heidenhain's haematoxylin was the best nuclear stain, Giemsa penetrated mature oocysts to show sporozoites, and P.A.S. gave a strong reaction throughout the cytoplasm of trophozoites, schizonts, gametocytes, gametes and zygotes, showing dense reserves of carbohydrate in these bodies, but not in maturing oocysts or sporozoites. Feulgen's nuclear stain gave indistinct reactions in gametic and zygotic nuclei. (b) Fresh smears were made in normal saline, and examined under negative phase contrast. This method was particularly useful for early stages which were too rare to find in sections. Iodine stained trophozoites, schizonts and gametocytes deep brown in fresh smears.

All drawings were made with the help of a squared eyepiece grid, on graph paper.

Description of the Neogregarine

In contrast to the other two neogregarines previously described from *T. castaneum*, the present species has only one type of schizogony. The description will trace the life cycle, from the infection of a new host larva.
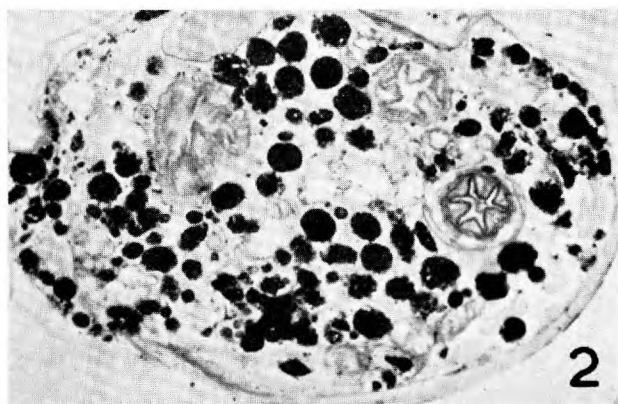
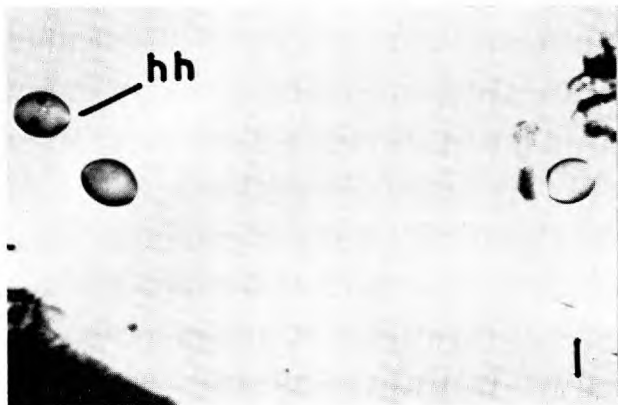*Infection.* Infection follows ingestion of mature

Fig. 1. *L. tribolii* spores in mid-gut of *T. castaneum*. hh.: spore with two sporozoites still contained. Heidenhain's haematoxylin; × 1,000.

Fig. 2. Section of *T. castaneum* larva, with moderate infection of *L. tribolii*. PAS stain shows distribution of parasite in host. × 200.

oocysts by a young larva. The gametocyst wall probably breaks down before or during ingestion. The caps at both poles of the oocyst are digested either by the host's digestive enzymes or from within, as a result of some stimulus from the host, allowing the sporozoites to escape into the gut (Fig. 1). The sporozoites (Figs. 7, 8) are vermicular and crescent-shaped, measuring 8 × 1 μ, and they emerge, often from both poles at once, by a gliding movement, without apparent change in shape. The cytoplasm of the newly emerged sporozoite is entirely non-granular.

Development of trophozoite. The sporozoite must at some stage penetrate the host's gut, and enter the haemocoele. There is no evidence that the Malpighian tubules are used in the route to the haemocoele. The sporozoite elongates to about 15 μ (Fig. 9), and then becomes broader at one end (Fig. 10). The cytoplasm becomes steadily more granular. The fully developed trophozoite (Figs. 3, 11) measures up to 30 × 15 μ, and is usually ovoid in shape with a clear terminal pseudomerite. The trophozoite cytoplasm contains numerous granules and globules of various sizes, which stain heavily with PAS, and its outer wall is marked

with a number of longitudinal striae (Fig. 13a) which appear as ridges in cross sections (Fig. 13b). These have a fine granular appearance under negative phase contrast. The striae do not stain in sections, only being visible as pellicular thickenings.

The nucleus of the trophozoite is often situated near the centre of the body, to one side of the long axis; it is large (5 μ diam.) and has a well marked endosome which may either be spherical, or connected by darkly staining processes to small granules on the nuclear membrane (Fig. 12).

No movement has been observed in the trophozoite; its penetration to all parts of the haemocoele (Fig. 2), including even the legs, may be helped by circulation of the haemolymph, or possibly by small movements of the pseudomerite.

Schizogony. Schizonts, which develop from trophozoites, are very variable in size: 10 × 5 μ to 30 × 15 μ. They are not common, and are hard to find, even in very heavy infections. Their cytoplasm is rather more heavily granulated than that of the trophozoite, and they have no pseudomerite or longitudinal striae (Fig. 14). The schizont nucleus divides into 2-8 daughter nuclei (Figs. 4, 15), and cytoplasmic division then takes place, releasing spherical merozoites, measuring 4 to 6 μ in diameter. These grow into further trophozoites and schizonts, or into gametocytes.

Gametogony. Gametocytes are spherical or ovoid in shape, and vary in size between 12 × 8 μ and 20 × 15 μ. They associate in uninuclear pairs (Figs. 5a; 16). During nuclear division of the gametocytes (Fig. 17), a thin membrane envelopes them, forming a gametocyst, 20 μ in diameter. Each gametocyte nucleus divides into 16 gametic nuclei which migrate to the periphery of the gametocyte (Figs. 5b; 18). These nuclei often contain 3 darkly staining granules, arranged on the nuclear membrane.

Gametes are budded off from the surface of each gametocyte (Figs. 5c; 19a, b, c). They measure 3-4 μ in diameter, and have lightly staining cytoplasm. There are usually a number of residual nuclei in the cytoplasm of the gametocytes, which now coalesces as residual cytoplasm. The volume of residual cytoplasm in the gametocyst is considerable.

In some gametocysts, some gametes will probably join with others from the opposite gametocyte, but zygotes are sometimes restricted to one end of the gametocyst, indicating that they have all developed from the same gametocyte (Figs. 20a, b, c; Fig. 5d).

Sporogony. The zygotes are ovoid or lemon-shaped, measuring about 6 × 4 μ, and form round the periphery of the gametocyst, often in two groups, one at each end. Their cytoplasm stains more darkly than that of any other stage, and their nuclei are more clearly defined than those of gametes, being sub-terminal, and containing numerous darkly staining granules. Shortly

after formation, the zygote becomes enclosed in a resistant wall which impedes fixation and staining. The development of the oocyst as observed under negative phase involves a concentration of the cytoplasmic granules into 1-4 large, highly refractile bodies in the mature oocyst (Figs. 21a, b). These bodies are unaffected by KOH, and usually disappear during hatching. Nuclear division and sporozoite formation are not visible by this method.

Giemsa staining of the mature oocyst shows two sub-terminal groups of four elongated nuclei, and eight faintly distinguishable sporozoites (Figs. 22a, b). The oocyst wall is invariably distorted during fixation..

The number of oocysts per gametocyst varies between 4 and 16 (average 9-10), depending on the number of gametic nuclei which form gametes. Twice the number of zygotes plus the number of residual nuclei is almost invariably 32.
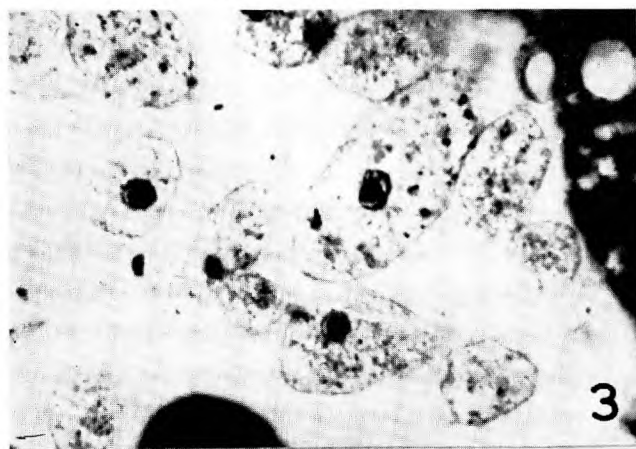
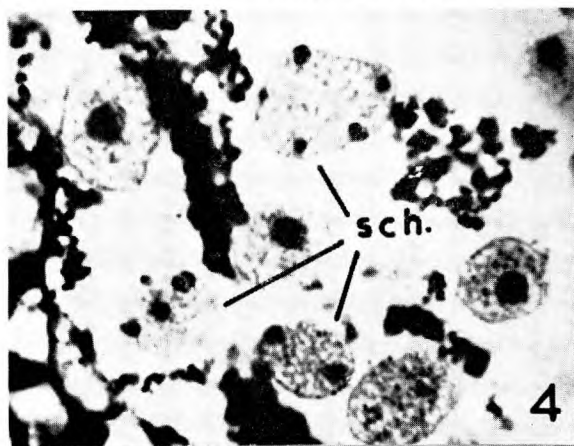As the oocysts develop, the residual cytoplasm disappears.

The mature oocysts are broadly lemon-shaped, and are slightly thickened and pointed at the poles (Fig. 21). The polar caps are not distinguishable in fresh smears from the rest of the oocyst wall. The oocysts measure 8-9 $\times$ 5-6 μ.

Relationships with Host

Visible effects. Larvae which have been infected for a long time are darkened, particularly in the posterior half. Moribund larvae are dark brown or black in colour; they are straight, immobile, and laterally shrunken. On death they rapidly dry out, become dorso-ventrally flattened, and dark brown in colour.

Effects of infection on the behaviour of the host have not been studied.

Host reaction. In old infections, a fresh smear of

Fig. 3. *L. tribolii* trophozoites in section of host. Heidenhain's haematoxylin; $\times$ 1,250.

Fig. 4. *L. tribolii* schizonts (sch.), in section of host. Heidenhain's haematoxylin; $\times$ 1,250.

Fig. 5. *L. tribolii* sexual stages in section of host. a. mono-nucleate, associating gametocytes. b. multinucleate gametocytes in gametocyst. c. gametocyst with gametes. d. gametocyst with zygotes. Heidenhain's haematoxylin; $\times$ 1,250.

Fig. 6. Mature spores of *L. tribolii* showing sporozoites. Giemsa stain; $\times$ 1,750.

the larva will often show round brown capsules surrounding trophozoites or gametocytes. The encapsulated parasites appear to degenerate. These capsules seem to be constructed of successive layers of cells which have encrusted the parasites. Encapsulation is rare in very heavy, but common in light infections, and possibly effects considerable resistance to small doses of the disease. No other defence reactions by the host have been observed.

Habitat and internal effects. The parasite occupies

the entire haemocoele of the host, packing it with mature gametocysts before causing death (Fig. 2). Death is certainly caused in part by the physical obstruction of the host systems.

The only tissue in which any change has been observed is the fat body, which atrophies and degenerates considerably. The parasite does not actually invade the cells of the fat body; it presumably uses nutrients in the haemolymph, causing demands to be made on the fat body, and leading to the degeneration

SCALE FIGS. 7-11 [5μ]
FIGS. 12-15 [10μ]
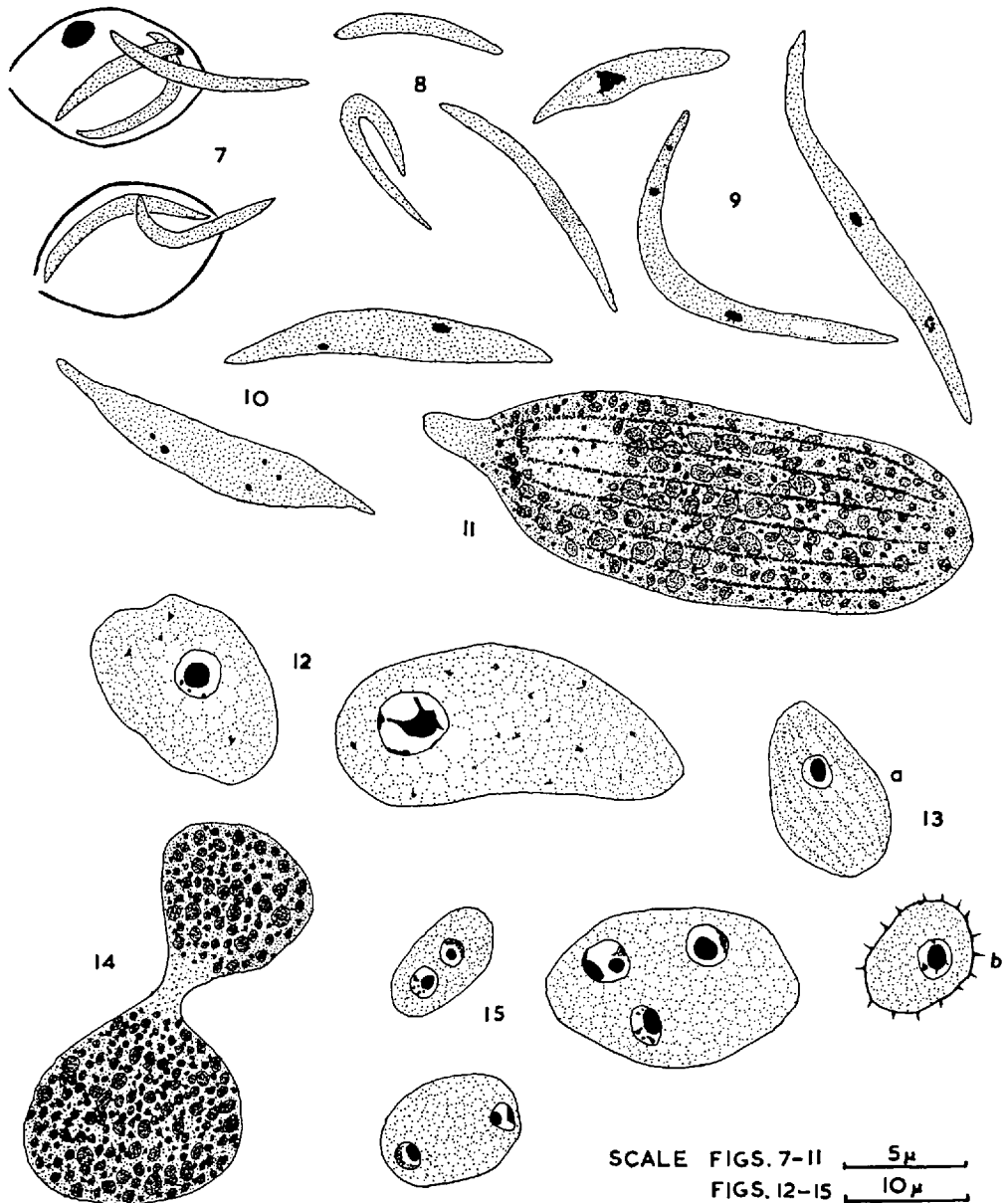
Figs. 7-15. *L. tribolii*, asexual stages. Figs. 7-11, 14. As seen in fresh smears with negative phase contrast. Figs. 12, 13, 15. In haematoxylin stained sections. Fig. 7. Sporozoites emerging from oocysts. Fig. 8. Sporozoites in gut of host. Figs. 9, 10. Developing trophozoites. Figs. 11, 12. Trophozoites. Fig. 13. Trophozoites showing striae. a. Lateral view. b. Transverse section. Figs. 14, 15. Schizonts.

of this organ. Stored food reserves in the fat body are lost, and not replaced. Starvation is probably an additional cause of death.

Infectivity and mortality. Lymphotropha tribolii is characteristically a parasite of larvae; it has very rarely been found in pupae, and only occasionally in adults. Young larvae are more susceptible than old; but those which receive a small dose at an early age may retain the infection for many weeks until they die, or recover and pupate. The life cycle of the parasite is completed in about 15 days, and death occurs from massive doses after around 20 days.

Preliminary experiments indicate that 10^4 oocysts per gram of foodstuff cause 50% mortality, in larvae reared from the egg in this medium, and 10^6 spores per gram cause 95% mortality. In one experiment, where 3×10^4 oocysts of *L. tribolii* per gram were mixed with those of *Farinocystis tribolii*, in the ratio
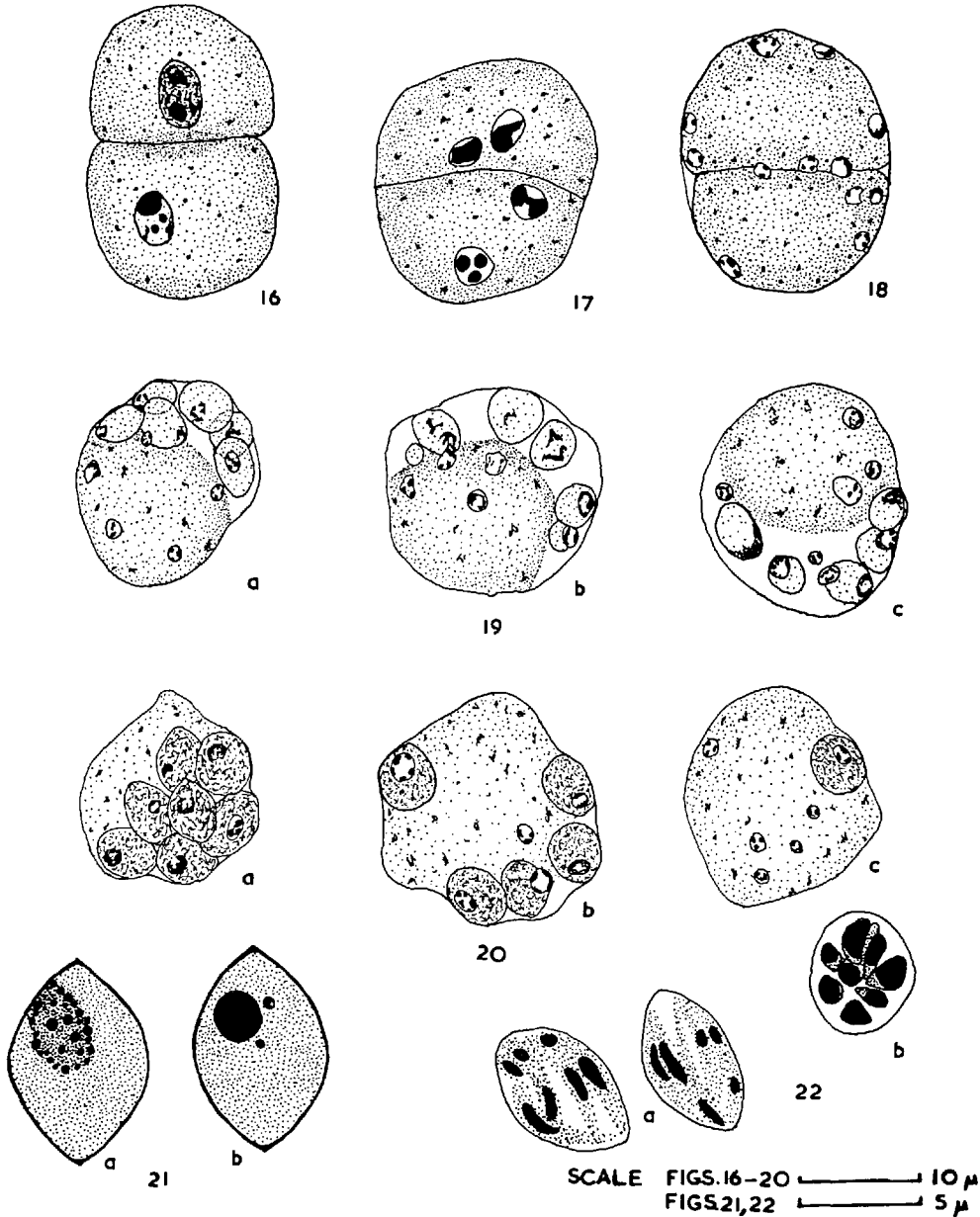
Figs. 16-22. *L. tribolii*, sexual stages. Figs. 16-20. Haematoxylin stained sections. Fig. 21. Fresh smear; negative phase contrast. Fig. 22. Giemsa stained section. Fig. 16. Associating mononucleate gametocytes. Fig. 17. Binucleate gametocytes in gametocyst. Fig. 18. Section of 32-nucleate gametocyst. Fig. 19. Serial sections of a gametocyst with gametic nuclei and gametes. Fig. 20. Serial sections of a gametocyst with residual nuclei and zygotes. Fig. 21. Oocyst development. a. Immature oocyst. b. Mature oocyst. Fig. 22. Sporozoites in mature oocysts. a. Lateral view. b. Terminal view.

of 40:1, 100% mortality occurred, and the ratio of *Farinocystis* to *Lymphotropha* oocysts in the resulting dead larvae was about 3:1. *Farinocystis* thus rapidly supersedes *Lymphotropha* in mixed infections, probably because it causes the death of the host before many *Lymphotropha* oocysts have matured, but *Lymphotropha* could be of importance in limiting populations where *Farinocystis* does not occur.

L. tribolii has been found to infect larvae of *Tribolium anaphae* as well as *T. castaneum*, but not *T. confusum* or *Tribolium madens*.

DISCUSSION

Various classifications have been proposed for the neogregarines, but in all there is difficulty in deciding on which factors to place most emphasis. The most recent classifications are those of Weiser, 1955(10,11), and Grassé, 1953(4). Weiser depends largely on the number of schizogonies, then the number and morphology of the oocysts. Grassé places more importance on the morphology of trophozoites and schizonts, and their habitat.

The present discovery of a new neogregarine in such a comparatively well studied insect as *T. castaneum*, would seem to indicate that only a small proportion of the living members of this group have been described, and in view of the possible complications of polyphyletic and convergent evolution within the group, it is considered that all existing classifications must be regarded as tentative. With this in mind, it is proposed to follow that of Grassé in the present work, as this is the one followed at the supra-familial level by the Committee on Taxonomy and Taxonomic Problems of the Society of Protozoologists(2), and as *Lymphotropha tribolii* can be fitted into this system more naturally.

Grassé divides the Neogregarinida into five families:

The Ophryocystidae are a compact group with mycetoid trophozoites, inhabiting the malpighian tubules of various Coleoptera. They have two schizogonic cycles, and each gametocyst has only one oocyst.

The Caulleryellidae have flask-shaped trophozoites, regularly bunched merozoites, and a thin-walled oocyst.

The Lipotrophidae show one or two cycles of intracellular schizogony, and differ from the present species in many other important ways.

The Syncystidae have large numbers of merozoites and oocysts, the oocysts being spined.

The family with which *L. tribolii* most closely agrees is the Schizocystidae which have large trophozoites with longitudinal striae. There are two known genera, *Schizocystis* Léger and *Machadoella* Reichenow(7). In *Schizocystis* the trophozoite quickly becomes multinucleate and nematoid and develops an adhesive organelle at the anterior end. The gametes are anisogamous.

The present species most closely resembles *Machadoella*. The name *Machadoella* was first used by Reichenow 1935(7) for a parasite *M. triatomae* from *Triatoma dimidiata*. *Schizocystis spinigeri* described by Machado (1913)(5) from *Spiniger* sp. was transferred to the genus *Machadoella* by Reichenow (1935). Reichenow observed the association of mononucleate gametocytes only of *M. triatomae* and pointed out that the multinucleate stages termed gametocytes by Machado were most likely schizonts. Mayer and Pifano (1949)(6) in their partial description of an unidentified species of *Machadoella* believe that the gametocytes are binucleate before association. They also show the nematoid shape of trophozoite characteristic of the genus. Weiser depicts *Machadoella* ". . . after drawings in original descriptions" as having multinucleate gametocytes, and creates a subfamily based on this phenomenon(10,11).

In *Lymphotropha* gametocytes are mononucleate on association and stages of sporogony follow a course similar to that of *Machadoella*.

However *Machadoella* has nematoid trophozoites which undergo nuclear division while still elongate and thus resemble the multinucleate trophozoites of *Schizocystis*. *Lymphotropha* trophozoites are ovoid and do not begin nuclear division until they become distinguishable as schizonts by their irregular form.

The two known species of *Machadoella* inhabit the Malpighian tubules of triatomid bugs whereas *Lymphotropha* is found in the haemocoele of a tenebrionid beetle.

It is therefore proposed to place the present parasite in a new genus, in the family Schizocystidae (classification of Grassé, 1953). *Lymphotropha* is an appropriate generic name in view of the habitat of the parasite; *tribolii* as a specific name indicates the host genus.

According to Weiser, the number of nuclei in the gametocytes on association is more important than similarities in the morphology of the trophozoites and schizonts. Thus *Machadoella* is placed in the family Ophryocystidae characterized by 2 cycles of nuclear division prior to association of gametocytes. *Lymphotropha*, having only one schizogony would belong to the other family, Caulleryellidae. A new sub-family would have to be created to include it.

Generic diagnosis: Trophozoites ovoid with terminal projection, and with faint longitudinal striae. Gametocytes mononucleate on association. Oocysts with 8 sporozoites.

Genotype: Lymphotropha tribolii gen. nov., sp. nov.

Specific diagnosis: Trophozoites measuring 20-30 μ by 10-15 μ. Schizonts giving rise to up to 8 merozoites. Gametocytes with 4-16 lemon-shaped oocysts with indistinct polar caps; oocysts measuring 8-9 μ by 5-6 μ. Sporozoites 8 μ by 1 μ in fresh smears.

Locality: Insectaries at Imperial College Field Station, Ascot, Berkshire.

Habitat: Haemocoele of *Tribolium castaneum* (Herbst).

REFERENCES

1. Bhatia, B. L. 1937. On *Adelina tribolii*, a Coccidian parasite of *Tribolium ferrugineum* F. *Parasitology* 29, 239-46.

2. Committee on Taxonomy and Taxonomic Problems of the Society of Protozoologists. 1964. A revised classification of the phylum Protozoa. *J. Protozool.* 11, 7-20.

3. Dissanaike, A. S. 1955. A new Schizogregarine *Tribolio-cystis garnhami* n. g., n. sp. and a new Microsporidian *Nosema buckleyi* n. sp. from the fat body of the flour beetle *Tribolium castaneum*. *J. Protozool.* 2, 150-6.

4. Grassé, P. P. 1953. Classe des Gregarinomorphes, in Grassé, *Traité de Zoologie*, 1 (fasc. II), Masson, Paris, 550-689.

5. Machado, A. 1913. Sobre o ciclo evolutivo de *Schizo-cystis spinigeri* n. sp. gregarina do intestino de uma especie de *Spiniger*. *Mem. Inst. Oswaldo Cruz* 5, 5-15.

6. Mayer, M. & Pifano, F. 1949. Sobre una Schizogregarina de *Entriatoma maculata* de Venezuela. *Arch. Venezolanos Patol. Trop. Parasitol. Med.* 1, 335-40.

7. Reichenow, E. 1935. *Machadoella triatomae* n. g., n. sp., eine Schizogregarine aus *Triatoma dimidiata*. *Arch. Protistenk.* 84, 431-45.

8. Thompson, H. M. 1960. A list and brief description of the Microsporidia infecting insects. *J. Insect Pathol.* 2, 346-85.

9. Weiser, J. 1953. Schizogregariny z hmyzu skodíchího zásobám mouky, 1. *Vestnik. Csl. Zoolog. Spolecnosti* 17, 199-211.

10. Weiser, J. 1955. A new classification of the Schizo-gregarina. *J. Protozool.* 2, 6-12.

11. Weiser, J. 1955. A new classification of the Schizo-gregarina: a correction. *J. Protozool.* 2, 88.

12. Weiser, J. 1963. Sporozoan Infections. In Steinhaus, E. A., *Insect Pathology*, Vol. 2, 291-334. Academic Press, New York and London.

13. West, A. F. 1960. The biology of a species of *Nosema* (Sporozoa: Microsporidia) parasitic in the flour beetle *Tribolium confusum*. *J. Parasitol.* 46, 747-54.