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***NOSEMA DISEASE OF THE BUMBLE BEE
BOMBUS TERRESTRIS (L.).***

A THESIS PRESENTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS
FOR THE DEGREE OF
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ABSTRACT

A *Nosema*-like microsporidian pathogen of the bumble bee *Bombus terrestris* (provisionally referred to as "NBT") is described in terms of its occurrence, its developmental cycle and morphology.

Variation in *Nosema* infection levels in *B. terrestris* queens from four sites in the central North Island suggested seasonal and climatic effects, with greater infection of queens from milder and/or urban habitats.

Experimental infection of bumble bee workers provided details of the morphology and life cycle of the microsporidian. Features of the somatic stages of NBT observed from stained, smear preparations of tissues were characteristic of the genus *Nosema* (e.g. predominantly binucleate somatic stages comprising meronts, sporonts and sporoblasts). Each group of stages were variable in size and had from 1-4 nuclei. The timing of the appearance of somatic stages was investigated and a life-cycle for NBT was proposed. The minimum generation time of NBT spores at 30°C was approx. 4 days, with 50% of spores appearing within 7 days.

Transmission electron microscopy indicated two spore forms were present, one *Nosema*-like with a polaroplast, diplokaryon, and 14-18 polar filament coils, the other having the same basic features but lacking the polar filament. NBT spores and somatic stages were found primarily in the Malpighian tubules and occasionally in the tissues of the midgut. The remaining tissues were free from infection.

In a cross-infectivity test NBT was found to be slightly infective to *Plutella xylostella*, a Lepidopteran species that feeds on brassica plants. As bumble bees visit brassica flowers it is possible that natural cross-infection of NBT may occur, with other insect species acting as reservoirs for the disease.

Comparisons of the morphology and life history of NBT with other *Nosema* species known from Hymenoptera indicated that NBT was different from all but *N. bombi* Fantham and Porter 1914. It is concluded that the NBT of this study is *N. bombi*.

Experiments were undertaken to examine the transmissibility of NBT and its maintenance in individual bumble bees. Fecal material from naturally and experimentally infected queens was examined and numbers of spores estimated with a haemocytometer. The time taken from the ingestion of heat-killed spores by queens, to their complete elimination in the faeces, was approximately 5 days.

Contamination of the nectar wick with faeces containing NBT spores caused the spore loads of individual bees to increase. Similar results were achieved by

feeding bees directly with faeces (mixed with sugar syrup) containing a known number of spores. No significant reduction in spore numbers was observed from infected bees treated with Fumidil-B (an anti-*Nosema* drug).

NBT infection did not appear to affect the nest initiation or egg-laying behaviour of bumble bee queens nor was there evidence for *Nosema*-linked mortalities.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE HISTORY OF BUMBLE BEES AND POLLINATION IN NEW ZEALAND

New Zealand has no native bumble bees or honey bees. Honey bees were first brought to New Zealand around 1839 and up to the late 1800's farmers relied on them for crop pollination (Gurr 1961). However there still remained persistent problems with low seed yields from red clover, *Trifolium pratense* L. and lucerne, *Medicago sativa* L.. Darwin (1858) focused attention on the bumble bee as an efficient pollinator of red clover in Europe. On the basis of this knowledge a small number of queen bumble bees were imported into New Zealand in the early 1880's (Nottidge 1890).

Bombus terrestris and *B. ruderatus* were liberated in the South Island of New Zealand in 1885 (Gurr 1957b). Both species survived well and became readily established. In 1906 at least four species were imported and released. Gurr (1964) reported that two of the four became established in the South Island, namely *B. hortorum* (L) and *B. subterraneus* (L) sub sp. *latreillellus* (Kirby). New Zealand now supports four known species of European bumble bees, the most widespread being *B. terrestris*.

Red clover flowers have long corolla tubes and this means only long-tongued insects are able to reach the nectaries (Gurr 1975). However, *B. terrestris* is the only one of the four species that does not have a long tongue, resulting in their tendency to 'rob' the flowers by biting holes at the base of the corolla tube so bypassing contact with the sexual parts of the flowers (Gurr 1975). Honey bees often become 'secondary robbers' by re-using the holes and pollination is further reduced (Prys-Jones & Corbet 1987).

Although *B. terrestris* may not be suited to the pollination of red clover it has since been found to be an efficient pollinator of many other crops (Gurr 1955, Palmer-Jones & Clinch 1966). Work has been undertaken in Massey University's Botany/Zoology Department, examining the use of *B. terrestris* as a pollinator of kiwifruit and muskmelons (Fisher & Pomeroy 1989a). In Europe, *B. terrestris*

colonies are produced on a commercial level and placed in glass-houses to effect the pollination of tomatoes and other crops. An important feature of bumble bees in general is that they will work more efficiently than honey bees in glass-houses. This is of potential market value where crops, such as tomatoes, are usually pollinated by hand at a considerable cost (in money and manpower) to the grower. *B. terrestris* was chosen as the study species primarily because it is the species used in other studies at Massey University and is abundant within the vicinity of Palmerston North. The renewed interest in the bumble bee as an alternative pollinator (especially in Europe) also implies the need for further research on the problems that are encountered when rearing such an insect on a commercial scale.

The controlled rearing of commercially viable bumble bee colonies is difficult. Even where the bees are able to free forage (outside the laboratory) small nests usually result (Tod 1986). There are many potential problems contributing to poor colony development and some of these, especially social ones, have been studied extensively (Pomeroy 1981, Tod 1986, Van Doorn 1986). However, one aspect that has not really been adequately researched is that of disease.

When bumble bees were introduced into New Zealand from Europe, many species-specific commensals and parasites entered with them. These ranged from a variety of internal pathogens to external mites. At the time of importation no records were kept nor is there at present a comprehensive review of the pathogens affecting bumble bees. Some progress has been made towards compiling a list of those organisms currently found associated with bumble bees in New Zealand (R.P. Macfarlane unpublished manuscript). However, little work has been directed towards gaining a more comprehensive understanding of the impact that these organisms have on the bees as individuals or the colony as a whole. In comparison, diseases of the honey bee *Apis mellifera* have been listed and studied in detail (Bailey 1981) and in particular the protozoan *Nosema apis* Zander which was discovered in 1906. In 1914 Fantham and Porter described a similar protozoan from the bumble bee naming it *Nosema bombi* (after its host *Bombus*). Its status as a new species though is disputed even today but there have been no recent studies reported which resolve this. In the past bumble bees have generally been regarded as "wild" insects and consequently little interest has been shown in their associated pathogens. Now that there has been a resurgence of interest in the bumble bee as a commercial pollinator I believe it is important to investigate the nature of the pathogens affecting them in New Zealand and the degree to which they may be considered detrimental to the health of the bee and its overall productivity. Considering this *N. bombi*

merits further study particularly as there has been no recent comprehensive research done on it. It is important also to determine whether its presence within a colony may produce similar problems as those encountered with *N. apis* in the honey bee and as a consequence hinder attempts at large-scale bumble bee production.

1.2 BUMBLE BEE NATURAL HISTORY

Young queen bumble bees, inseminated during late summer, hibernate through late autumn to spring. In Europe, this may be "anything from 6-8 weeks or even 9 months" (Alford 1975). However, a more temperate climate, as found in New Zealand, reduces the hibernation period to as little as 2-3 months (Cumber 1953a), and in some cases, may eliminate it all together. The queens dig into loose, well-drained soil to a depth of up to 10 cm, and mould a small cavity in which they remain during the hibernation period (Sladen 1912, Alford 1969). During spring when soil temperatures rise, the queens emerge and spend a few weeks flying, sunning and foraging while their ovaries are still small and undeveloped (Heinrich 1979a, Alford 1975). Once their ovarioles have begun to develop and contain eggs and nurse cells, the queens commence the search for nest sites (Alford 1975, Cumber 1949a).

Typical sites chosen by queens are nests abandoned by small mammals, although this varies with the species of bumble bee and its situation in urban or rural regions. *B. terrestris* queens nest predominantly under ground, although nests have been found in carpet felt, fiberglass Pink Batts (housing insulation), compost heaps, wood piles and under houses in the region of Palmerston North (personal observation). The site chosen usually has material (paper, plant fibre) that can be manipulated by the queen to form a cavity in which she will lay her first eggs. The brood needs to be maintained at a temperature of 28-30 C, so a suitable nest material is important (Hasselrot 1960).

The queen shapes a pollen clump on the floor of the nest and builds on it a cell of wax and pollen. It is in this that she lays her first batch of eggs, with *B. terrestris* queens depositing up to 16 eggs in their first brood batch (Alford 1978). The larvae hatch about 4 days after oviposition and grow rapidly, feeding initially on the pollen below them and later on a pollen/nectar regurgitate from the queen. The larvae remain in a common cell for approx. 10 days and on reaching the fourth instar, spin flimsy, silken cocoons and become separated from one another (Alford 1978). After

10-20 days they each spin a tough, cylindrical cocoon into which they deposit fecal material (meconium). This is incorporated into the wall of the cocoon (Prys-Jones & Corbet 1987). The larvae then pupate.

Workers emerge from 18-26 days (personal observation on *B. terrestris*), after egg laying. In the first 24 hours the young workers (termed callows) do not perform any duties as they are still damp, have soft wings and have not yet gained their distinctive yellow/black colouration (Pomeroy 1977). Eventually the first workers take over all of the foraging and nest duties while the queen remains in the nest. She lays more eggs on top of the cocoons of pupated brood. (Brood rearing is regulated to meet the needs and economy of the colony. The presence of pupal cocoons is usually a signal for the queen to build egg cells and lay eggs (Alford 1978). Empty cocoons are utilised as pollen or nectar storage pots.

The comb develops upward and outward in a somewhat staggered manner, with egg cells formed on the cocoons from each previous laying. Comb shape is often hemispherical, but ultimately depends on the size and shape of the nest site. The worker number increases rapidly (Plowright & Jay 1966) until, at a switch over point males and new queens (collectively known as reproductives) are produced. Once this stage has been reached it is rarely reversed and worker numbers diminish as a consequence.

Once the young queens emerge from their cocoons they stay in the nest for about 5 days after which they embark on a mating flight (Cumber 1953a). Once they have mated they return to the nest and spend time feeding, resting and building up their fat body (necessary for survival during hibernation). Their ovaries remain underdeveloped during this time, only developing slowly during the hibernation period. The young queens then search for sites in which to over-winter and the cycle is repeated.

Males leave the nest when they are 2-4 days old and generally never return. Their primary objectives in the ensuing period are foraging and inseminating young queens (Alford 1975). They die soon after this has been accomplished.

1.3 MICROSPORIDIA IN INSECTS

The members of the order Microsporidia are predominantly obligate,

intracellular pathogens (Kellen 1976). They are generally small (1.5 μm - 20 μm) and are characterised by resting spores which contain a single infective sporoplasm and polar filament. The genus *Nosema* is the largest of all the microsporidian genera and contains approximately 200 species. Most of these are parasitic in insects but representatives occur in almost all the major animal groups and have a world-wide distribution (Sprague 1982).

Microsporidia of the family Nosematidae may be transferred between hosts in two general ways ; by direct contact or indirect contact (Kramer 1976). The former mechanism involves the microsporidian being transferred directly from the original host to a susceptible host. The latter requires the microsporidian spores to be dispersed by physical agents or carried by a vector. Examples of direct contact are a) the transfer of spores from a parent to its offspring during reproduction (transovarial/vertical transmission), b) the consumption of decaying infected cadavers by susceptible hosts, and c) emerging larvae consuming spores adhering to the surface of their egg (horizontal transmission) and d) the injection of spores into the host by infected parasites. Examples of indirect contact are a) mechanical disturbances carrying particles of soil, excreta etc. into the air that may contain clumps of spores and b) predators/scavengers that consume the spore-filled tissues of the host and eventually discharge the unchanged spores with their feces.

There are three possible portals of entry for the microsporidian into the insect host ; the oral, cuticular and ovarial portals (Andreadis 1987). Some microsporidia employ more than one (e.g. *N. heliothidis* from Lepidoptera, Brooks 1973) whereas others (e.g. *N. apis*, Bailey 1963) are restricted to one, commonly the oral portal. Once the microsporidian spore has entered the body of the insect host it undergoes a cycle of development and reproduction. Under the appropriate stimuli (poorly understood in most species) the spore will extrude under pressure a hollow polar filament, the length of which varies between species. The filament is a firm structure capable of penetrating both the peritrophic membrane and the gut epithelium (Weiser 1961). Testing of the peritrophic membrane in the honey bee, by way of dyes, showed that only the large colloidal dye particles (Congo red) were arrested by it (Wigglesworth 1972). However, little ultrastructural work has been done on the peritrophic membranes of the Hymenoptera so it is difficult to know how differentially permeable they may be. When the filament penetrates a cell (of the gut, fat body etc.) a sporoplasm (nuclei and cytoplasm bounded by a membrane) is ejected and this either remains in the cell or becomes amoeboid and migrates to other parts of the body progressively infecting other tissues. The sporoplasm then

undergoes two distinct cycles of growth and division termed merogony and sporogony. This ultimately results in the formation of spores, the number of which varies according to the species. Infected cells containing the spores eventually burst and they are released into the gut, malpighian tubules or body cavity prior to dispersal. These spores are the infective agents in the microsporidian life-cycle, capable of survival in the extra-corporeal environment if necessary before contacting a new, susceptible host.

The infection of individual insects by microsporidia produces physical effects ranging from acute to sub-lethal/chronic to minor/benign. The bacterial flora of the gut determines in part whether a given dose of spores will cause an acute or chronic infection (Weiser 1976). Massive doses of spores can promote either acute infections or septicemias without any development of the microsporidian. Bacteria entering the wounds (made by filaments) in the gut wall can instigate a septicemia in the host prior to the incubation period of the microsporidian (Weiser 1976). Acute infection (and septicemias) result in the premature death of the insect and tend to limit spread of the disease as the spores remain in the cadavers. Sub-lethal or chronic infection is the most common form and may result in a loss of vigor, reduced fecundity, mating success and overall longevity in the insect (e.g. *N. carpocapsae*; Mercer 1981). It also leads to the spread of infection through the horizontal (between host generations) and/or vertical transmission (within host generations) of spores. Minor or benign infection may not produce any obvious symptoms, the insect surviving and reproducing as well as a non-infected individual. If the physiological functions of the host are not seriously damaged by the microsporidian the insect will often survive the infection. This has been indicated in nosematosis of the honey bee - the mid-gut epithelium has great regenerative powers and a complete recovery can follow an infection originally localised in the gut (Veber & Jasic 1961).

Many factors can influence the overall effects that the microsporidian has on its insect host. The development of the protozoan in the insect can be restricted by the tissue it infects, the infective dose the insect originally ingested and the instar of the insect affected (Weiser 1976). The physiological state of the insect at the time of infection will also have an effect (e.g. if it is a female with brood). Stressors, when considered as inadequate food, abnormal temperatures and humidity, can influence infected insects by shortening the duration of the infection and emphasising the inability of the infected individual to adapt to the change in conditions (Weiser 1963).

1.3.1 Control measures

The introduction of various insect antibiotics in recent years has helped to reduce much of the effort required to maintain disease-free groups of insects. Perhaps the most detailed studies have been on the containment of *N. apis* of the honey bee. Experimental work incorporating various antibiotics (including Urotropin, Apimycin, Nosematose and derivatives of fumigillin) have highlighted the potential value of fumigillin as a treatment for *N. apis*. Fumigillin has also been applied as a general control for many other microsporidian parasites (Lewis & Lynch 1970).

Fumigillin is an antibiotic with amoebicidal properties (McCowan et al 1951). Early trials testing the drug produced striking results in the honey bee, substantially reducing numbers of nosema-infected individuals. Bailey (1953) determined that the fumigillin acted on the microsporidians mainly by arresting or destroying the vegetative stages. Later studies, (Hartwig 1971) indicated that the antibiotic appeared to act at the DNA level by inhibiting the DNA replication by the parasite but without actually affecting the DNA replication within the cells of the host. An added benefit proved to be the restoration of RNA synthesis in the host cell after treatment with fumigillin.

A readily available form of fumigillin is Fumidil-B (Abbott Laboratories, Chicago) and this is a preparation used extensively by apiarists. The antibiotic was used over the course of the study in an effort to maintain disease-free bees and/or reduce their susceptibility to infection. This was based on the positive effects of treatment as seen in the honey bee (Kunst & Tomasec 1965, Furgala & Maunder, 1978) and also on the successful treatment of queen bumble bees with Fumidil-B (De Jonghe 1986).

1.4 SOCIAL INSECTS

Studies on insect-associated microsporidia are often directed towards a) gaining an understanding of the pathogen and its relationship with the insect and/or b) the possible application of the microsporidian as a biological insecticide. These are the approaches used where the study insect is considered a noxious pest. In direct comparison, studies on commercially valuable insects (e.g. *Bombyx mori*, *Apis*

mellifera) are undertaken to determine a measure of control for the pathogen itself in order to maintain healthy, disease-free insect populations. When investigating the microsporidians that affect social insects any of the three study types may be implemented. It is important to realise that a general understanding of pathogens (in terms of pathogenicity, transmission etc.), as developed through studies on other insect groups, will include features not directly applicable to social insects and their pathogens. Various characteristics of the insect society, such as division of labour, rearing of the young and cleaning the colony, all influence the level of infection and transmission of the pathogen within the population (Maddox 1987). Because the colony resembles in many ways an individual as well being comprised of many inter-related parts, studies can be done between or within colonies as a consequence.

Some of the insects on which work has been done include ants, termites, honey bees and bumble bees. Within these groups microsporidian infection may range from chronic to benign, attack a variety of body tissues and be transmitted ovarially or orally.

ANTS : At least seven different species of microsporidian have been described from ants. One of these, *Thelohonia solenopsae* of the fire ant (*Solenopsis*) affects the ovaries of the queens and the fat tissue of workers and sexuals. It is not transmitted orally implying a probable transovarial route. It tends to produce chronic pathogenic effects, reducing the longevity of the infected colony (Knell et al 1977). Few long-term studies on these microsporidia have been undertaken but it is believed that infection levels vary from one season to another.

TERMITES : Very little is known regarding the microsporidian parasites of the *Isoptera*. Kalavati (1976) added four new species to the small list of three previously known. One of these, *Gurleya spraguei*, was found to infect the termite *Macrotermes estherae*, invading the fat tissue and consequently was not transmitted via fecal material. Knowledge of the pathology of the microsporidian is poor but it is believed that the social activity of the colony results in seasonal fluctuations in prevalence.

HONEY BEE : Of all the social insects the honey bee has received the most research attention. Many books, reviews and scientific papers have been published on the honey bee *Apis mellifera* and its associated pathogens and commensals, a large proportion of which are devoted to the parasitic protozoan *Nosema apis* Zander. First described in 1906, *N. apis* was found to primarily affect the midgut epithelium of the adult honey bee and was occasionally found in the Malpighian tubules. Claims have been made concerning other sites of infection (Steche 1960 ,

Sokolov & Grobov 1963), however in most cases they have not been substantiated (Bailey 1981). Generally, infected bees show no outward symptoms and even heavily infected bees are seen to behave normally. The larvae are not susceptible to infection and only horizontal transmission is known. Worker bees are the primary hosts although drones and to a lesser degree queens, may become infected. It is the infection of the queen that leads to the decline of the colony. This is effected by damage incurred to her ovaries resulting in the failure of eggs to hatch, eventual cessation of oviposition and ultimately her supersedure or death (Hassanein 1951). Infected bees generally live only half as long as healthy bees and afflicted colonies are weakened, although they are rarely decimated by the disease.

Nosema infection disrupts the age-duty sequence and upsets the division of labour of workers within the colony. The infected workers appear to be physiologically older than healthy bees of the same age and perform various duties earlier as a consequence (Wang & Moeller 1970). There is also a correlation between infection with *Nosema* and brood rearing. The stress on the metabolism of workers caused by brood rearing leads to conditions that favour the development of the disease (Butler 1976). These changes within the individual bee also affect the colony as a whole and result in an overall increase in *Nosema* levels.

A notable feature of *N. apis* is the consistent seasonal variation with infection levels highest in the spring and declining over the summer (Weiser 1961). Various explanations for this have been suggested, especially the idea that the bees during winter are more likely to defecate on the comb and healthy workers cleaning the combs in early spring (in preparation for brood-rearing) may become infected if spores are present (Bailey 1955).

N. apis has a global distribution with levels of infection varying with geographical distribution (Weiser 1961, Bailey 1981). In New Zealand, *N. apis* is a significant problem in the maintenance of honey bees and it is estimated that nearly all apiaries are currently infected at some level (L. Malone pers. comm.).

Of all the insect-pathogen associations the protozoan diseases of social insects are perhaps the most complex. Basically, it is the social behaviour of the insect colony as a whole that (probably more than any other factor) that influences infection levels, the development of seasonal fluctuations and epizootics.

1.5 REVIEW OF LITERATURE ON NOSEMA IN BUMBLE BEES

Throughout studies on microsporidia there has been one recurring problem - the lack of a universally acceptable classification. Today, perhaps the most comprehensive taxonomic studies on the microsporidia are those proposed almost simultaneously by Sprague (1977) and Weiser (1977). Both have similarities at the higher taxonomic level but many differences are apparent especially at the genera and family levels. Sprague's system, updated in 1982, is the more widely used. As advanced technologies (e.g. SEM and TEM) reveal new information on microsporidian morphology the old bases for classification become redundant (Larsson 1986). These include classifications based on spore shape and size, site of infection in host tissues and host range. The terminology used to describe life-cycle stadia has also altered with time often making comparisons of studies on various microsporidian species difficult (Jacobs 1976). In the case of *Nosema bombi*, first described in 1914 by Fantham & Porter, the above mentioned problems apply. Only the light microscope was utilised with the taxonomic status determined partially on the host species (*Bombus*) and on features of the life-cycle and spore size and shape. These facts alone give cause for questioning the true identity of *N. bombi* and this emphasised by the number of conflicting studies concerning *N. bombi* since 1914.

Research on the microsporidia affecting bumble bees has been limited. Early studies on *N. apis* (Fantham & Porter, 1913) indicated that it was possible to infect a number of species of *Bombus*, including *B. terrestris* L., with the microsporidian. In the following year Fantham & Porter identified a new species of *Nosema* which they named *N. bombi* after its host *Bombus*. They described it as being distinctly different from *N. apis* in terms of spore morphology and tissue specificity (features no longer considered suitable for taxonomic purposes). This may explain why the few studies concerning nosema disease of the bumble bee are mostly contradictory and cast doubt on the true status of *N. bombi*.

Many authors claimed to have succeeded in cross-infecting bumble bee species and the honey bee *A. mellifera* with *N. apis* and *N. bombi* respectively (Fantham & Porter 1913, Kudo 1924, Showers et al. 1967). Yet others argue that the bumble bee is not susceptible to infection by *N. apis* (Uspenski 1949, Weiser 1961). Showers et al. (1967) ventured the opinion that in the case of some studies where there was difficulty in accurately identifying the nosema (e.g. Skou et al. 1963), the infection was probably caused by *N. apis* and not *N. bombi* as stated. Weiser (1961) believed that the morphological differences between the two nosemas were not

sufficient to warrant claiming *N. bombi* as a new species and implied that the two were probably synonymous. This opinion is held by various authors (Showers et al. 1967, R.P. Macfarlane unpublished manuscript) and is reinforced by evidence that not only are microsporidia are often cross-infective between hosts but the morphology of the parasite may actually be altered in different hosts (Canning 1977).

Skou et al. (1963) noted that the seasonal variation of nosematose in the bumble bee appeared similar to that seen in the honey bee. However, the seasonal variation idea is perhaps not valid for the bumble bee as the colony is annual and not perennial like the honey bee. For the same reason it is perhaps uninformative to make direct comparisons with honey bee colonies that show high levels of nosema infection during late winter and early spring. The observation that *Nosema* incidence increases (in both the honey bee and the bumble bee) with a decrease in the ambient temperature is perhaps more important (Showers et al. 1967). Although the bumble bee as a colony lasts only a few months it is the new queens produced during that period that overwinter, emerging the following spring to reproduce. The bumble bee queens that are infected with *Nosema* during the summer are more likely to die prior to or during hibernation as indicated in the study by Skou et al. (1963) although many infected queens do survive to emerge in the spring (Betts 1920, personal observation on *B. terrestris*). The bumble bee queen appears to be the one individual that maintains the infection within the bumble bee population from season to season.

Other than the studies mentioned above, little or no work has been done on elucidating the exact nature of *N. bombi* and whether it can be classified as distinct from *N. apis*. Most of the studies have concentrated on cross-infection between the honey bee and the bumble bee the results of which, even today, have not been agreed on. The productivity of *Nosema* infected colonies has been examined (Fisher & Pomeroy 1989b) but the overall effect of the microsporidian on individuals not been studied. As large, healthy colonies of bees are needed for their commercial application, any factor(s) that may reduce colony productivity or longevity will need to be identified.

A study of *B. terrestris* and one of its pathogens has presented a chance to elucidate some of these problems. The pathogen, a microsporidian of the genus *Nosema*, is referred to from here as "NBT" (= *Nosema Bombus terrestris*) after the convention of Watanabe (1976).

1.6 OBJECTIVES OF STUDY AND THESIS PLAN

This project describes a microsporidian observed from the bumble bee *Bombus terrestris*. Firstly its distribution, incidence and taxonomic status are determined and then aspects of the pathogen/host interaction are examined.

Chapter 3 examines the distribution of the pathogen and its frequency in the populations sampled.

Chapter 4 describes the pathogen in terms of its life cycle and spore morphology. Comparisons to other microsporidia are made and its taxonomic status determined.

Chapter 5 examines the relationship between the pathogen and its host in terms of its infectivity and transmissibility.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. ESTABLISHMENT AND MAINTENANCE OF *BOMBUS TERRESTRIS*.

Queen bumble bees were collected from various sites in and around Palmerston North (Chapter 3). They were then screened for any detectable natural infection with NBT through fecal sampling. Faeces were collected by placing a clean glass slide under the mesh floor (of the front compartment) of a colony-starting box¹ housing the bee. When sufficient material had accumulated it was then examined for *Nosema* spores using the light microscope. This method provided only a gross estimate of infection levels because of the variations in fecal volume examined and the difficulty in detecting spores amongst high densities of pollen grains. Where no spores were detected in the faeces, the queens were fed a Fumidil-B/sugar solution (see section 1.3.1). The fumigillin was prepared at 1.3g powder (25mg fumigillin activity) / 1 litre sugar syrup (50:50 v/v), and supplied to the bee in a small screw-top jar with an absorbant wick. These 'clean' bees were then used in the experiments and also maintained as disease-free colonies. Any infected queens that were found were used directly in some experiments or as a source of NBT spores.

All bees in starter boxes and those used in experiments were supplied daily with pollen and had access to a continuous supply of sugar syrup. The pollen was supplied by various apiarists and was in the form of pollen pellets (as removed from the legs of the honey bee in commercial pollen traps).

2.2 SPORE INOCULA.

2.2.1 Origin of Spores.

Spores of NBT used in the preparation of the inoculum were obtained from infected queens. These bees were caught in the field and dissected specifically to obtain spore infected tissues. Some bees were also screened (as mentioned section 2.1) and these queens were maintained in the laboratory as a source of infected material. Other bees used as a source of NBT spores were those adults reared from

¹ This is a modification of the heated observation hive as designed by Pomeroy and Plowright (1980) and is scaled down for single bees and small colonies.

colonies naturally infected with the parasite.

2.2.2 Preparation of Spore Inocula.

The selected bees were anaesthetised with CO₂ and their abdomens were removed. These were placed in a small glass container singly or a few at a time, depending on their relative sizes and then crushed using a glass rod. Distilled water was added in sufficient quantities to produce a spore/tissue suspension that could be filtered through two layers of muslin. This process removed large fragments of cuticle and pieces of tissue. The extract obtained was then centrifuged at 2000rpm for 5 minutes. A crude pellet of spores was formed leaving some pieces of cuticle and tissue, pollen and bacteria in the supernatant. The supernatant was discarded and pellet was resuspended in distilled water. This was then centrifuged for a second time, the supernatant discarded and the pellet of spores resuspended. This process of resuspending the pellet and centrifuging 3 or more times had the effect of washing the spores and progressively removing any unwanted material. It produces a semi-pure sample suitable for *per os* inoculations. This routine is the most commonly used method of extracting microsporidian spores from invertebrate hosts. The preparation of a pure sample necessitates the use of a Ludox differential gradient column. This is an expensive process and was not considered necessary for the type of experimental work that was carried out.

The inocula preparations were also checked for the presence of other microorganisms and potential pathogens by microscopic examination of the stained film.

2.2.3 Spore counts

The spore concentrations of the inocula used were estimated using a 0.1mm deep haemocytometer (Neubauer Improved Brightline blood cell counting chamber), after Wigley (1980).

Numbers of spores in the fecal material from adult bees were also counted using the haemocytometer.

2.3 EXPERIMENTAL INFECTION OF INSECTS.

2.3.1 Infection methods.

Infectivity experiments frequently focus on the larval stage of the insect rather than the adult form. This is generally because they are the main feeding stage and, as many pathogens are transmitted through food, are more open to infection

(Burgess, 1981). Susceptibility to infection appears to decrease through subsequent larval instars to adults (Vavra & Maddox, 1976). Most insects studied usually have an independent larval stage that is easily handled and manipulated (e.g. *Bombyx mori*, codling moth, potato tuberworm). Bumble bee larvae however, are communally enclosed in a wax cell which, as they develop, is enlarged to accommodate them. At no stage can they be considered independent. Also, other insect larvae may be raised on artificial diets but the bumble bee larva relies on being fed a honey/pollen regurgitate at regular intervals through a hole in the wax cell. This would make hand-rearing a difficult and time-consuming task. Such is the life-cycle that it puts constraints on the use of the bumble bee larvae in experiments. Instead of larvae, the adult bees were predominantly used in the experiments. They comprised a selection of queens and workers.

Inoculation of the individual bees with the spore suspension was achieved using three separate techniques depending on whether larvae or adults were used. The first method involved enclosing each worker (starved for 12 hours), in a separate plastic jar with a screw-top lid. (Prior to this each bee had had its wings clipped in order to make the handling of them more efficient). The inoculum was dispensed as a 10 μ l drop onto the floor of the container, using an automatic pipette (Gilson, 2-20 μ l). This had been determined the maximum amount that the smaller bees in the sample would completely consume. The inoculum was diluted with sugar syrup as the bees preferred this over a similar solution of distilled water only. The bees were then left in the containers until the majority of them had consumed the inoculum. This took anywhere from 15 minutes to 2 hours depending on the hunger level of the bee. Those bees that did not consume the inoculum were discarded. Control bees were given an identical amount of sugar water only.

The second method was used to dose queen bees rather than workers or drones. This required the spore inoculum (usually 0.5 ml) to be placed in the wax honey pot that the queen had built in the starter box (see Fig. 52). Prior to this the queens were starved for 12 hours to ensure they would readily consume all the inoculum. When the honey pot had been emptied the queen was again given access to sugar syrup.

The third method involved the inoculation of larvae. Because the larvae are enclosed in a wax cell they can only be fed through a small hole on the upper surface. The larvae are positioned such that their body forms a semi-circle below this hole. A small amount of sugar/inoculum solution was dropped onto the surface of the larva through the hole. This elicits a feeding response from the larva and it

'laps' the solution up quite rapidly. However, determining the exact amount to feed any one larva of a particular instar is difficult as they can vary significantly in size. It is also very easy to 'drown' them if too much solution is fed at one time. Because of these features the work undertaken using larvae was limited.

2.4 MICROSCOPICAL TECHNIQUES

2.4.1 Light microscopy (LM).

The wet mounts of spores, stained smears and paraffin sections made from various insect body tissues were examined with either an Olympus CH light microscope or a Leitz fluorescence/light microscope (inclusive of a phase contrast system of objectives). Photographs were taken using Tech-Pan (low contrast) film in a camera fitted to a Leitz light microscope (with phase contrast objectives).

2.5 HISTOPATHOLOGY

2.5.1 Smears.

Generally, smears were made from representatives of each stage of bumble bee development - eggs, larvae, pupae and adults. Where possible the abdomens of individuals were dissected and the gut removed. This and the remaining tissues in the body cavity were dabbed repeatedly across the length of a glass slide. The eggs were simply pierced and their fluid contents then spread over a clean slide. Removal of the gut from the pupae proved difficult and so the abdomen was detached, cut open and the exposed tissues then smeared on the slide.

Inoculum smears were achieved by spreading a small aliquot of the solution over the length of a slide. To aid in adhesion of the material to the slide, it was first covered in a thin layer of glycerin albumin (Gurr, Lot 2416). This technique was used in the making of all slides. In all cases, the smears were left to air-dry before being fixed with 95% methanol and then stained with buffered Giemsa (Appendix 2).

2.5.2 Light Microscope - Paraffin sections.

Eggs, larvae, pupae and adults were all fixed in modified Carnoy's Fixative (Appendix 3). To allow for complete infiltration of the fixative into the insect tissues, an incision was made down the midline of the insect through the cuticle.

This opened the abdomen of the adult bees and the pupae to the fixative and assisted penetration of all tissues. The larger larvae were dissected along their dorsal surface the entire length of the body. The smaller larvae did not require any dissection.

The specimens were placed in 5 ml of fixative and left for 24 hours for fixative penetration. The use of Carnoy's solution meant that the fixed specimens could be placed directly into 70% alcohol on the carousel (Histokinette, Hendry Slough Bucks Relays) without requiring a step-down through alcohol.

The specimens were then processed - dehydrated and infiltrated with paraffin wax (56°C) (Appendix 3). They were then embedded, blocked and sections cut using a rotary microtome (Reichert, Austria). The thickness of the sections ranged from 5 - 8 μm . As an aid to the sectioning of cuticular material Mollifex was utilised. Prior to sectioning the block was placed with the cut face submerged in Mollifex* for up to 1 hour. (*Mollifex is a chemical solution used to soften tissues that are otherwise difficult to section). This helped to soften the cuticle and thinner, smoother sections could be obtained. Sections were kept at intervals along the length of the specimen. These were then placed on a slide, floated and heated on a warm plate at 45°C until the wax cleared and any wrinkles had smoothed out. After this stage the sections could be de-waxed and stained (Appendix 3).

2.6 STATISTICAL METHODS

The statistical package MU-TAB PC version 3.01 (Copyright I.F. Boag, Massey University), run on an IBM-compatible XT, was used for data analysis.

CHAPTER 3

OCCURRENCE OF NOSEMA-INFECTED QUEEN BUMBLE BEES
FROM SITES IN THE MANAWATU AND OHAKUNE.

3.1 INTRODUCTION

One important aspect of insect-pathogen relationships is the occurrence of epizootics. This becomes quite complex in social insects as the behaviour within the colony directly affects infection levels and epizootics. Ideally when studying such relationships, information on both the host population and the percentage host infection should be obtained in order to better understand the epidemiology of protozoan diseases (Maddox 1987), yet few studies are this detailed. However, it may be possible to make inferences on gross trends in cases where the data is incomplete (e.g. where the host population size is unknown).

Extensive research on the honey bee and *N. apis* has indicated that changes in the parasite populations are seasonally related. Globally it has been shown that the prevalence of *N. apis* (whether it is expressed as % colonies infected or % infected bees per infected colony) has a consistent seasonal variation (Maddox 1987). Levels of infection tend to be highest in the spring and decrease over the summer. This is believed to result from the prolonged confinement of infected bees over the winter. Bees are more likely to defecate on the comb during this time, and in the spring when brood-rearing commences, healthy, susceptible bees clean the combs and in turn become infected (Bailey 1955). The infection persists at a low level over the summer months and is probably maintained during the winter (when bees are confined) by the infective spores in the fecal material on the comb (Bailey 1981). Other sources of infection have been proposed, including flowers covered in excrement, drifting drones from infected hives, air currents and fecally contaminated drinking water (Fantham & Porter 1912, 1914), but none of these have been rigorously examined as important vectors (Bailey 1981).

The possible seasonality of *Nosema* infection in bumble bees has received little research attention. Betts (1920) noted spring queens with high levels of *Nosema* infection and Skou et al (1963) had similar findings in Autumn queens (prior to hibernation). Alford (1978) believed that the only way the protozoan could survive was in overwintering queens harbouring low levels of infection. With a more

temperate climate in New Zealand seasonal trends in *Nosema* incidence are likely to be less pronounced, because bumble bee queens are found almost all year round. R.P. Macfarlane (unpublished manuscript) surveyed sites in Canterbury (South Island) from September - November (spring) and found that less than 1% of *B. terrestris* queens were infected with *Nosema*. He noted that the ovarian development and weights of infected queens were similar to those from healthy queens. Two other species of bumble bee examined at that time did not show evidence of infection. Fisher & Pomeroy (1989b) observed that 61% of *B. terrestris* field-placed colonies (from November-January = spring/summer) and 10% of queens (caught August - November = winter/spring) in the Manawatu, were infected with *Nosema*. They noted that *Nosema* did not appear to cause significant mortality of queens or young nests (some of the most productive colonies were infected with the protozoan).

From the few studies on *Nosema* incidence in the bumble bee it appears that infection levels are not tightly synchronised with the seasons (compared to *N. apis* in the honey bee). A survey of 3 areas in the Manawatu (and samples from sites in Ohakune) frequented by bumble bee queens, was undertaken in tandem with the collection of these bees for later study. This provided estimates of *Nosema* infection levels in the queen bumble bee populations at these sites at certain times of the year and allowed for some comparisons to be made between sites.

3.2 METHODS

Queens were collected from 3 sites in the Manawatu (1988 - 1990) and Ohakune (early 1990). The plants from which the bees were captured were tree lucerne (*Cytisus proliferus* L.) which flowers from early July to September and various species of Rhododendron, which flower from August to February in these areas. Queens only were sampled as they alone overwinter and are the most likely source of *Nosema* infection for the next generation. The sampling times coincided with the spring emergence of queens and the period prior to the hibernation of the new season's queens.

The queens were then either 1/ dissected and smears were made of their gut and its contents, or 2/ contained in starter boxes and samples of their faeces were taken. Both gut and fecal smears were examined for the presence of *Nosema* spores. In areas of questionable infection the slides were stained with Giemsa (Appendix 2), a

technique that differentially stains the *Nosema* spores.

3.3 RESULTS

NBT was found in queens from all but one of the sites sampled (refer Table 1). The highest incidences of infection were found in queens from the "Esplanade" in the town of Palmerston North. These levels were high throughout the year with the highest levels at the end of summer (1988), in spring (1988) and in late winter (1989). The high levels observed over the sampling periods provide no evidence for any seasonal trends. At the Kimbolton and Keebles sites (sampled less frequently) infection levels generally decreased from 1988 - 1989 and the levels were lower than found in queens from the Esplanade site. The Ohakune sample contained no infected bees.

Pooling the results for each site gave infection frequencies of 36.7% (n=154) at the Esplanade, 20.45% (n=56) at Keebles, 17.36% (n=119) at Kimbolton and 0.0% (n=192) at Ohakune. These frequencies appear to decrease as the sites become more rural and/or distanced from urban areas and also as the topography varies. The combined infection frequency averaged over all sites and dates was 26.1% (n=521).

3.4 DISCUSSION

Only the queens were sampled as 1/ they may be considered a primary source of infection for the first generation, 2 /they are the most visible of the castes, and 3/ there is difficulty in finding nest sites to screen other castes. Measurement of bumble bee field populations would have been a major and complex task which was beyond the scope of this project.

The infection frequencies as calculated may not truly represent the level of infection present in the queen populations due to 1/ the small sample sizes from some sites and 2/ the insensitivity of the techniques used at detecting low levels of infection, (those less than 1000 spores/insect). However, the overall level of infection for all sites was quite high, with approx. one in every four bees infected with NBT. This level is higher than that found in queens from Canterbury (R.P. Macfarlane unpublished manuscript) and in the bees sampled by Fisher & Pomeroy (1989b). The results of this survey imply that within the Manawatu region NBT is quite prevalent and variable.

High levels in the late winter indicate that queens emerging from hibernation are infected or become infected soon after emergence. Infected queens in the late summer, provided they do not die prior to hibernation, are possibly the source of *Nosema* for the spring outbreaks. The infection levels are fairly high throughout the sampling periods (the limited number of sampling periods obscuring any clear trends) and provide no evidence for seasonal trends.

The location of the sites may have an effect on the level of infection found. Maddox (1987) noted that honey bees in rural areas had lower rates of infection than those placed in urban areas and suggested it was because hives in urban areas were moved more frequently (increasing stress levels within the colony). Another possibility is that a higher density of honey bees in one area would increase the chances of infected bees contacting susceptible bees, directly or indirectly.

The sites sampled in this survey would have differed climatically although precise site data are not available. The Esplanade site was flat and fairly sheltered within the city, the Keebles site was more exposed on farm-land, Kimbolton was a higher altitude site in hilly country and the Ohakune sites were near Mount Ruapehu. As these sites became progressively more "rural" they featured colder winter temperatures and a shortened plant growth season. This seems to be associated with a more compact queen flight season and tighter synchrony of bumble bee colony cycles (N. Pomeroy pers. comm.). In the more mild areas there would have been more temporal overlap between different stages of the colony cycle and hence a possible increase in opportunities for spore transfer between queens, and workers from other colonies.

Little is known of the interactions (if any) between the honey bee and the bumble bee and it is possible that contact between the two and higher population densities in urban areas could influence levels of *Nosema* infection. Fantham & Porter (1914) claimed they found bumble bees infected with both *N. apis* and *N. bombi* near honey bee hives and also honey bees infected with *N. bombi* and believed such conditions to arise from inter-racial robbing. Because the *Nosema* in the bumble bee is poorly defined and understood, there have been varied reports on the likely/unlikely cross-infection between the honey bee and the bumble bee. Until these differences have been resolved, there still remains a possibility that *Nosema* infection is passing between these two species and is one of the ways in which infection levels are being maintained in bumble bee populations.

TABLE 1 Frequency of *Nosema* infection in *Bombus terrestris* queens caught from various locations in and near the Manawatu.

Date		Site Sampled ^a	Percentage Infection	Number in Sample
February	1988	Esplanade	30.7	39
March	"	"	54.7	42
July	"	Keebles	33.3	13
August	"	Esplanade	28.5	21
October	"	"	40.9	22
November	"	Kimbolton	35.7	28
July	1989	"	0.0	24
August	"	Keebles	7.6	26
August	"	Esplanade	42.8	17
October	"	Kimbolton	16.4	67
Feb/Mar	1990	Ohakune	0.0	192
July	"	Esplanade	23.1	13

^a Further locality details given in Appendix 1.

CHAPTER 4

THE STRUCTURE AND REPRODUCTION OF *NOSEMA* IN
BOMBUS TERRESTRIS.

4.1 INTRODUCTION

The taxonomy of the microsporidia has been widely studied (e.g. Wieser 1977, Sprague 1977) but there exist few comprehensive guides for their identification. Because new species and genera of microsporidia are frequently being found, and the old re-assessed, taxonomic reviews and identification keys constantly require updating.

Identification of a microsporidian to genus depends in part on the features visible with the light microscope which, in some situations (such as field work), is the only conveniently available tool (Hazard et al 1981). The most important taxonomic features at this level are spore shape and size, the number of nuclei in spores and somatic stages, the particular modes of merogony/sporogony (binary or multiple fission, plasmotomy), the presence or absence of a sporophorous vesicle, the number of spores in the vesicle, tissue affinity (histopathology) of the microsporidian and the identity of the host (Larsson 1986, Canning 1990). Although some characters have changed in emphasis over recent years (e.g. spore size and shape are considered less important now due to the wide degree of variation, that recent studies have described, even within single species) all of the characters mentioned are still applicable at various taxonomic levels (Vavra et al 1981).

Infrageneric taxa cannot always be separated by LM alone and it is often necessary to incorporate observations using electron microscopy, particularly transmission electron microscopy (TEM) of resin-embedded sections. Larsson (1988) reviewed the ultrastructural characters considered important for the identification of microsporidian species. These characters include structural features of the exospore, polaroplast and polar filament, the number of filament coils, the diameter of these coils and the form of the sporophorous vesicle (if present).

The application of EM has resolved some of the early problems of defining genera/species and interpreting parasite morphology, spore structure and some host-parasite interactions (Canning 1990). However, LM is still an essential tool for routine identification and the clarification of developmental sequences and for the

determination of stages recorded in electron micrographs (Hazard et al 1981).

Fantham & Porter (1914) were the first to describe a microsporidian in bumble bees (from various sites in England), which they named *Nosema bombi*. The type host was *Bombus agrorum*, but the parasite was also seen in *B. terrestris* and other *Bombus* species and also in *Apis mellifera*. To describe the morphology and life-history of *N. bombi* they made use of stained smear preparations and histological sections of bumble bee gut tissues. They also examined the succession of the developmental stages through a time trial experiment, infecting the host specimen's *per os* and then microscopically examining them at set time intervals. From these procedures, the diagnostic features they observed for *N. bombi* included meronts that were variable in size, shape and number of nuclei, binucleate sporoblasts and broad, oval spores (approx. $5.2 \times 3.7 \mu\text{m}$). They also determined that *N. bombi* development commenced within 48 hours of ingestion by the host and observed that the infection was sited primarily in the Malpighian tubules with occasional additional infection of the host's midgut. *N. bombi* was found to infect bumble bee queens and workers in equal proportions and drones to a lesser extent. Larvae were also found with the infection although it was believed they became infected through contaminated pollen and not transovarially. In *N. bombi*, as with many microsporidian infections of the gut, infective spores were released with fecal material and ultimately contaminated the food of the host.

Fantham and Porter's observations on *N. bombi* and their final conclusions were acceptable under the standards at the time. Today, however, the definition of a microsporidian to the level of species would require added ultrastructural knowledge, obtained only through the application of the EM. Considering this, it is reasonable to be a little sceptical of the validity *N. bombi* as a distinct species when it was originally defined only on the basis of LM observations.

In *B. terrestris* from New Zealand, in preliminary observations in the present study, the microsporidian was found in the gut of the bees and in severe infections the gut became pale and distended. When the diseased gut was perforated, a milky fluid containing numbers of spores was released. The spores, when examined in wet mounts, were small, refractive, oval structures similar to those observed by Fantham and Porter. Stained spores had characteristics of spores of *Nosema* species in general, having two distinct nuclei and a terminal crescent of cytoplasm. Infected bees did not exhibit any obvious difference in behaviour compared to healthy bees and infected queens commenced nest-making and reared apparently healthy brood.

In comparison, Fantham and Porter noted that infected bees frequently lost their power of flight, that they were easily irritated and often died as a result of the infection.

The purpose of this study was to examine the microsporidian observed from *B. terrestris* (in NZ), particularly to detail its life history and ultrastructure. On the basis of these observations comparisons with *N. bombi* and other microsporidia known from Hymenoptera, were made in order to assess the taxonomic status of NBT. The first part of the study was conducted using the same general methods as used by Fantham and Porter in describing *N. bombi*. This involved the application of smear preparations and histological sections, from both naturally infected and experimentally-inoculated bees, to elucidate the life cycle and developmental stages of NBT. The use of TEM enabled observations to be made on the ultrastructure of the microsporidian and comparisons to be made with other microsporidia at that level. The infectivity of NBT to other insect species was also examined in a limited trial.

4.2 METHODS

4.2.1 Microscopy

a) Smears

Smears were made of the gut and tissues from the abdomens of infected and healthy individuals representing each developmental stage of *B. terrestris* (i.e. adults, pupae, larvae and eggs) (see section 2.5.1). The resulting smears were fixed in 95% methanol and stained in buffered Giemsa solution (Appendix 2). These individuals were sampled randomly from infected and healthy laboratory colonies of *B. terrestris*.

b) Light microscope sections - Paraffin method.

Eggs, larvae, pupae and adults were haphazardly sampled from infected and healthy laboratory colonies of *B. terrestris*. Specimens were fixed in modified Carnoy's solution either *in toto* or after varying degrees of dissection, embedded in paraffin, sectioned and examined (Appendix 3).

c) Light and electron microscope sections - Epoxy resin method.

The gut from one infected worker bee (obtained from the nest of an artificially

infected queen), was removed and prepared as in Appendix 4, embedded in Spurr's resin and sectioned using both glass and diamond knives on a Reichert-Jung Ultracut E ultramicrotome. Both the midgut and Malpighian tubules were examined for spores and life-cycle stages. Unstained 0.25 - 0.50 μ m sections of plastic-embedded tissue were examined with phase-contrast optics under the light microscope. Sections of 60 - 100nm thickness were collected on formvar-coated 50-mesh or slot grids, stained with uranyl acetate and lead citrate, and examined with a Philips transmission electron microscope.

d) Measurements of spores and vegetative stages

Life cycle stages were measured from Giemsa stained smears (Appendix 2) made from the guts of adult bees. Measurements were made using a Leitz filar eyepiece micrometer (Leitz Wetzlar Germany) in an Olympus CH microscope at 1000x magnification.

Spores from adult bees were measured from water mounts after the method of Vavra and Maddox (1976) using the filar eyepiece micrometer at 1000x magnification. Spores were also measured from Giemsa stained smears made from individuals representing each developmental stage of *B. terrestris* (i.e. larvae, pupae and adults).

Mean spore sizes from individuals were then compared using a two-sample (two-tailed) t test (Williams 1984).

4.2.2 Time-course Experiment

a) Method of infection and sampling

The inoculum used for infecting the bees was prepared as in section 2.2.2. One hundred worker bees were infected *per os* from a suspension of 6.7×10^6 spores/ml. Each bee received a dose of 10 μ l of suspension containing approx. 6.7×10^4 spores. Only the bees that completely consumed the inoculum within 2 hours were used in the experiment (see section 2.3.1). The bees were held in small wooden boxes with mesh floors and maintained at $30 \pm 1^\circ\text{C}$ and $40 \pm 5\%$ relative humidity. They were provided with a continuous supply of sugar-water and were fed pollen daily for the period of the trial. Forty control workers were fed sugar water instead of the spore suspension. They were placed under identical conditions to those of the inoculated bees.

At 12 h intervals, from the time of consumption of the inoculum to the

appearance of new spores in the subject insects, samples of 4 workers were taken. After spores appeared, samples were taken at 24h intervals. The bees were anaesthetized with CO₂, dissected, and smears were prepared from the excised gut. Every 24 h two control individuals were removed and treated as above. Also at 24 h intervals, two inoculated workers and one control worker were removed and prepared for histological sectioning (see section 2.5.2). Sections were taken at intervals along the entire length of the individual.

The changes in the numbers of vegetative stages observed over the period of the time course experiment were determined by a series of sample counts on the smears from infected bees. At each count, the number of each stage seen in a randomly chosen field of view (at 1000x magnification, oil immersion) was noted. The counting continued over all of the slides from a particular time point until half an hour had elapsed.

Minimum generation times were determined by the methodologies of Kramer (1965) who defined 'minimum generation time' as the time taken from inoculation to the first appearance of spores of the next generation and Milner (1972a) who defined it as the interval between inoculation and the first smears made in which 50% of the stages are spores.

4.2.3 Cross-infection studies

Larvae of the lepidopteran species *Plutella xylostella* and *Epiphyas postvittana* were exposed to spores of NBT and *N. apis*. The larvae were obtained from disease-free stocks maintained at the Horticulture Department at Massey University. *P. xylostella* larvae were maintained on potted cabbage plants and *E. postvittana* larvae were fed an artificial diet. 4mm² pieces of cabbage leaf and 4mm³ sections of the artificial diet were inoculated with 10 µl of a 5 x 10⁶ spore suspension of either NBT or *N. apis*. This suspension was left to dry to allow a film of spores to form on the food. Only the larvae that consumed all the inoculum were kept for the trial. Some larvae were fed equivalent sized pieces of cabbage or diet inoculated with distilled water only and were kept as controls against the possible presence of species-specific pathogens. Dead larvae found after the onset of the trial were examined microscopically in wet mounts and stained smears for evidence of infection. Adults emerging after pupation were anaesthetized with CO₂, dissected and prepared for LM examination. On termination of the trial all remaining live insects were sacrificed and examined.

Healthy *B. terrestris* larvae were inoculated with NBT or *N. apis* spore suspensions (section 2.2.2). On termination of the trial all larvae and pupae were dissected and examined for evidence of infection (section 2.5.1).

4.3 RESULTS

4.3.1 Morphology of NBT

a) Qualitative aspects

The most heavily infected region of the gut was the Malpighian tubules (Figs 36,40). Where the infection was extreme the structure of the tubule was destroyed and mature spores were found in the lumen. The midgut epithelium was also infected though not as heavily as the tubules (Fig. 31). Spores were found in the regenerative cell layer of the epithelium near the basement membrane and also in the muscle layer surrounding the gut (Fig. 38). No other tissues examined appeared to be infected. Eggs that were removed from infected colonies and sectioned provided no evidence of internal infection.

Meronts were seen in the epithelial cells of the Malpighian tubules and in the midgut, in the outer muscle layer and in the regenerative layer of the epithelium. They were bounded by a simple unit membrane in direct contact with the cytoplasm of the host cell (Figs 40,44,46). They were predominantly round in outline and were variable in size with a range of 3.36 - 7.35 μ m length x 2.66 - 6.51 μ m width, n=120 (see Table 2). Many of the meronts were binucleate (Figs 2-4,26,27,40,41,44,46) with condensed and commonly, closely opposed nuclei (diplokarya). There were also numbers of smaller, densely stained, apparently uninucleate meronts with large nuclei (Figs 1,29). A trinucleate meront and larger tetranucleate forms were also present (Figs 6,7,8). Tetranucleate meronts were observed cleaving into two binucleate forms (Figs 9,31). An apparently binucleate meront undergoing cytoplasmic cleavage was observed (Figs 5,27). Occasionally the nuclei of cells in smear preparations were not very distinct, being more diffuse and/or crescent-shaped than those observed in the majority of meronts.

A few intact epithelial cells (from either the midgut or the Malpighian tubules) were observed from smears of larval gut. These contained large numbers of small, compact meronts and other developmental stages (Figs 32,33). The nuclei of many of these meronts were indistinct. A section of intact Malpighian tubule from a larval

FIGS 1 - 25 Diagrammatic representations of NBT somatic and reproductive cells as seen in air-dried, alcohol-fixed and Giemsa-stained smears.

- Fig. 1 Uninucleate meront.
- Figs 2,3,4 Binucleate meronts.
- Fig. 5 Binucleate meront undergoing cytoplasmic cleavage.
- Fig. 6 Trinucleate meront.
- Figs 7,8 Tetranucleate meronts.
- Fig. 9 Tetranucleate meront undergoing cytoplasmic cleavage.
- Fig. 10 Binucleate fusiform sporont with vacuole.
- Fig. 11 Binucleate sporont.
- Fig. 12 Binucleate sporont undergoing cytoplasmic cleavage.
- Fig. 13 Uninucleate sporont.
- Fig. 14 Uninucleate fusiform sporont.
- Fig. 15 Tetranucleate sporont.
- Fig. 16 Tetranucleate sporont undergoing cytoplasmic cleavage.
- Fig. 17 Uninucleate oval sporoblast
- Fig. 18 Binucleate oval sporoblast.
- Fig. 19 Vacuolated binucleate sporoblast.
- Figs 20,21,22 Immature spores.
- Fig. 23 Mature spore.
- Fig. 24 Mature spore - wet mount.
- Fig. 25 Large immature spore.



1 2 3 4 5



6 7 8 9



10 11 12 13 14



15 16 17 18



19 20 21 22 23



24 25

5µm

TABLE 2 Measurements (μm) of the life-cycle stages of NBT as seen in Giemsa stained smears from adult bumble bees.

Reproductive Stage	Sample Size	Length		Width	
		Mean \pm SD	Range	Mean \pm SD	Range
Uninucleate meront	40	4.19 \pm 0.58	3.36 - 5.25	3.53 \pm 0.49	2.66 - 4.34
Binucleate meront	40	4.71 \pm 0.61	3.78 - 6.51	3.93 \pm 0.33	2.80 - 4.76
Tetranucleate meront	40	5.73 \pm 0.61	3.85 - 7.35	4.82 \pm 0.60	3.71 - 6.51
Uninucleate sporont	40	6.40 \pm 0.49	5.32 - 7.70	3.32 \pm 0.36	2.80 - 3.99
Binucleate sporont	40	6.61 \pm 0.48	5.67 - 8.05	3.65 \pm 0.34	3.15 - 4.41
Sporoblast	40	5.24 \pm 0.26	4.64 - 5.76	3.37 \pm 0.21	3.03 - 3.94

smear contained meronts (Fig. 34) as did one from an adult smear (Fig. 35). No chains of meronts were observed from any of the preparations.

Sporonts were oval in outline and, in smears, had more diffuse, pale-staining cytoplasm than was typical of meronts. The nuclei in many of the sporonts were positioned towards one end of the cell (Figs 11,13,14). It was often difficult to determine the number of nuclei in the sporonts - many were apparently uninucleate while others were distinctly binucleate. They were generally large, and uninucleate forms did not differ significantly in size from binucleate forms ($P > .05$, $n=80$). Binucleate sporonts were also observed undergoing cytoplasmic cleavage (Fig. 30). Fusiform sporonts were observed (Figs 10,16,29) but sporonts of this shape were not very common. The few tetranucleate sporonts seen were large, elongated cells with pairs of nuclei positioned at either pole and a few were observed dividing (Fig. 31). They were faintly staining and had highly vacuolated cytoplasm (Fig 15).

Sporonts observed in EM sections of Malpighian tubule were characterised by a layer of electron-dense material on the surface of the plasmalemma (Figs 40,42). No sporonts were observed from EM sections of the midgut epithelium.

Sporoblasts were similar in shape to the oval sporonts but were smaller and frequently more darkly stained (Figs 17,18). In some sporoblasts it was possible to distinguish two nuclei, and in others there appeared to be only one nucleus. A few sporoblasts were clearly binucleate and were vacuolated, this state possibly representing a later stage during sporogenesis (Figs 19,30).

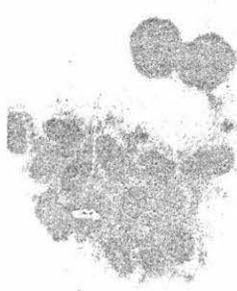
Transformation of sporoblasts in the Malpighian tubules was accompanied by further development of the polar filament, the isolation of the cell from the host cell cytoplasm (evidenced by a clear space) and a change in cell membrane structure resulting in stellate forms (Fig. 42). The sporoblasts also became quite electron-dense. In midgut sections, however, no sporoblasts were found with the stellate form nor did any show polar filament development although developing polaroplasts were observed (Figs 44,48,49).

The spores, refractive in wet preparations, were oval in outline and smaller than the sporoblasts. In smear preparations some spores stained quite faintly and it was difficult to determine the number of nuclei present in these cases. Darker staining spores had an outer edge of clear cytoplasm and two distinct nuclei in the stained central region (Figs 20,21,22). These spores were considered to be immature as the nuclei were still apparent. The mature spores were distinguished by only a terminal

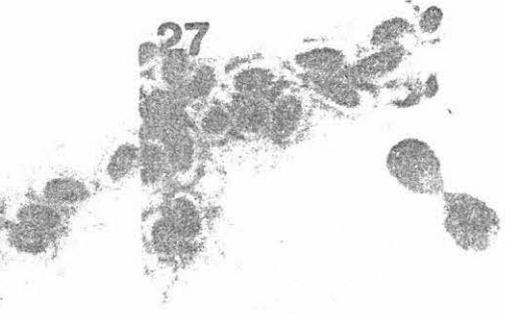
FIGS 26-35 NBT : Light micrographs of smear preparations.

- Fig.26 Tetranucleate meront and a cleaving binucleate meront (adult gut smear), 1400x.
- Fig.27 Cleaving binucleate meront (adult gut smear), 1200x.
- Fig.28 Tetranucleate meronts (larval gut smear), 1500x.
- Fig.29 Apparently uninucleate meront, a binucleate sporont (arrow) and a binucleate fusiform sporont with a vacuole (adult gut smear), 2200x
- Fig.30 Binucleate sporont undergoing cytoplasmic cleavage (adult gut smear), 1500x.
- Fig.31 Tetranucleate sporont undergoing cytoplasmic cleavage (adult gut smear), 2500x.

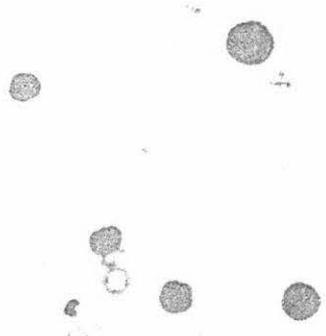
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27



28



29



30



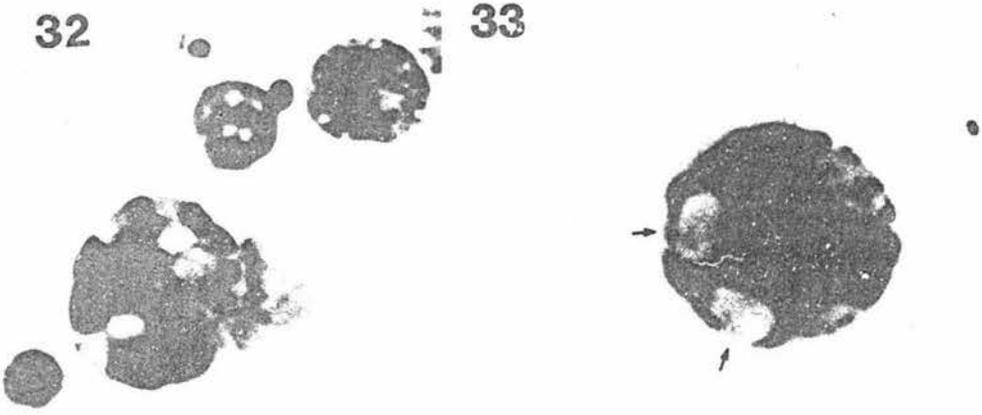
31



- Fig.32 Epithelial cell containing meronts and sporonts (larval gut smear). 1300X.
- Fig.33 Epithelial cell containing sporonts and immature spores (arrows) (larval gut smear). 2500x.
- Fig.34 Malpighian tubule containing spores and somatic stages (larval gut smear). Note tetranucleate meront (arrow). 800x.
- Fig.35 Ruptured Malpighian tubule with spores and meronts (adult gut smear). 800x.

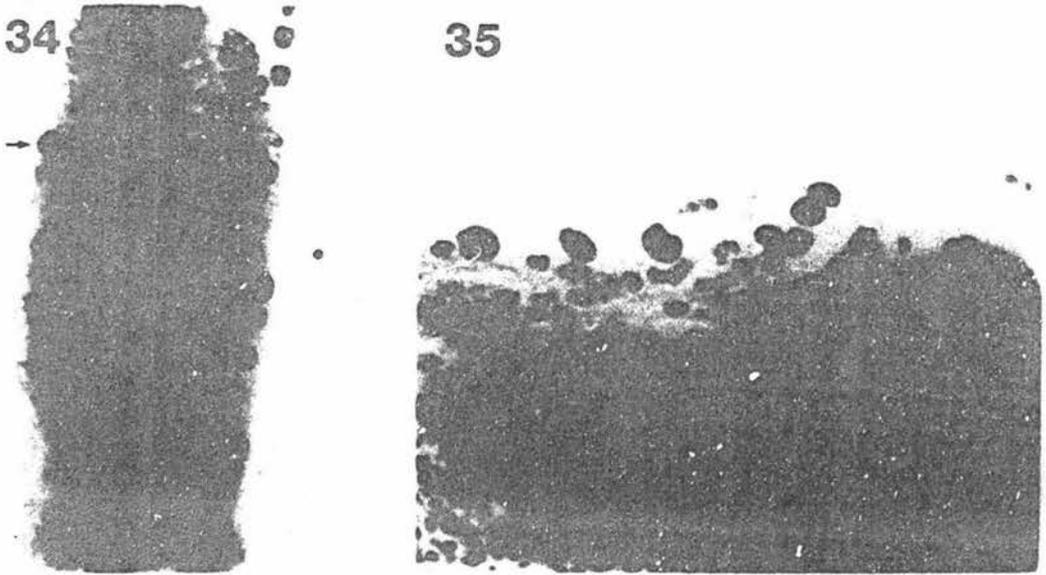
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FIGS 36-49 NBT : Light micrographs of unstained resin-embedded sections,
(phase contrast optics).

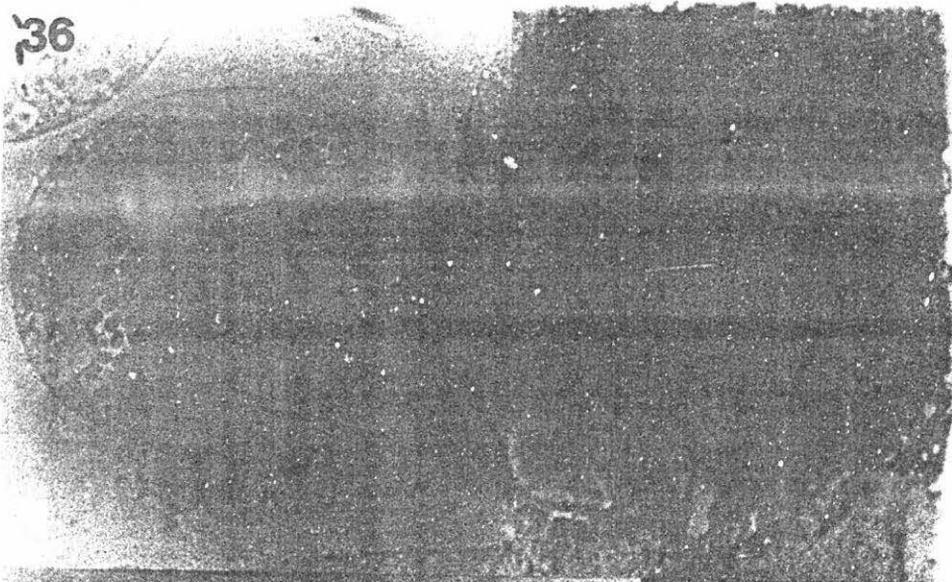
Fig. 36 Malpighian tubule sectioned to show spore infiltration. 1000x.

Fig. 37 Section of midgut epithelium containing a group of spores (s) and
somatic stages (large arrows). Note brush border (arrow). 2000x.

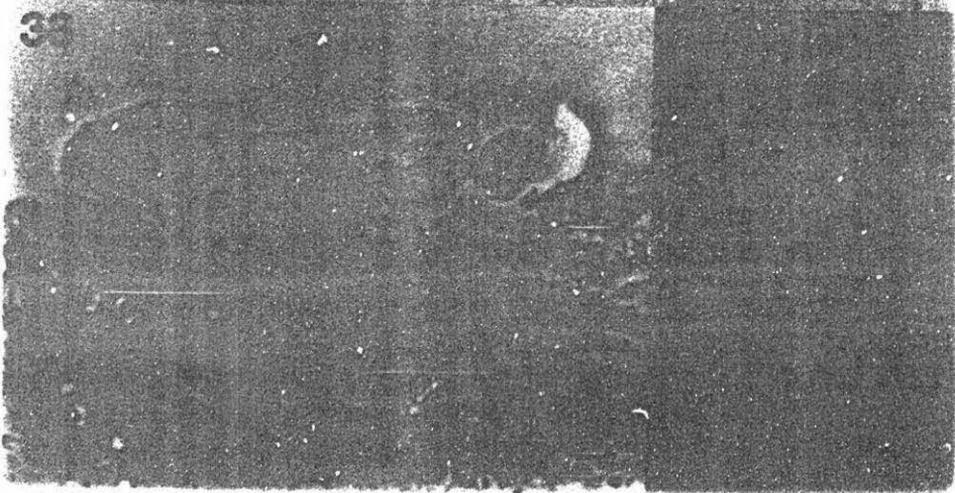
Fig. 38 Section of midgut tissue (e) and associated muscle layer (m)
containing spores and somatic stages (arrows). 1500x.

Fig. 39 Tracheole with spore (arrow) in associated connective tissue.
1800x.

36



37



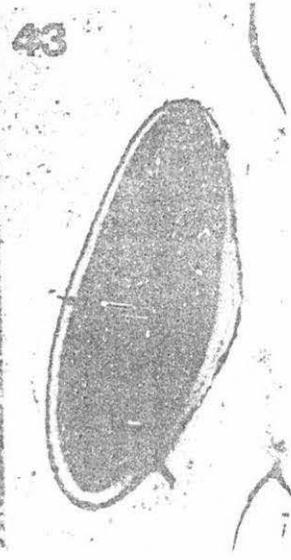
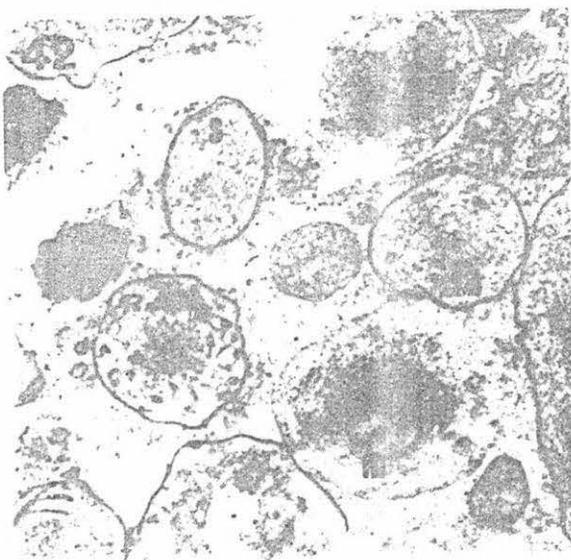
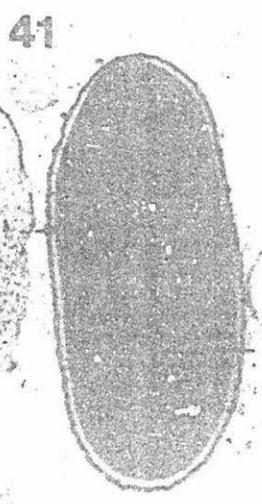
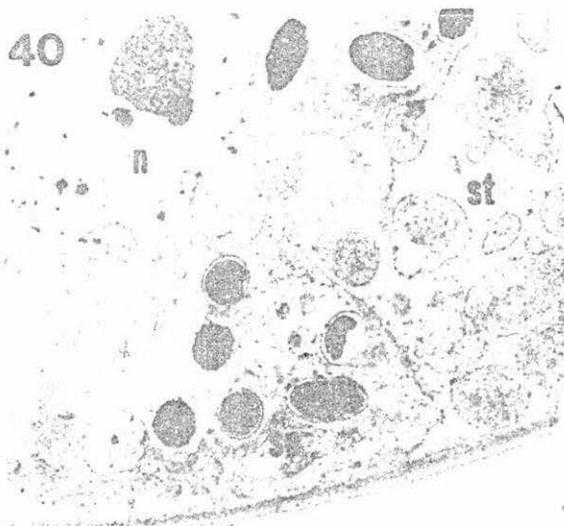
FIGS 40-43 NBT : Electron micrographs of cells in Malpighian tubules.

Fig. 40 Section of Malpighian tubule containing mature spores (sp) and somatic stages (st). Note host cell nucleus displaced (n). 10000x.

Fig. 41 Mature spore LS showing diplokarya (n), polaroplast (pp) and sections of polar filament (arrows). 21300x.

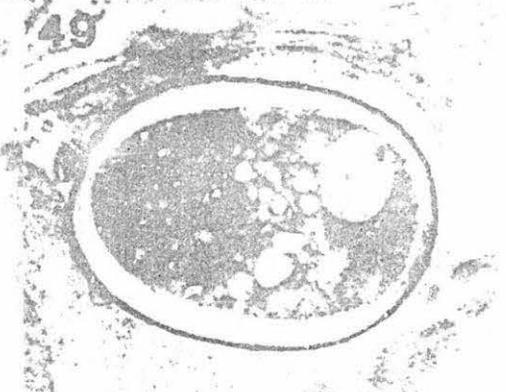
Fig. 42 Sporoblasts (sb) showing a developing polar filament (arrow) and thickened membrane, speronts (s) and meronts (m). 10000x.

Fig. 43 Immature spore, oblique section, showing strands of ribosomes (large arrow) and a developing polaroplast (pp) and polar filament (arrow), (n) is spore nucleus. 15000x.



FIGS 44-49 NBT : Electron micrographs of cells in midgut tissue.

- Fig. 44 Section of midgut with sporoblasts (sb), spores (sp) and other somatic stages (arrows) showing diplokarya, found in the epithelium (e) and muscle tissue (m). 3400x.
- Fig. 45 Maturing spores enclosed in an epithelial cell (arrow) of the midgut, (n) host cell nucleus. 3400x.
- Fig. 46 Meront with diplokarya in muscle tissue (m), (n) cell nucleus. 15300x.
- Fig. 47 Spore enclosed in a midgut epithelial cell, (bb) brush border. 5200x.
- Fig 48 Mature spore from midgut showing diplokarya (n), polaroplast (pp) and strands of ribosomes (arrow) but no polar filament. 31900x.
- Fig. 49 Sporoblast. 21300x.



portion of the cytoplasm staining and no obvious nuclei (Fig. 23). Large spores (possibly macrospores) were seen, approx. half as large again as normal spores (Fig. 25). They stained similarly to the immature spores and had two nuclei.

Mature spores observed in EM sections of Malpighian tubule were elongated oval structures, had two nuclei (diplokarya) and 14-18 polar filament coils (Figs 41,43). The spores occurred singly and in contact with the cytoplasm of the host cell. Spores found in the mid-gut were more rounded in outline and lacked any trace of a polar filament (Figs 48,49). Similar spores were seen apparently migrating up through the epithelium of the mid-gut and were enclosed within epithelial cells (Figs 45,47). A spore was also found in the epithelium of a tracheole which may have been attached to the connective tissue of the midgut (Fig. 39).

Spores were measured from smears made from an infected individual representing each developmental stage of *B. terrestris*. Spore length varied significantly between some individuals (see Table 3); spores from the queen the smallest, those from the worker the largest and the larva and pupa with spores of an intermediate size. The widths of the spores were less variable between individuals and had smaller size ranges. Large spores (comprising less than 2% of spores measured) were observed from all developmental stages of *B. terrestris*. They were approx. 1.5 times as long, and 1.1 times as wide, as 'normal' spores. Their mean size was $6.56\mu\text{m} \times 3.10\mu\text{m}$ with a range $5.60 - 7.70\mu\text{m} \times 2.73 - 3.50\mu\text{m}$ ($n=50$).

The mean size of NBT spores as measured from a fresh preparation of adult *B. terrestris* gut was $4.88\mu\text{m} \times 2.88\mu\text{m}$ with a range $4.20 - 5.39\mu\text{m} \times 2.31 - 3.29\mu\text{m}$ ($n=50$). This was significantly smaller ($P < 0.001$, $n=50$) than for spores of *N. apis* from an *A. mellifera* worker, measured under the same conditions ($5.59\mu\text{m} \times 2.94\mu\text{m}$ with range $4.97 - 6.51\mu\text{m} \times 2.17 - 3.29\mu\text{m}$, $n=50$). No "large" spores were found in the fresh preparations.

b) Quantitative aspects

Table 4 summarises the percentage composition of stages seen at various time points during the time course experiment.

Meronts (uni-, bi- and tetranucleate) were observed 24 h after inoculation of the subject insects, with tetranucleate forms comprising the smallest proportion. At 36 h the first sporonts were observed and included both uni- and binucleate forms. At 48 h a large number of sporonts and sporoblasts appeared together, although these stages were observed in only one worker, the others sampled at this time point being

TABLE 3 Measurements (μm) of spores of NBT taken from all stages of *B. terrestris* development.

Insect Stage	Sample Size	Length		Width	
		Mean \pm SD	Range	Mean \pm SD	Range
<u>Stained smears</u>					
Queen	50	4.34 \pm 0.38 A ^a	3.43 - 5.11	2.74 \pm 0.24 B	2.17 - 3.15
Worker	50	4.64 \pm 0.33 B	3.92 - 5.39	2.91 \pm 0.29 A	2.31 - 3.29
Larva	50	4.58 \pm 0.32 B	3.71 - 5.25	2.73 \pm 0.27 B	2.17 - 3.22
Pupa	50	4.56 \pm 0.33 B	3.78 - 5.32	2.79 \pm 0.24 B	2.31 - 3.29
Combined All	200	4.53 \pm 0.34	3.43 - 5.39	2.79 \pm 0.26	2.17 - 3.29
Large spores	50	6.56 \pm 0.59 C	5.60 - 7.70	3.10 \pm 0.21 C	2.73 - 3.50
<u>Wet mounts</u>					
<i>B. terrestris</i> worker NBT	50	4.88 \pm 0.28 D	4.20 - 5.39	2.88 \pm 0.28 D	2.73 - 3.50
<i>A. mellifera</i> worker <i>N. apis</i>	50	5.59 \pm 0.39 E	4.97 - 6.51	2.94 \pm 0.22 D	2.17 - 3.29

^a Values without a letter in common differ significantly at P = 0.05 ; values without a letter were not compared.

TABLE 4 Time trial results : Percentage composition of the life-cycle stages of NBT at set time intervals, as determined from Giemsa-stained smears.

Time (h)	No. of infected bees ¹	Total stages counted	Percentage							
			Meronts			Sporonts			Sporo-	Spores
			N	2N	4N	N	2N	Tot.	blasts	
12	3	0								
24	3	55	40	40	20					
36	3	145	65	30	1	3	1	4		
48	2	74	39	15	4	16	26	42		
60	1	239	10	5	1	45	13	58	26	
72	4	120	18	7	3	50	21	71	<1	
84	3	116	12	4	4	40	30	70	10	
96	3	119	9	12	5	22	42	64	10	<1
120	3	195	4	4	1	33	28	61	25	5
144	3	244	12	2	<1	40	20	60	14	11
168	3	242	9	7	1	10	10	20	23	40
192	4	294	8	3	3	10	11	21	11	54
216	2	144	11	6	-	22	15	37	22	24

¹ Four bees in each sample.

free of infection. The number of sporoblasts at 60 h was significantly reduced from that observed at 48 h and numbers fluctuated in the following periods. From 60 h onwards all of the above-mentioned stages were present in varying proportions although, overall, sporonts comprised the highest percentage. At 96 h the first immature spores were observed. Mature spores (comprising two size classes) were first seen in large numbers at 120 h.

Fig. 50 illustrates the fluctuations in the numbers of a particular stage at any one time point. The numbers of meronts and sporonts reached an identifiable peak and then declined as sporoblasts and spores increased in proportion. The sporoblasts varied in number at a low level throughout the experiment, a notable feature being an early peak at 48 h (resulting from the single infected individual examined). The spores gradually increased to a peak of 60% of the total stages seen and then declined.

The minimum time required for the development of one spore into a spore of the next generation was approx. 96 h. The interval that elapsed before 50% of the stages seen in the smears were spores was approx. 192 h.

At no time were any of the above stages found in the smears from the control insects.

4.3.2 Infection experiment

Both experimental groups of *P. xylostella* and *Epiphyas post-vittana* suffered premature mortalities although most of the larvae did complete pupation. Of the larvae examined at the time of death, none were infected with either NBT or *N. apis*. Meronts and sporonts looking like those of NBT were found in the gut smear of one adult *Plutella xylostella* moth (Table 5). No other microorganisms were noted from the control or inoculated larvae.

Sixty percent of *B. terrestris* larvae inoculated with NBT showed heavy infections and had all vegetative stages and spores present in the gut smears, but none became infected with *N. apis*.

None of the control (non-inoculated) larvae died prematurely and none showed any evidence of microsporidian infection when examined in smears.

FIG. 50 Changes in percentage of four life cycle stages with time.

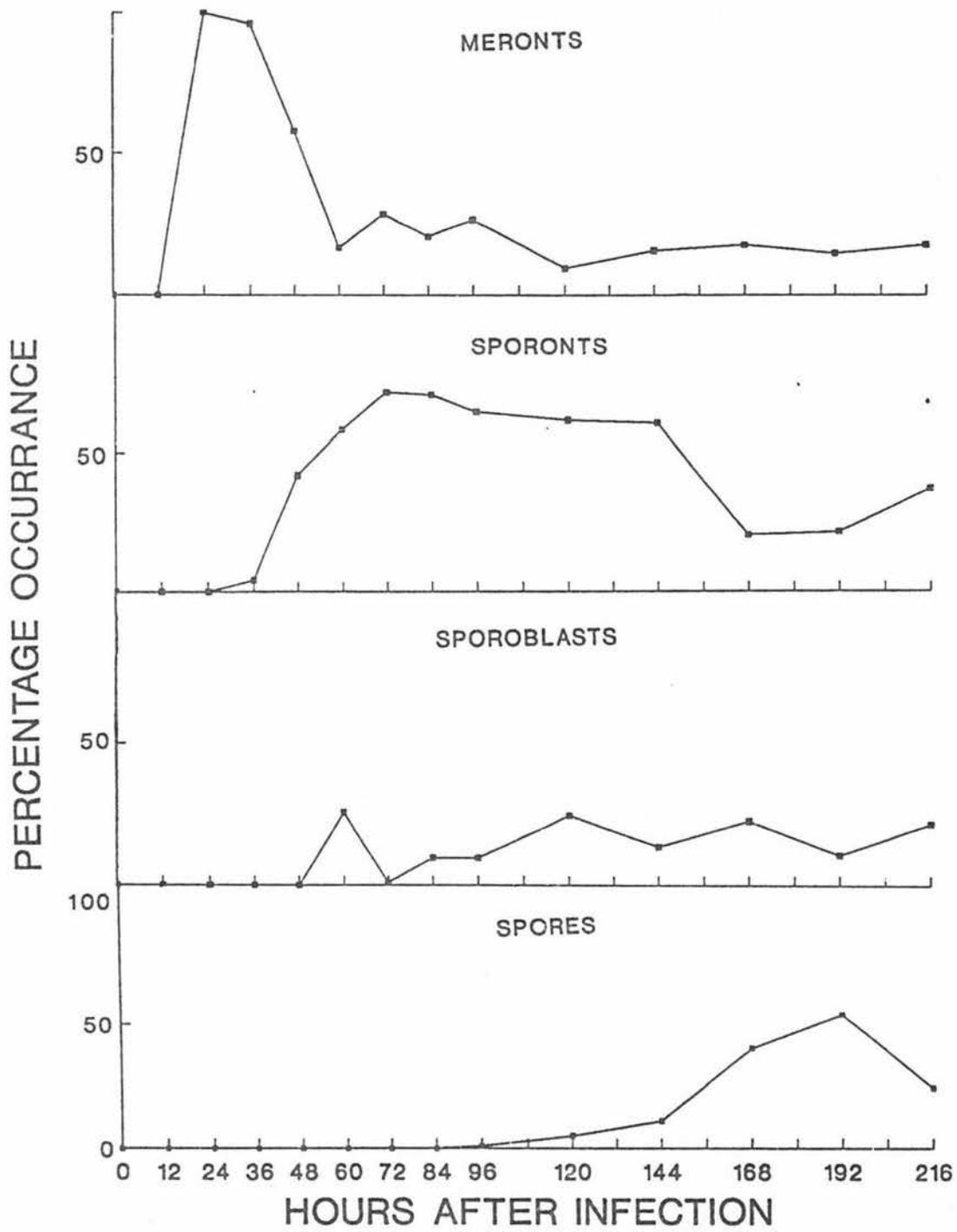


TABLE 5 Infection of Lepidopteran and Hymenopteran larvae with NBT and *N. apis*.

Family / Species	Inoculum	Sample Size	Mortality	Number Infected
TORTRICIDAE				
<i>Epiphyas postvittana</i> (Walker)	NBT	6	2	0
	<i>N. apis</i>	6	3	0
	Control	6	0	0
HYPONOMEUTIDAE				
<i>Plutella xylostella</i> L.	NBT	6	3	1
	<i>N. apis</i>	6	2	0
	Control	6	0	0
HYMENOPTERA				
<i>B. terrestris</i> L.	NBT	12	4	6
	<i>N. apis</i>	12	4	0
	Control	12	0	0

4.4 DISCUSSION

Many features of the NBT life history were found that resembled those observed from *N. bombi* as described by Fantham & Porter (1914). Perhaps the most striking similarity was that of the infection sites in tissues of the bumble bee host. Fantham & Porter observed microsporidia "mainly in the Malpighian tubules and to a lesser extent in the alimentary tract," and none in the reproductive organs or any other tissues. NBT was found to primarily infect the Malpighian tubules and sparsely infiltrate the epithelium of the gut. The Malpighian tubules were, in many cases, completely filled with spores and stages and this was observed from both adults and larvae in this study. Where the infection was heaviest, the structure of the tubule was obliterated, a feature also noted from *N. bombi* infections. No reproductive structures were found to contain somatic stages or spores of NBT implying that NBT was not transmitted transovarially, also noted by Fantham and Porter for *N. bombi*.

Fig. 51 presents the life cycle of NBT as deduced from LM and EM observations.

The life cycles of NBT and *N. bombi* are very similar. In both developmental cycles, apparently uninucleate meronts were observed undergoing both nuclear and cytoplasmic division and uninucleate and multinucleate meronts were seen together. Meronts were found to vary in size and shape and, as sporonts were not described from *N. bombi* there is the possibility they had been confused with meronts. The sporoblasts of *N. bombi* were binucleate and each gave rise to a binucleate spore as in NBT (although this feature is common to *Nosema* species in general). *N. bombi* spores were described as broad, oval structures approx. $5.2 \times 3.7 \mu\text{m}$ with occasional large spores $6.5 - 7 \mu\text{m}$ in length. NBT spores were observed in two size ranges from stained smear preparations with mean sizes of $4.53 \times 2.79 \mu\text{m}$ and $6.56 \times 3.10 \mu\text{m}$ (see Table 3). These ranges for NBT include the spore lengths as noted by Fantham and Porter but not the spore width. However, as they do not give spore size ranges for *N. bombi* it is difficult to know if the $3.7 \mu\text{m}$ width stated is at the extreme end of the size range or an average. The large spores of NBT comprised less than 2% of total spores and this is consistent with Fantham and Porters observations of "occasional" large spores of *N. bombi*.

Fantham & Porter also noted that younger bees appeared to have a larger spore form of *N. bombi* than the older bees. This was also observed for NBT where the spores from a worker were larger than those from a queen. However, the reliability

of this result for NBT is doubtful as only one individual from each developmental stage was examined in this study and there is difficulty in separating differences between individuals from those between developmental stages.

Various authors have noted that spore size can differ 1/ between the developmental stages of the host (Walters 1958), 2/ within in the same tissue of different stages of the host and also in different tissues (Blunck 1954), 3/ between host localities (Malone & Wigley 1981), and 4/ between individuals of the same age (Mercer 1981). Staining of spores also produces a degree of shrinkage as noted by the difference in spore size from the treated and untreated tissues. As Fantham & Porter did not indicate the conditions of their spore measurements or sample sizes, it is difficult to know the actual variability of spore sizes of *N. bombi*.

Fantham and Porter (1914) observed *N. bombi* to be equally infective to workers and queens, and to a lesser extent in drones. They also noted infected larvae in cases where the pollen mass on which the eggs had been laid had initially been contaminated by parental excrement. Some of these features were noted for NBT which was also infective at all stages in *B. terrestris* development. However, no stage appeared to be any less susceptible to infection than any other, this also being distinct from *N. apis* in which the larvae do not become infected (Bailey 1981).

The only significant differences between NBT and *N. bombi* appeared in the life cycles. All the somatic stages of *N. bombi* that Fantham & Porter observed came from stained smear preparations of tissues. Although they described merogony and sporogenesis they did not mention sporogony apparently dismissing it as a period during which "complicated changes accompany the formation of a spore of *N. bombi* from a meront" (pg. 629, Fantham & Porter 1914). It is possible that they confused sporonts with meronts and consequently did not distinguish the separate phases. None of the figures they drew illustrated diplokarya, an obvious and important feature of *Nosema* development, although at that time it may not have been a known feature of microsporidia. This feature was observed frequently in developmental stages of NBT.

N. bombi, as described by Fantham and Porter, and NBT have many features in common observed at the LM level. The few differences noted may be accounted for by the microscopical techniques and a limited understanding of microsporidia in the early 1900's. On the basis of the LM observations *N. bombi* and NBT may be considered synonymous.

FIG. 51 Proposed life-cycle for NBT based on qualitative and quantitative observations.

MEROGONY A/B

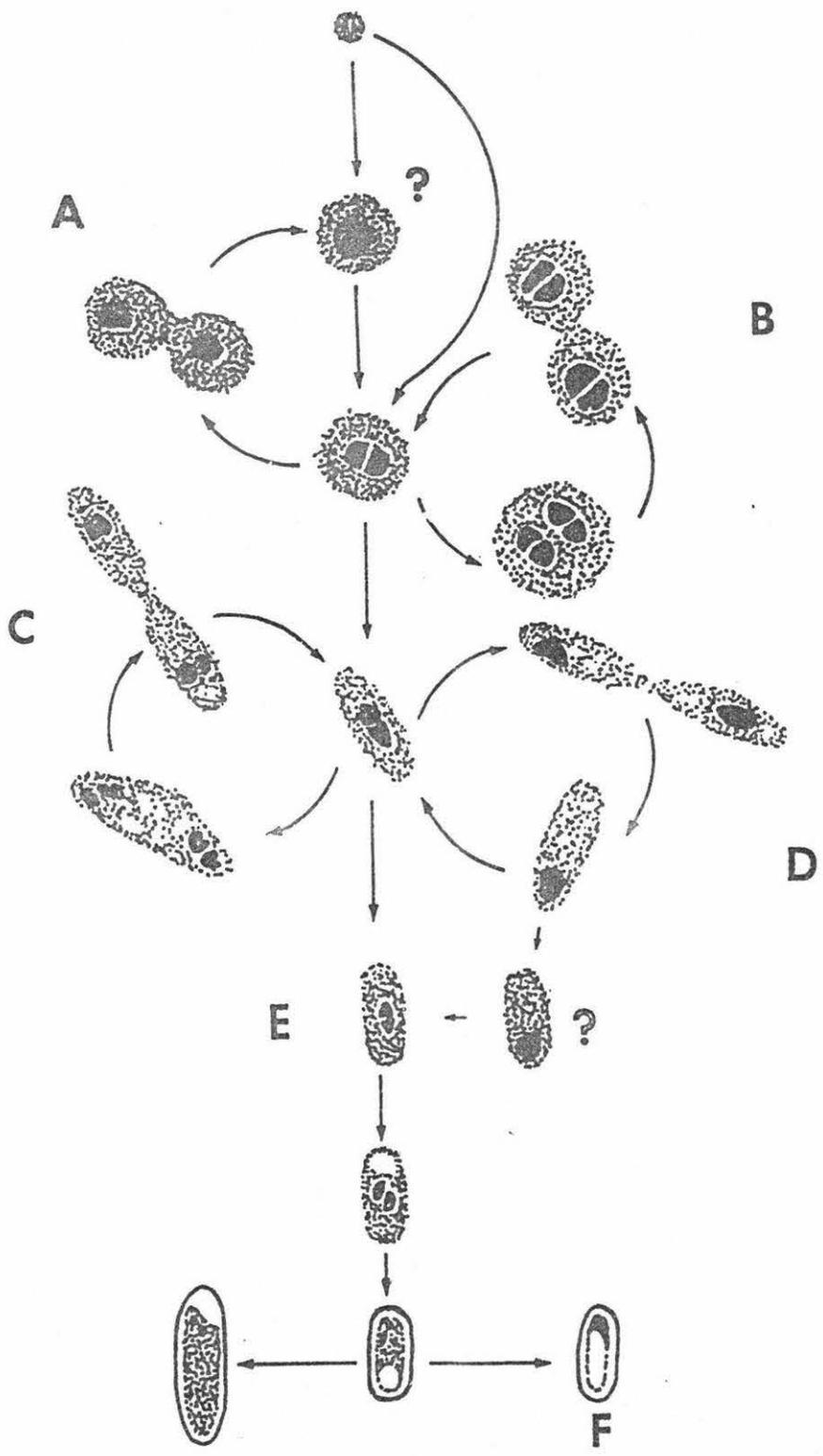
Binucleate meronts and possible uninucleate meronts arising from a binucleate sporoplasm undergo two possible cycles, A or B, to produce numbers of uni-/binucleate and tetranucleate meronts. No chains of meronts are apparent.

SPOROGENY C/D

Binucleate meronts develop into binucleate sporonts and undergo two possible cycles to produce numbers of apparently uninucleate sporonts, binucleate and tetranucleate sporonts. Both fusiform and ovoid sporonts are apparent during both cycles of this phase of development.

SPOROGENESIS E/F

Binucleate and possible uninucleate sporonts develop into binucleate sporoblasts which mature into spores. Macrospores are occasionally formed.



5 μ m

As all of the observations on *N. bombi* were based on the LM it was not possible to make any comparisons at the ultrastructural level. The application of EM to tissues infected with NBT revealed that, in the individual examined, there were two spore forms present - one in the Malpighian tubules and one in the midgut. The spores observed from the Malpighian tubules were oval in outline and had *Nosema*-related features (e.g. two nuclei, polar filament). Spore morphogenesis was accompanied by polar filament development, observed in the sporoblasts. However, those spores and sporoblasts observed from the midgut tissue were more rounded in outline and, although they had diplokarya, they did not appear to have polar filaments. The absence of a polar filament from the second form of spore implied that it was probably a different species to the first.

The observation of two spore forms from one host is not unusual as it is possible for two or more microsporidian species to co-exist in a host, with each infecting different tissues (Larsson 1988). However, two spore forms may not always mean that there are two separate species present. It is possible that they are morphs of the one species that preferentially develop in different host tissues (Weiser 1976). Lui (1984) believed that *N. apis* spores probably developed in the midgut because they were adapted to the metabolism of the epithelial cells and this association with cell suitability may have an effect on where the microsporidian is to be found in the host.

On the basis of observations from one infected bumble bee it was not possible to determine the significance of the two spore forms. Without examining more individuals it is difficult to know if the spores are morphs of one species or the result of a mixed infection.

N. bombi is known to occur in the honey bee *A. mellifera* but no other insect species have been examined in cross-infectivity tests (Fantham & Porter 1914). *N. apis* has been reported to infect a wide range of insects including *Bombus* and Lepidoptera (Fantham & Porter 1913, Showers et al 1967). NBT may not be species-specific and other insect species may act as potential reservoirs for the disease in the wild. For example, NBT was possibly infective to larvae of *Plutella xylostella*, which feed on brassica plants. Bumble bees forage from brassica flowers and this may allow for the interspecific transfer of spores. Further infectivity trials would need to be done to determine the extent of the host range of NBT as one infected larvae is too small a sample size from which to make definitive claims.

NBT was also compared to other species of microsporidia known to infect Hymenoptera either naturally or experimentally (Table 6). Only four such species were found, all of which are also infective to Lepidoptera. Where possible the

original descriptions were consulted, otherwise annotated lists were used (Thomson 1960a). All species had sufficient data to allow them to be compared to NBT.

Both *N. polyvora* and *N. destructor* are considered distinct from NBT on the basis of features of their life cycles and host ranges. Spore size is not a useful feature for comparison because of the overlapping ranges. There is little ultrastructural detail from these microsporidia so comparison could not be made at this level.

N. bombi could not be distinguished from NBT on the basis of the published description of LM observations. A comparison of NBT with *N. apis*, however, highlighted a number of differences. At the LM level, they differed in their tissue affinity, spore size and cell forms present during merogony (Gray et al 1969). At the EM level, *N. apis* did not have stellate forms of sporoblasts as observed for NBT (from Malpighian tubules) and it had more polar filament coils in the mature spores. Various authors have noted for *N. apis* spores polar filament coils numbering from 18 - 44 (Lui 1973, Scholtyssek & Danneel 1962) with the most common number cited being 44. The number of coils seen in NBT spores from the Malpighian tubules were 14-18, this number only just abutting on the range noted above and being less than half the number generally noted for *N. apis*.

It is concluded that NBT is indistinguishable from *N. bombi* and, that it can also be considered distinct from *N. apis* and the other microsporidian species noted in Table 6.

Many of the features of NBT, as observed through light and electron microscopy are identifiable with the characteristics of the microsporidia in the genus *Nosema* Naegeli 1857. These include binucleate ovoid spores, a single polar filament, binucleate stages in merogony and sporogony, diplokarya, one or two binucleate sporoblasts from each sporont and the absence of a pansporoblast. However, anomalies do exist in the observations of 1/ uninucleate as well as the usual binucleate stages during schizogony and 2/ the presence of two apparently different types of spores, including one that apparently has no polar filament.

There has been confusion over the characterisation of some of the species assigned to the genus *Nosema*, including the type species *Nosema bombycis*. One of the problems lies in the accurate determination of the number of nuclei present in the vegetative stages. Where once it was thought that *N. bombycis* had some uninucleate stages in its life cycle it has since been reported that all stages are exclusively binucleate (Cali 1970).

TABLE 6 *Nosema* species pathogenic in Hymenoptera

Species	Spore Size (μm)	Tissues Infected ^c	Polar filament coils	Host Range
NBT	3.43-5.39 x 2.17-3.29 ^a 5.60-7.70 x 2.73-3.50 ^a 4.20-5.39 x 2.73-3.50 ^b (oval)	Mpt, Mg	14 - 18	<i>B. terrestris</i> , <i>P. xylostella</i> .
<i>N. apis</i> (Zander 1906)	5.0-7.0 x 2.8-4.0 ^a (oval)	Mg, Mpt	18 - 44 (various authors)	Hymenoptera incl. <i>Bombus</i> , <i>Apis</i> <i>Vespa</i> , and some Lepidoptera.
<i>N. bombi</i> (Fantham & Porter 1914)	5.2 x 3.7 ^a (oval)	Mpt, Mg	unknown	Various <i>Bombus</i> , <i>Apis</i> , and some Lepidoptera.
<i>N. destructor</i> (Steinhaus & Hughes 1949)	4.0 x 2.8 ^a (oval)	FB, Mpt, SG, all tissues	no information	Many Lepidoptera & experimentally in Hymenoptera and Neuroptera.
<i>N. polyvora</i> (Blunck 1952)	4.8 x 1.9 ^a (elongate oval)	Mg, Mpt, SG	no information	<i>P. brassicae</i> , <i>P. rapae</i> , <i>A. crataegi</i> , <i>A. glomeratus</i> .

^a fixed and stained spores, ^b fresh spores.

^c FB = fat body, G = gonads, M = muscle, Mg = midgut, Mpt = Malpighian tubules, N = nervous tissue, SG = salivary /silk gland.

Other studies though, have documented the existence of uninucleate cells in the life cycles of some *Nosema* species. Canning & Sinden (1973) examined *N. algerae* (from the mosquito) using TEM and found that uninucleate cells could occur although diplokarya were the rule. This observation also led to the suggestion that sporonts could arise from uninucleate cells. Uninucleate cells have also been observed by light microscopy in other species of *Nosema* (Canning & Vavra 1975, Canning 1977) and by EM from *N. apis* (Yousef & Hammond 1971). There are also observations from light and electron microscopy that report the fusion of diplokaryotic nuclei and the division of a single nucleus to give rise to two nuclei (Maurand 1966, Canning 1977). Hazard et al (1985) observed that some species of *Nosema* infecting mosquitos undergo plasmogamy (where the cytoplasm but not the nuclei fuse) to form diplokaryotic stages. Yet Canning (1990) states that the genus *Nosema* and some other genera are diplokaryotic in merogony and sporogony and that as meiosis is not a regular part of their development, uninucleate forms are not produced. Any substantiated evidence of uninucleate forms in species of *Nosema* would indicate that meiosis is occurring and could ultimately necessitate a re-evaluation of the genus *Nosema*. In view of the above literature and the few detailed studies done on the genetics of the microsporidian life cycle, I believe, through my observations, that uninucleate stages may be present during the life cycle of NBT. This claim does, however, invoke the possibility that NBT is not, after all, a *Nosema* but a member of another genus that has meiosis as part of its life cycle. It could also imply that the definition of *Nosema* needs to be expanded to include species that do have meiosis as part of their life cycle.

It must be noted though, that there are obvious problems with relying completely on observations from the light microscope and sometimes even the electron microscope. The uninucleate stages seen of NBT were generally darkly stained and had large nuclei. It is possible they may have been binucleate with the nuclei so closely paired that they were indistinguishable at the LM level. This was noted by Cali (1970) who observed that closely opposed nuclei can appear to be one unit under light microscopy, when through EM they are actually distinct units. Also, the smear technique caused some vegetative stages to be more flattened and lighter staining rendering their nuclei more distinct than the intact, darker staining stages. Another possibility is that when the smears are made, the diplokarya are observed from the polar end. This would give the appearance of a single nucleus. This may also occur in EM where sections through the stages may only include one nucleus. One way of resolving this is to take serial sections through the cells and then construct 3-D models (C.J. O'Kelly pers. comm.). In this way it would be possible to determine the number of nuclei present if one was accidentally obscured.

The lack of a polar filament in one of the spore types suggests that some other organism may be present in *B. terrestris*. This spore type is something of an anomaly, although again many basic features are reconcilable with *Nosema* species, especially *N. bombi* (as infective stages in the gut tissue). Further studies on the frequency with which these two forms are observed in *B. terrestris* and the identification of any differences in the life cycles is possibly the only way to fully determine their taxonomic status.

CHAPTER 5

OBSERVATIONS ON THE TRANSMISSIBILITY AND INFECTIVITY
OF NBT.

5.1 INTRODUCTION

Three ways in which microsporidia gain access to host tissues are by oral or transovarial transmission or directly through the cuticle (Kramer 1976). Of the microsporidia known from social insects, a large number are orally infective only, i.e. the spores are ingested with food (e.g. *N. apis* Fantham & Porter 1913, *N. bombi* Fantham & Porter 1914). Typically, the spores are eliminated in the faeces of the host of origin (Kramer 1976). Between hosts spores are more resistant to temperature changes, dessication and UV rays when bound in fecal matter and can remain viable for months (Weiser 1976, Kramer 1976).

The pattern of defecation by infected insects has a significant bearing on the contamination of food and the amount of infective material available for re-cycling within the population. In social insects, features such as brood-rearing behaviour and colony sanitation will influence infection levels. Butler (1976) stated that honey bees will not usually defecate inside the nest and Seeley (1985) believes it is this action that reduces the spread of infection through the faeces of infected bees. Only when confined for sustained periods (during winter, inclement weather) or placed under stress will bees defecate in the nest and any infected bees will also eliminate infective spores (Bailey 1955). The workers that clean the combs after this period are likely to become infected through ingesting the spores and this process has been suggested to account for increased *Nosema* outbreaks in spring nests (Bailey 1981).

Various methods have been devised to screen honey bees and colonies for the presence of *Nosema*. For whole colonies, a representative sample of the population is removed and the bees are microscopically examined either individually or as a group (Clinch 1976, Laere et al 1980, Fries et al 1984). For the screening of individual queen bees, where sacrifice is not a viable option, the examination of fecal material has been found to provide a reliable means of estimating levels of *Nosema* (Roberts 1966, Loskotova et al 1980). These levels have been found to compare favourably with spore numbers in the gut of the bee (counted using a haemocytometer, Roberts 1966).

No research has apparently been done to examine the relationship between fecal contamination and spread of disease in bumble bee colonies. *N. bombi* and NBT from *Bombus* spp. are protozoans with similar life histories to *N. apis* and both are orally infective (Fantham & Porter 1914, pers. observation on NBT). It is likely that these protozoa are also primarily spread through faeces. A study by Williams (1987) examined some of the stimuli that induced *B. terrestris* individuals to defecate and it was found that (under enclosed, artificial conditions) the bees would defecate up to 10cm or more away from the nest, in cooler areas and on a textured surface. Under natural conditions, the *B. terrestris* nests are often enclosed within a small area and have long access tunnels (Alford 1978). The foraging bumble bees tend to defecate along this passage and around the outer entrance. House-bees defecate at the interface between the nest material and the floor of the chamber and occasionally on the nest material itself (personal observation from wild *B. terrestris* nests). In most cases there is adequate drainage and the fecal material disperses quite quickly. However, as fecal material is generally eliminated near the nest (not away from the hive in 'cleansing fights' as with honey bees), any spores eliminated from infected bees are more easily contacted by other susceptible bees.

Donovan & Weir (1978) consider one of the four features necessary for the successful rearing of a bumble bee colony to be the dispersal of fecal material. Sladen (1912), Plowright & Jay (1966) and Plowright & Pomeroy (1980) each designed artificial hives for bumble bees that reduced the amount of fecal build-up and provided adequate ventilation to dry the fecal material. Fecal contamination is a potential problem in the large-scale rearing of bumble bee colonies and will play a role in determining the design of portable bumble bee 'hives'. The problems associated with fecal build-up, especially the harbouring of potential pathogens (such as *Nosema*), need to be addressed in the rearing of bumble bees for commercial use.

The following experiments were devised to observe the natural transmission and re-cycling of spores and the conditions that result in an increase/decrease in infection levels in individual bees.

5.2 METHODS

5.2.1 Experiment 1 - Observations on natural fluctuations in spore levels in individual queen bees.

Eight field caught queen bumble bees, found by fecal sampling to be naturally infected with NBT, were placed in starter boxes (similar to Fig. 52) and maintained with a supply of sugar-water and pollen for the period of the trial.

Fecal material was collected from the outer compartment of the starter box which for this trial was shallow and had a plastic mesh-covered floor. Clean glass slides were also placed under the mesh to trap the fecal material that passed through it. The faeces removed from the outer compartment and the glass slides were combined and mixed with 0.5 - 2ml of distilled water (dependent on the amount of fecal material obtained). Dilution was necessary for the examination of fecal material with the LM due to the high density of pollen grains in the samples. Samples were initially taken every day for 9 days, then pooled over three days for another 9 days and then pooled weekly for 4 weeks to cover a total sampling period of 47 days.

A drop of the fecal suspension was placed on a clean glass slide for examination at 400x magnification. Infection was rated on a four point scale based on the number of spores averaged over five fields, i.e. 1 (0-5 spores), 2 (6-30), 3 (31-60) and 4 (60+), similarly to the method of Roberts (1966). This simple ranking was used because variations in fecal volume and thickness of spread on the slide meant the actual spore counts were not precisely comparable between samples.

After 9 days, the pooled samples were kept in a freezer until they could be examined. As well as the above method, spores were counted in a haemocytometer. A small drop of fecal solution (approx. 0.01ml) was placed at the edge of the coverslip/haemocytometer interface and drawn across the grid by capillary action. The sample was then examined at 400x and spores were counted from the four large corner squares of the grid.

5.2.2 Experiment 2 - Examination of conditions that may affect NBT levels in individual queen bumble bees.

From the information gained in Experiment 1 conditions that could influence

differences in NBT levels between individual bees were further examined.

Sixteen NBT infected queens were randomly assigned to three groups :

- 1/ "Dirty" - where spores were recycled through the fecal contamination of food,
- 2/ "Drug clean" - where Fumidil-B (see 1.3.1) was administered in the sugar-water,
- 3/ "Control clean" - where queens are maintained as in experiment 1.

The "dirty" conditions were achieved by spreading a small portion of the faeces from each queen on the tip of the wick supplying the sugar-water, twice weekly. The natural dirtying of the wick had been observed prior to the experiment from other laboratory bees in starter boxes.

Bees in the second group were supplied with sugar-water containing Fumidil-B, an anti-protozoan drug used to control *Nosema* in honey bees, using the commercially recommended honey bee concentration (see section 2.1).

Bees were maintained in separate starter boxes with mesh-floored outer compartments. Fecal samples were pooled weekly over a period of three weeks. All fecal material from each queen was removed daily, examined *en masse* at the end of the week and assessed for spores as explained in 5.2.1. (Spore numbers were not counted in fecal material using the haemocytometer).

At the end of the trial all queens were sacrificed, their guts were removed, macerated and their contents examined for spores. Spore counts were made using the haemocytometer. This data was then compared to that obtained from the fecal estimates in order to determine the validity of assessing infection levels on the spore content of the faeces (see Roberts 1966).

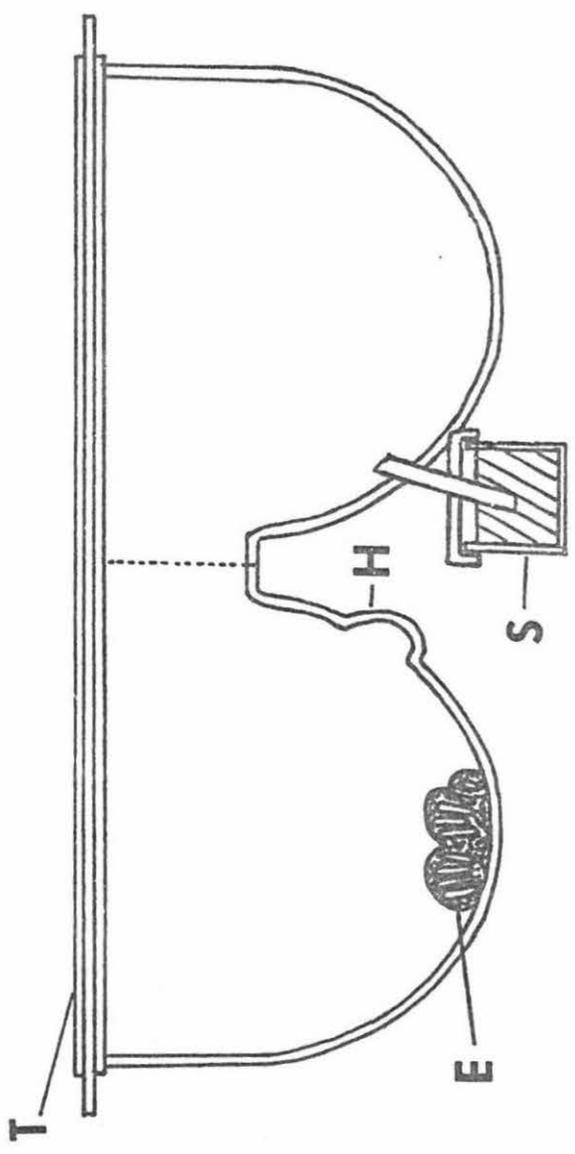
Qualitative observations were also made on the nest-starting behaviour of the queens from each group.

5.2.3 Experiment 3 - Quantitative study on the recycling of spores in artificially infected bumble bee queens.

This experiment required a modification of the starter box to allow for the quantitative sampling of fecal material. As illustrated in Fig.52, the outer compartment no longer had a mesh floor, but was instead identical to the nesting compartment. The bowl-shape directed all the fecal material to collect in the

FIG. 52 Longitudinal section of starter box modified for the collection of fecal material.

- B Rear, heated section of starter box.
- F Front section modified for the collection of fecal material.
- E Eggs/Brood.
- S Sugar syrup dispensing bottle and wick.
- H Depression for wax honey pot (built by queen).
- T Transparent fitted cover.



F

B

depression at the base and the whole compartment could be removed when necessary and replaced with a clean one. The fecal material remained quite liquid and was removed from the compartment with a graduated syringe, this also allowing for the volume of faeces to be measured at the same time.

Fifty egg-laying queens were screened daily for a week for evidence of *Nosema* and of these thirty were consistently found free from infection. These thirty queens were randomly assigned to two groups - 1/ Control and 2/ Fecal recycling. All queens were placed in separate modified starter boxes and supplied with sugar water and pollen for the period of the trial.

Queens were artificially infected with NBT one week before the start of fecal sampling. The inoculum was prepared as in section 2.2.2 in sufficient quantity to allow for three treatments per bee. (Staggered doses are preferred over one large dose as they reduce the likelihood of bacterial infection, Weiser 1976). Each bee was starved for 12 h prior to the inoculations by restricting their access to the sugar-water. The inoculum was then placed directly in the honey pot in each starter box and topped up with sugar solution. Each bee received approx. 2.5×10^6 NBT spores in total over a course of three inocula applications (each of 0.1ml). As all queens consumed the entire contents of the honey pots after each dose none were excluded from the trial.

Sampling of the fecal material commenced one week after the first inoculation. Faeces were removed every second day for a period of two and a half weeks and then every four days for a further two weeks. When compartments containing faeces were removed they were replaced with clean ones (washed and sterilised). The volume of fecal material from each bee was measured, diluted to twice its volume (necessary for haemocytometer work) and its spore content determined with the haemocytometer. Four squares on the grid were initially used for spore counts, but as the spore numbers decreased it became necessary to use sixteen squares for the counts. These counts were used to determine the approx. spore content of the faeces collected from each bee. Those bees assigned to the spore recycling group were fed 10% of their own fecal solution (containing a known number of spores) at the end of each sampling period. This was added to the contents of the honey pot and access to the sugar bottle was restricted until all the solution in the honey pot had been consumed (usually overnight). The control bees did not receive any fecal material. This process was repeated for all the bees at each sampling period for the course of the trial.

5.2.4 Experiment 4 - Spore transit time.

As a control for determining if spores seen in the fecal material were actually new spores and not the original inocula spores a simple 'spore transit' trial was devised. Spores of *N. apis* were killed by heating them at 80°C for 10 min (L. Malone pers. comm.). *N. apis* spores were used primarily because of their large size and because when viable, they had not been found infective to *B. terrestris* larvae (see 4.3.2). A dose of known spore concentration (estimated from counts using the haemocytometer) was administered to each of 5 healthy queen bees.

Daily, fecal samples were examined for spores (see 5.2.1) and spore counts were made. This was continued until spores were no longer found in the fecal material over three successive days. When this point was reached it was assumed that most of the spores had passed through the bee and had been eliminated.

5.3 RESULTS

5.3.1 Experiment 1.

Fig. 53 illustrates the variation in spore levels between and within individual queens as determined from fecal samples. As one queen died during the trial she was excluded from the results.

Spearman rank correlations of spore number with time were significantly negative for three queens, positive for one queen and not significant for the other three (Table 7).

The quantitative spore counts from the haemocytometer showed directionally similar fluctuations to the 1-4 grouped data, but over the reduced time span from day 12 onwards, showed no significant correlation with time.

5.3.2 Experiment 2.

Fig. 54 illustrates the mean spore index (determined from scaled data as explained in 5.3.1) against time for the three treatments. Spore levels of the queens

FIG. 53 Index of NBT spore abundance in the faeces of seven queens, versus time.

SPORE INDEX

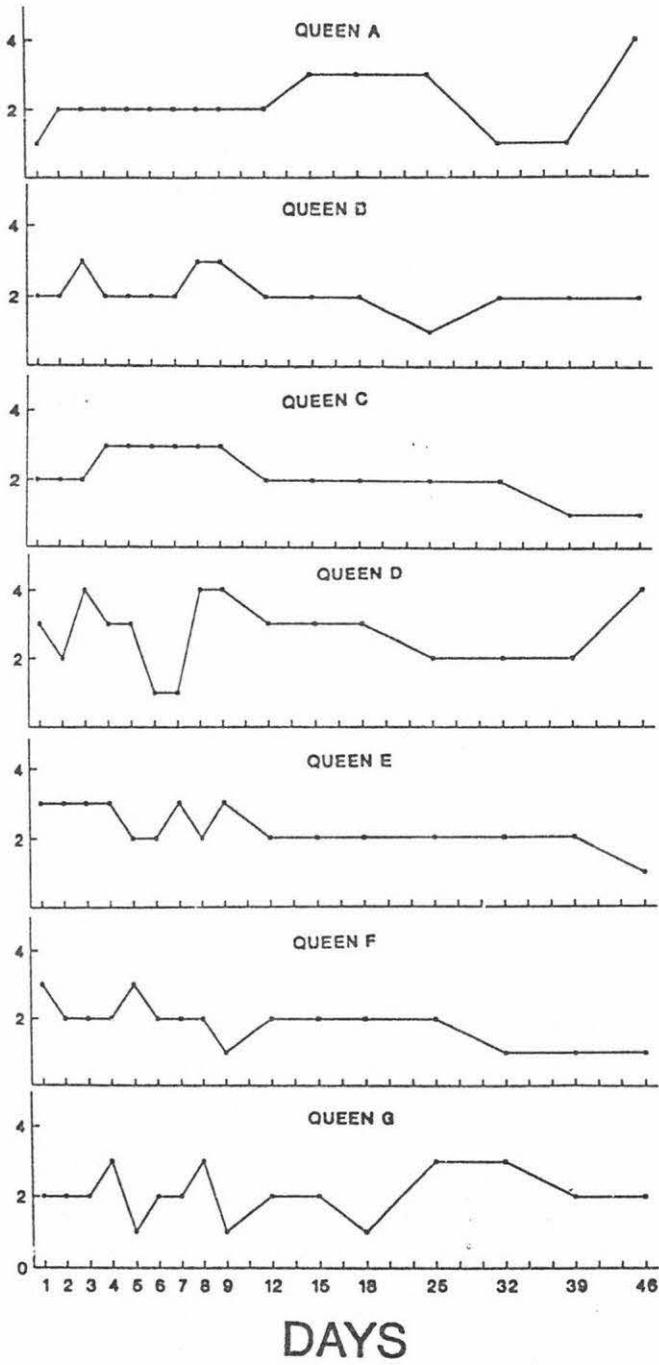
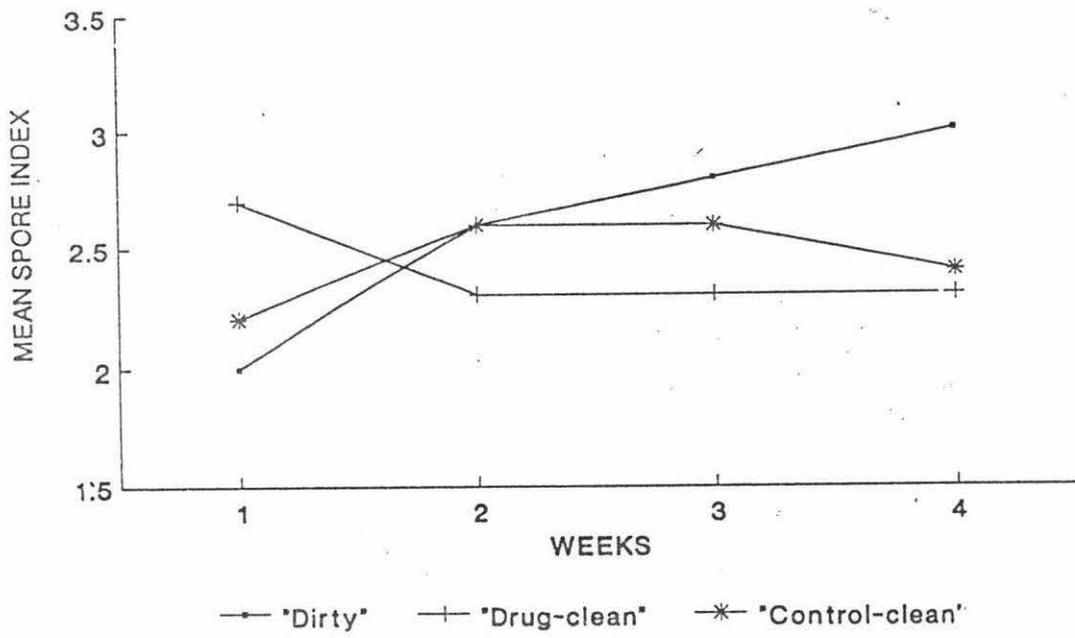


FIG. 54 Index of NBT spore abundance in the faeces of queens versus time under three treatments :

"Dirty" - having faeces smeared on the feeding wick,

"Drug clean" - without fecal contamination and with the drug Fumidil-B in their food,

"Control clean" - without fecal contamination or Fumigillin treatment.



in the drug-clean and control-clean groups, when compared, were not found to be significantly different from one another. The index of difference between the "Dirty" group and each of the "clean" groups though, rose significantly with time (Spearman's rank corr. = 1.000, df=7, P<.05 (1-tailed)).

At the end of the trial, all queens were sacrificed and the spore content of their guts examined. All queens from the clean-control group had laid eggs and had thriving brood. The average spore load of these queens at the end of the trial was 4.3×10^6 . Only two queens from both the "dirty" and Drug-clean groups laid eggs. The mean spore loads of queens for these groups were 1.4×10^7 and 3.4×10^6 respectively. These means were not significantly different at the 5% level (one-tailed t test).

5.3.3 Experiment 3.

Fecal spore output declined rapidly from day 2 to 8 (Table 8). These spores were probably the inoculum being passed through the gut. After this period the numbers appeared to stabilise with no extreme fluctuations. Fig. 55 illustrates the spore numbers for both groups from day 10 onwards. For the first few sampling periods in this time a significant proportion of the spores recovered from the treated individuals were estimated to be attributable to elimination of the 10% of the fecal sample that had been fed to the bees, but from day 20 this comprised only a very small proportion of the total spore output. During this final four week period the levels of infection in the treated bees, when corrected for the elimination of fed spores (the cross-hatched areas in Fig. 55), were approximately twice that of the control bees (1133 compared to 584 spores eliminated per queen per day: $P < 0.05$, $F = 9.12$, d.f. = 1/6, two way ANOVA).

5.3.4 Experiment 4.

Spore numbers were high in the faeces of individual queens a day after the inoculation, and steadily decreased until the fifth day of sampling. After this time, over three consecutive days, no more spores were observed from the fecal material of the queens. The mean daily spore counts are illustrated in Fig. 56.

The total spores administered per queen in the inoculum however, were not recovered. On average, only 30% of the spores were recovered from each queen.

FIG. 55 NET spore abundance in the faeces of queens versus time:

"Treated" - consuming 10% of their faeces in sugar syrup,

"Control" - consuming a similar volume of added water.

The columns represent the estimated spore numbers in the faeces directly attributable to the treatment intake.

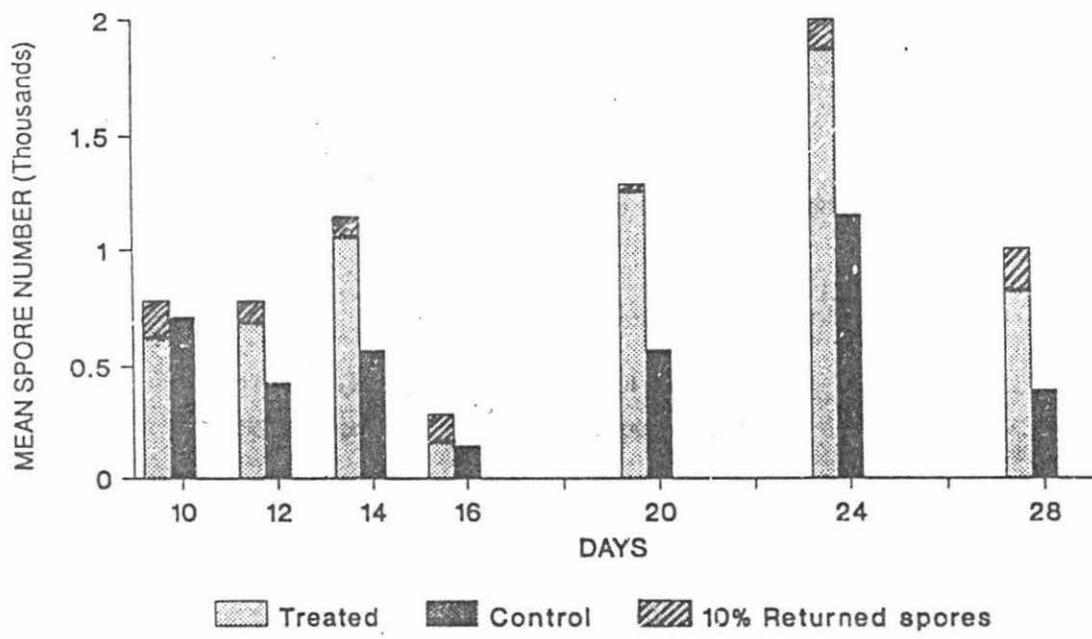


TABLE 7 Spearman rank correlations of spore number versus time for seven queens.

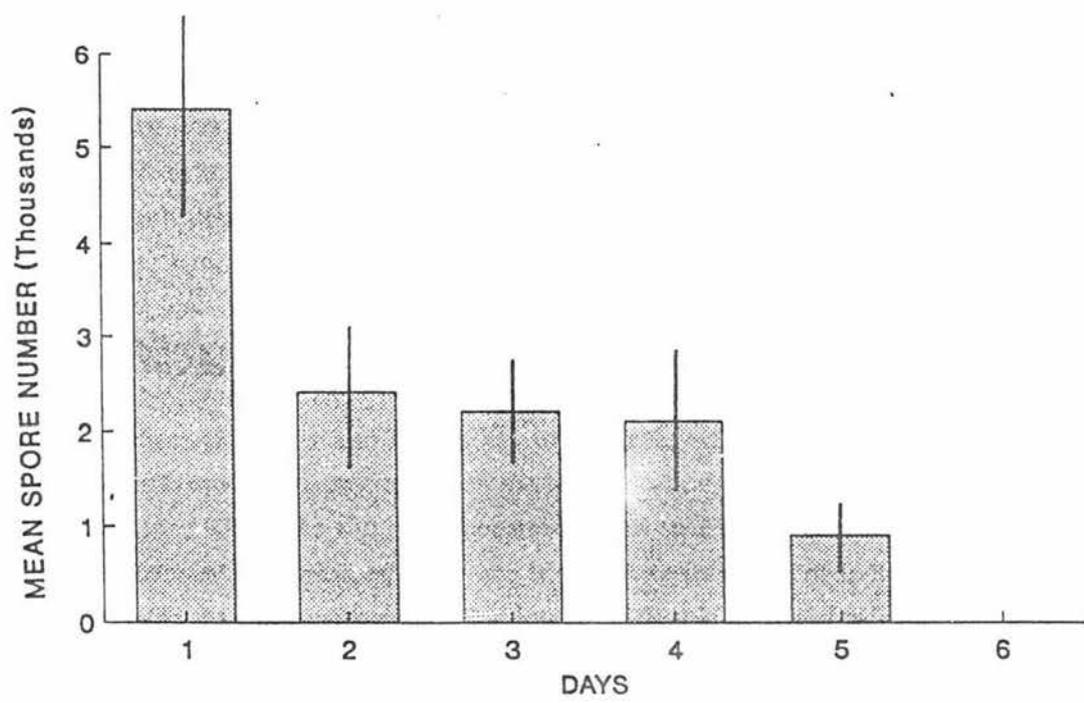
Queen	A	B	C	D	E	F	G
r^S	+0.668	-0.294	-0.488	-0.061	-0.756	-0.704	+0.059
	**	n.s.	*	n.s.	**	**	n.s.

** $P < 0.01$, * $P < 0.05$, n.s. $P > 0.05$

TABLE 8 Mean spore numbers from fecal samples of Control and Treatment groups.

Days	Treated Group mean spores/bee	Control Group mean spores/bee
2	78050.0	46850.0
4	37957.1	23424.9
6	17878.6	7200.0
8	1785.7	714.3
10	785.7	714.3
12	785.7	428.6
14	1142.0	571.4
16	285.7	142.9
20	1285.7	571.4
24	2000.0	1149.2
28	1000.0	392.2

FIG. 56 Number of NBT spores in bumble bee faeces on successive days after being fed a dose of 1.5×10^4 spores/bee. Vertical lines represent S.E.M. (N= 5).



5.4 DISCUSSION

As queen bumble bees may be considered the primary source of infection for the next generation, they were used in the four trials. Adult insects are generally more resistant to infection than juveniles and consequently they require larger doses of spores to become infected (Vavra & Maddox 1976). It appeared that spore levels varied widely between queens and over time. Similar variability between individuals was reported by Fisher & Pomeroy (1989b). After spores are ingested many factors determine their infectivity to the host, including spore viability and internal host conditions (Vavra & Maddox 1976, Weiser 1976). These, and possible variation in host resistance may account for the different levels of infection encountered in individual field-caught queens. This may also help to explain why queens, when originally inoculated with similar spore doses, maintained differing spore loads as indicated by the spores eliminated in their faeces (e.g. Expt. 3). The idea of host resistance has been examined for *N. apis* in *A. mellifera* and is believed to be feasible by some authors (Furgala 1962, L'Arrivee 1965). Others state that it is not a significant feature (Loskotova et al 1980). Physical aspects such as re-infection through faeces and the ability of the infected host tissues to regenerate, may also influence the degree to which the infection levels increase or decrease over time (Weiser 1976). As the most common trend in Experiment 1 was for spore levels to decrease it is assumed that there was little re-infection of individual queens occurring, and that they were supporting low, containable levels of infection.

From experiment 1 it can be concluded that levels of NBT infection varies between field-caught queens and in individuals and that the general trend is for spore numbers to decrease naturally over time. This variability was noted by L'Arrivee (1965) who observed that spore counts from fecal deposits of honey bee queens differed excessively during their lifetime.

As NBT is considered to be orally infective, it would be expected that increasing the queens' exposure to infection through contaminated food, would result in higher levels of infection. This was observed in the second experiment where queens whose wick supplying sugar water was contaminated with a smear of faeces showed a significant increase in fecal spore output over time. This "dirtiness" factor may have accounted for the increasing spore levels observed in a few queens in Expt.1. They may have been naturally re-infecting themselves through their own fecal material. How frequently this occurs in the wild is unknown but, as colonies

develop, greater fecal output and subsequent build-up of faeces could increase the chances of exposure of healthy individuals to any spores present in the fecal material.

The queens fed Fumidil-B in their sugar water showed no significant decrease in the number of spores eliminated, although this chemical is a recognised prophylactic against *N. apis* infection in honey bees (Katznelson & Jamieson 1952, Tibor et al. 1987). In retrospect, it would have been instructive to treat some queens with both dirty wicks and Fumidil-B to see if there was a reduced spore buildup. Any efficacy of the drug for *Bombus terrestris* seems to be masked by the bees' natural tolerance of moderate infection levels. It is possible that higher dosages may be needed to reduce infection levels in bumble bees.

Experiment 3, an attempt to be more quantitative about both the spore intake and output, did not show the same rise in spore output of reinfected over 'clean queens'. The spore output was probably dominated by original inocula being passed out. This is consistent with the transit time trial (experiment 4) which showed most spores eliminated within 5 days. Subsequently, spore output fluctuated over time but generally averaged twice as many from reinfected as from clean queens. As the spore-to-spore generation time is about a week (Chapter 4) the spores observed from day 8 onwards were probably second generation spores liberated from infected tissues of the bees.

The absence of a marked increase in spore output, even though the spore consumption level must have been much greater from 10% of the faeces than from the wick smears in experiment 2, was surprising. Perhaps the spores lacked viability due to never having been dried, or at least being directly exposed to air. This drying period may be an important requirement for the germination of NBT spores and has previously been noted as important for other microsporidia (Weiser 1976).

All queens from the trial laid eggs and of those that had emergent workers, neither group's offspring were found to be infected with NBT. This reinforces the observation that NBT is purely contaminative (rather than transmitted in the egg) and also implies that it is not highly virulent. None of the queens appeared to suffer any ill-effects from infection, although one queen from each group died part-way through the trial and, when examined, had heavy bacterial infections. This may have resulted from the initial inoculations, as septicemias are often related to high spore dosage and the resultant damage to the gut wall of the host (Weiser 1976).

Generally, it appears that certain physical conditions may influence the spore levels of individual bees after they become infected with NBT. Regardless of spore load, the infection does not appear to affect the reproductive capabilities of the bumble bee queen, which is in complete contrast to the effect of *N. apis* on honey bee queens. Infection of the honey bee queen is uncommon but if present can lead to degredation in egg-laying abilities, supersedure and death (Hassanien 1951, Shimanuki et al 1973). Generally, *N. apis* is not believed to be a major cause of mortality of whole honey bee colonies, though smaller hives can be weakened through the loss of infected workers (Bailey 1981). Such dramatic effects were not observed from infected bumble bee queens and there were few mortalities that could be attributed to NBT infection. Fisher & Pomeroy (1989b) also noted that *Nosema* did not appear to affect the overall productivity of the bumble bee colonies. Because the NBT infection appears to produce only sub-lethal/inapparent effects in its bumble bee hosts, it may not prove to be a significant problem in bumble rearing. However, as no study was undertaken on NBT's impact at the colony level, it may be premature to dismiss it completely.

CHAPTER 6

GENERAL DISCUSSION

The present study appears to be the most comprehensive work on the life history and morphology of *Nosema* in the bumble bee since *N. bombi* was first described in 1914 by Fantham and Porter. Since then, there has been a considerable amount of conflicting information in the literature concerning the distinction between *N. bombi* and *N. apis*, a much more thoroughly studied pathogen of honey bees. Some authors believe that they are separate species and that they are not cross-infective between their *Bombus* and *Apis* hosts (Uspenski 1949, Weiser 1961). Yet others have claimed to have succeeded in cross-infecting the two (Fantham and Porter 1913, Showers et al 1967).

Observations from this study at the LM level, suggest the "NBT" organism is in fact *N. bombi* Fantham and Porter 1914. This is quite possible in view of the fact that bumble bees in New Zealand originated from European stocks.

EM detail has also provided a record of NBT's ultrastructure which, until now, has not existed. When LM details of NBT were compared to those of *N. apis*, a number of significant differences became apparent. These included spore size, merogony and the site of tissue infection. Ultrastructural detail on NBT revealed a smaller number of polar filament coils than was generally found in *N. apis* spores and also a difference in sporoblast development.

The epidemiology of *Nosema* in *Bombus* also differs from that in *N. apis*. Infection of the queen in *Apis* is rare and ultimately results in her death (Shimanuki et al 1973) and the decline of the colony. In *Bombus*, the queen appears to be the primary source of infection for the next generation and consequently she can have heavy *Nosema* infection with no apparent effect on her nest initiation or egg-laying abilities. The annual bumble bee life-cycle seems to be associated with a less virulent, generation to generation parasitic life-style (Fisher & Pomeroy 1989b), whereas in *Apis* (with perennial colonies), *Nosema* tends to be endemic and cyclic in its virulence (Bailey 1981).

When all these observations are considered it is obvious that the differences between *N. bombi*/NBT and *N. apis* are sufficient to consider them as being distinct species.

The indications from this study are that NBT is only sub-lethal/inapparent in its effects on its bumble bee hosts as individuals. However, as NBT-infected

colonies were not studied, it is difficult to know how the colony as a whole might be affected if at all. Fisher and Pomeroy (1989b) failed to find any significant reduction in colony size due to *Nosema* infection of colonies placed in the field. However, Fisher and Pomeroy's spore recording method of taking a small proportion of faeces from each expired nest was not very quantitative or sensitive and they were reluctant to assert that *Nosema* never weakens colonies. In neither their nor the present study was there evidence of *Nosema*-induced deaths of individual queens or young nests.

Further detailed studies comparing healthy and infected colonies are required to confirm whether *Nosema* infection has an effect on overall colony productivity or influences the timing of the production of reproductives (this effectively shortening the life of the colony in terms of commercial productivity).

One way of reducing or eliminating the *Nosema* problem in commercial bumble bee rearing would be to screen all field-captured queens by fecal sampling before nest-initiation to ensure that heavily infected bees were kept out of the system. Alternatively, disease-free queens could be obtained from disease-free colonies and mated with "clean" drones similarly reared. These queens could then be artificially hibernated under controlled conditions, induced to emerge the following season and initiate colonies. This has potential, although there are still minor problems in reproducing the conditions under which queens choose to hibernate and in getting them to initiate nests (especially if they are forced to emerge from hibernation early, pers. observation).

Although de Jonge (1986) showed a *Nosema* reducing effect of Fumidil-B on infected queen bumble bees, this was not apparent in the present study. More work should be done on this subject, especially using increased dose rates and in situations where fecal contamination of food raises infection levels.

The present study was primarily concerned with describing the morphology and life history of NBT rather than measuring the damage it may do to colony development. So far there is no evidence for such damage; but in anticipation of such a possibility it would seem prudent to maintain colonies under conditions which minimise the contamination of food supplies with bee faeces. The work by Williams (1987) may be useful in the design of "clean" hives for use in commercial mass-rearing of bumble bees for pollination purposes.

APPENDIX 1

Locality Details of Catching Sites :

"Esplanade"

Fitzherbert Ave.,

PALMERSTON NORTH.

Keebles Farm,

Massey University,

Highway 54,

PALMERSTON NORTH.

Kimbolton Rhododendron Park,

Haggarty St.,

KIMBOLTON. (Approx. 50km from Palmerston North).

OHAKUNE (various sites),

Near Mt. Ruapehu.

APPENDIX 2

Basic Giemsa staining for Microsporidia

1. Air dry smear and fix for 5 mins. 95% methanol.
2. Pour off methanol.
3. Stain for 15 mins. in 10% (v/v) Giemsa (Gurr's R65 Improved) stain in 0.02M phosphate buffer, pH 6.9.
4. Rinse off gently in tap water.
5. Blot the slide dry.
6. Leave to air dry and view.

Sorenson's Phosphate Buffer (0.02M)

Solution A (0.2M); Take 28.39g of NaH_2PO_4 and add distilled water to make 1 litre.

Solution B (0.2M); Take 31.21g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and add distilled water to make 1 litre.

0.02M Solution; Add 55 mls of Solution A to 45 mls of Solution B and make up to 1 litre with distilled water. The buffer should be approx. pH 6.9.

APPENDIX 3

Fixation, dehydration and embedding of specimens for light microscopy.

Fixation: The fixative used for the sections was Modified Carnoy's fixative made up as follows:

Modified Carnoy's fixative:

- 60 ml absolute isopropyl alcohol
- 30 ml chloroform
- 10 ml formic acid

Dehydration:

1. 70% alcohol, 1 hour
2. 90% alcohol, 2 hours.
3. 100% alcohol, 1 hour.
4. 100% alcohol, 1 hour.
5. Xylene, 1/2 hour.
6. Xylene, 1/2 hour.
7. Paraffin wax (58°C) at 60°C, 2 hours.
8. Paraffin wax (58°C) at 60°C, 2 hours.

Embedded in paraffin wax (58°C).

Sections (5-10 μm) cut on a rotary microtome (Leitz, Wetzlar).

Slides placed overnight on a heated element at 40°C.

Staining of sections for light microscopy :

1. Giemsa (Gurr, 1957) :

1. Xylene, 2 mins.
2. Xylene, 2 mins.
3. 95% alcohol, 2 mins.
4. 95% alcohol, 2 mins.
5. 90% alcohol, 2 mins.
6. Rinse in distilled water.
7. Place in 30% (v/v) Gurr's improved R66 Giemsa in 70% distilled water, overnight.
8. Leave slides in stain for 3-4 hours.
9. Rinse in tap water.
10. Dip slides briefly in acidified water (0.5 ml glacial acetic acid in 100ml distilled water), until sections become deep pink.
11. Remove excess water by blotting the slides with filter paper.
12. Dip slides briefly in acetone to "blue" and dehydrate the sections.
13. Clear in xylene.
14. Mount in DePex.

2. Hematoxylin staining :

1. De-wax xylene, 5 mins.
2. Xylene, 5 mins.
3. Absolute ethanol, 2 mins.
4. 70% ethanol, 2 mins.
5. Tap water, 3-5 mins.
6. Mayers Haemalum, 10-12 mins.
7. Tap water, Rinse.
8. Scott's Tap water, 3 mins.
9. Tap water, Rinse.
10. 1% Eosin, Rinse.
11. Tap water, Rinse.
12. 50% ethanol, Rinse.
13. 70% ethanol, 1 min.
14. Absolute ethanol, 1 min.
15. Absolute ethanol, 2 mins.
16. Xylene, 5 mins.
17. Xylene, 5 mins.
18. Remove each slide from tray, blot dry by pressing between a piece of paper towel, run DPX along slide, lay cover slip on DPX and let weight of cover slip spread the DPX.
19. Can be viewed immediately under microscope, but must allow about a week to dry properly.

APPENDIX 4

Fixation, Dehydration and Embedding of specimens for Electron Microscopy

1. Place dissected tissue in Gluteraldehyde/Phosphate buffered saline (PBS) (2.5%/0.1M).
2. Immediately section the material into 1-5mm pieces to allow quicker fixative penetration.
3. Sections are left in fixative for 1 hr.
4. Rinse in 0.1m PBS, three times 10 mins.
5. 1:3 OsO₄ in PBS for 1 hr.
6. Distilled H₂O, three times 10 mins.
7. Leave in distilled water until ready to dehydrate.
8. 20% acetone, 20 mins, room temperature.
9. 40% acetone, 20 mins, room temperature.
10. 60% " , 20 mins, room temperature.
11. 80% " , 20 mins, room temperature.
12. 100% dried acetone, three times 10 mins, room temperature.
13. 1:3 resin (Spurr's)/acetone for 24 hrs.
14. 2:3 resin/acetone for 24 hrs.
15. Full resin for 24 hrs prior to embedding.
16. Embed in fresh Spurr's resin and polymerize in 60°C oven overnight.

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