INFECTION OF THE HONEY BEE, *APIS MELLIFERA* L. (INSECTA, HYMENOPTERA) BY *NOSEMA APIS* ZANDER (PROTOZOA, CNIDOSPORA) AND ITS RELATION TO ENDOCRINE FUNCTION

DISSERTATION

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By

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INTRODUCTION

Parasitic protozoa are widely distributed in association with insects (Kudo, 1924; Weiser, 1961). Some species are highly pathogenic causing the death of infected hosts while others are almost symbiotic living within the host but not causing overt pathologies. The Microsporidian, Nosema apis Zander, is a highly evolved parasite of the honey bee, Apis mellifera (L.) (White, 1919). It attacks only the adult insect and develops exclusively in the ventricular (midgut) cells (Bailey, 1955; Steche, 1960). All castes are susceptible and the parasite is known to adversely affect hosts in which it is found. Longevity of infected individuals is reduced (Hassanein, 1953; Moeller, 1962; Burnside and Revell, 1948), their hypopharyngeal glands do not develop normally (Hassanein, 1952) and the water accumulation in their bodies is higher than normal (Hassanein, 1953). Infected queens may stop laying eggs and are often superseceded (Farrar, 1947; Moeller, 1962). Apparently the parasite does not cause the death of its host directly but may reduce its viability and vigor.

In the temperate climatic zones, the highest rates of infection occur during the spring months with a gradual decline during the summer and fall months (Oertel, 1964). Parasite incidence is apparently very low during the winter months when brood is not being produced. When egg-laying and brood-rearing are resumed in the early spring, the incidence of N. apis increases rapidly (Oertel, 1964; Steche, 1960;

Hassanein, 1963, Morgenthalen, 1939). The factors responsible for the increase of parasite abundance are not known but transmission from diseased to healthy individuals appears to increase and very heavy infections develop in most of the infected individuals. The increased intensity of infection appears to be correlated with an increased rate of protein metabolism associated with pollen digestion and brood feeding. The amount and nutritional quality of pollen seems to have an effect on the incidence of the parasite both in individuals and in the colony (Beutler et al., 1949; Gontarski and Mebs, 1964; Steche, 1961).

The epizootiology of N. apis is not well understood. The obligate parasite seems to be present in most colonies at all times of the year (Oertel, 1960). Transmission from individual to individual occurs within the colony but it is not certain that there is much transmission between Apis mellifera and other species of bees. Within a single colony, the activities of individuals are integrated and directed toward the survival of the colony at all times of the year. The environment changes considerably during the span of a year and the colony must respond to these changes. As the environment is dynamic so the physiology of individual bees and the whole colony is dynamic. The bees in the colony during the winter are quite different physiologically (e.g. much increased longevity) than those occurring in the colony during the summer (Maurizio, 1959; Clark and Rockstein, 1964). The bees in early winter seem to be less susceptible to N. apis infection; perhaps changes in host physiology are such that the parasite does not

succeed in invading cells or if it is successful, it may encounter conditions unfavorable to its development.

Physiological functions in insects are controlled and integrated by hormones (Novak, 1966). Protein metabolism is known to be under the control of hormones (Engelmann, 1968). The present study shows that egg production in Apis mellifera queens is under hormonal control. This control may be influenced by the presence of N. apis in the ventriculus with the result that yolk deposition in the oocytes is prevented (Hassanein, 1951). It is not known how this influence is mediated but the result is similar to that observed in Rhodnius prolixus following removal of the corpora allata (Wigglesworth, 1948, 1964). Apparently, the hormonal balance is shifted in favor of reduced protein metabolism either by increasing the titre of one hormone or blocking the production and/or release of another.

The hormonal balance in many species of insect may be changed by the presence of a parasite (Fisher and Sanborn, 1964) and the parasite may be influenced by the hormones of the host (Salt, 1941). The life cycle of Perilampus hyalinus (Hymenoptera) is modified by the host it inhabits. The larva must emerge from its host at the time of pupation. If it attacks Limnerium validum, which does not pupate until spring, the parasite remains undeveloped in the host during the winter. If the parasite attacks Varichaeta aldrichi, which pupates in the autumn but does not emerge until spring, the parasite becomes ectoparasitic in the autumn but does not develop further until spring. If the parasite is

in two other species of Limnerium and two braconids, which not only pupate in the autumn but also proceed to emerge in the same season, the parasite likewise develops at once and hibernates as a full-grown adult (Salt, 1941). Another case is in Apanteles glomeratus (Hymenoptera), a common parasite of the cabbage caterpillar, Pieris brassicae. This host usually pupates in the autumn and the larvae of Apanteles become full grown and leave it just as it prepares to pupate. If the host is not ready to pupate in the autumn and hibernates as a larva, the Apanteles then hibernates as a young larva inside the host caterpillar (Salt, 1941). Apparently a similar situation occurs when the Microsporidian parasite Nosema carpocapse (Paillot) and N. bombycis (Nageli) are found in diapausing hosts. The parasite stops multiplying during the diapause of the host insect (Paillot, 1938).

When Aphis craccivora (Homoptera) was parasitized by Aphidius platensis (Hymenoptera) the normal developmental physiology was changed. Different stages of growth of the parasite have opposite effects on the host; unhatched parasite eggs acted like a local source of juvenile hormone which produced metathetely, whereas parasite larvae sometimes cause the premature appearance of adult characters (Johnson, 1959).

Failure to pupate is a response of some insects to microsporidian infections. This has been reported in simulium larvae (Strickland, 1911), Pieris rapae (Laigo and Paschke, 1966) and Tribolium confusum (West, 1960; Fisher and Sanborn, 1962). In further studies, Fisher

and Sanborn (1964) found that when Tribolium larvae are infected with Nosema, they undergo supernumery molts that produce giant larvae. Thus, the infection mimicked the effects of an active corpora allata in prolonging larval life and preventing maturation. When Nosema-infected tissue from Tribolium was implanted in cockroaches, it caused supernumery molts. Further experimentation indicated that Nosema produced a substance with juvenile hormone-like activity that acted on the host tissues directly rather than by stimulating the host's corpora allata to produce the hormone (Fisher and Sanborn, 1964):

The age, instar, or stage of an insect may affect its resistance to disease. Some bacterial and viral diseases become established more readily in larvae than in adults (Stephens, 1963). Terzian et al. (1956) showed that the older the adult mosquito Aedes aegypti L. became, the more resistant it was to the malarial parasite Plasmodium gallinaceum Brumpt. In the case of honey bees and Nosema apis, only the adults are infected, and they become more susceptible with increasing age (Hanko, 1963). The literature suggests that the relationship between age and resistance may be associated with hormone production in the host (Stephens, 1963).

Four different basic types of host-parasite relationships of Thelohania (Nosematidae, Microsporidia) in 16 species of mosquitoes were reported by Kellen et al. (1965, 1966). In some cases sporogony takes place in both sexes but males are more apt to have more serious infections than females. Sometimes the infections are fatal to both sexes but some females may transmit parasites transovarially. In

other species sporogony occurs in both sexes but the areas of infection are limited and the hosts usually survive.

In the wood feeding roach, Cryptocercus sp., the diverse genera of symbiotic intestinal flagellates change from asexual to sexual forms of reproduction each time their host molts. Each protozoan genus has its own place in the molting period when a sexual cycle is initiated. Extirpation of the neurosecretory cells in Cryptocercus suggested that the presence of these cells was necessary for the initiation of the sexual cycles and the continued development of the flagellates (Cleveland and Nutting, 1955).

Cleveland et al. (1960) then showed that injections of ecdysone into an intermolt nymph or adult roach induced gametogenesis in the flagellates although the host itself never underwent ecdysis. Further experiments showed that if the host produces too much ecdysone, or produces this hormone too rapidly, its protozoa cannot adjust to such a condition and die (Cleveland and Burke, 1960).

Pflugfelder (1948) found in the isopod, Asellus aquaticus L., an extensive growth of the parasitic Haplosporidian Aselli (sporozoa) in the coelom after the removal of the rudimentary nephridia of the antennae. These organs are regarded as endocrine glands. He concluded that the removal of the gland resulted in a decreased resistance to the parasite in the host.

Similarly, excystment of Gymnodinioides sp. and G. inkystans occurs shortly before the actual molting of their crustacean hosts (Trager, 1957).

The encystment of Opalina ranaram can be induced by injecting testosterone into parasitized Rana temporaria (Mofty and Smyth, 1960). This opalinid multiplies asexually by binary fission during most of the year. However, during the frog's breeding period the reproductive pattern of the parasite changes to a sexual one.

Steche (1964) has reported that Nosema apis multiplies asexually during the spring and summer and sexually during the fall.

Neurosecretory material accumulated in abnormally high amounts in the neurosecretory cells of adult female alfalfa plant bugs (Adelphocoris lineolatus Goeze) when the insects were infected by a fungus (Entomophthora sp.) (Ewen, 1966).

Since Nosema apis only infects adult honey bees, it would not be expected to have the effects of a juvenile corpora allata. It might, however, show some of the effects attributed to the adult corpora allata, such as water regulation, protein metabolism, and a feed-back relationship with the neurosecretory cells (Lea and Thomsen, 1962).

Changes in corpora allata activity are difficult to detect by conventional light microscopy techniques but changes in neurosecretory cell activity can be determined fairly easily because at least one of the secretory products of the cells stains selectively with paraldehyde fuschin (Ewen, 1962). This procedure has been used in the present study to determine the effects of N. apis on the endocrine balance in the bee.

Neurosecretion in the animal kingdom has been extensively reviewed by Gabe (1966). Neurosecretory cells are widely distributed in the

nerve centers of Metazoa and the neurosecretory product of these cells is now recognized as one of the fundamental coordinating mechanisms in animals having anatomically distinct nervous systems. The hormones from the cells are released into the circulatory system and carried throughout the whole organism. The target tissues respond according to their own genetic program and thus morphological and physiological integration and coordination are accomplished (Scharrer and Scharrer, 1963).

In the Pterygota or winged insects which includes the majority of insect species, the position of the neurosecretory cells which stain with paraldehyde fuschin is identical in all cases. They are situated bilaterally in the pars intercerebralis on both sides of the mid-dorsal line (Gabe, 1966). Some of the physiological functions in adult insects which have been shown to be under endocrine control and may be correlated with the activity of this group of cells are diapause, egg-laying, water balance, and protein-lipid mobilization and utilization (Novak, 1966).

The first definite description of neurosecretory cells in an insect were reported in Apis mellifera L. (Weyer, 1936). He compared this structure to the crustacean X-organ described by Hanstrom (1934). The presence of neurosecretory cells in Bombus sp. were described by Scharrer (1937) and in Bombus fervidus (Fabricus) by Crosswhite and Medler (1966).

M. Thomsen (1954) described the neurosecretory cells in some Hymenoptera and concluded that "among insects, the Hymenoptera are

exceptionally well suited for a histological study of neurosecretion, for in some of the species neurosecretory material occurs in unsurpassed quantity and distinctness." However, he found that there was a very small amount of neurosecretory material in Apis workers caught and fixed in the autumn and concluded that the apparent lack of material might represent a temporary situation perhaps due to age or season. He suggested that the neurosecretory cells and other endocrine organs of the honey bee deserved a more careful study since he did not look at these organs at other seasons of the year.

Formigoni (1956) and Biedermann (1964) reported a parallel between neurosecretory activity in Apis mellifera workers and their social function. They noted that neurosecretory activity in workers dropped to a very low level during the winter and Formigoni (1956) found that the activity of the neurosecretory cells increased in the workers one week after the queen started laying in the spring.

Biedermann (1964) reported on neurosecretion in worker and queen honey bees under natural and experimental conditions. The quantity of neurosecretory material remained large throughout the period of oviposition in queens, indicating a high level of activity. The young queens showed a pattern of neurosecretory activity that was related to a behavior schedule from emergence until egg-laying started, after which it showed little variation. Light stimulated neurosecretory activity and treatment with carbon dioxide caused a sharp increase especially in young workers (Biedermann, 1964). No work has been reported on the

neurosecretory activity in queens during the fall and winter months.

The purpose of the present study was to gain an understanding of the relationship between changes in neurosecretory cell activity, corpora allata activity, egg-laying, brood rearing, and the development of Nosema apis infection in the honey bee Apis mellifera (L.). It was also of interest to see if there was any correlation between enodcrine activity and the pathogenesis of the disease in the individual bee and the epizootiology of the disease in the colony.

MATERIALS AND METHODS

Facilities for Research on *Apis mellifera*

The Ohio State University has an exceptionally well equipped Bee Laboratory where genetically known lines of bees are used for studies in bee pathology. The facilities include about 300 full-sized colonies and about 150 nucleus-sized colonies distributed in 10 apiaries. There are five observation-hive shelters with 20 attached flight cages as well as incubators and other indoor laboratory facilities. The professional staff is a ready source of information and equipment and the author has relied heavily on their help and advice in the present studies.

Maintenance of Greenhouse Colonies

Two colonies of bees were maintained in 5-frame hives under natural light conditions in separate rooms in the Ohio State University Botany and Zoology greenhouse. One colony had a high incidence of *Nosema apis* among the adult workers active in the colony. This colony was considered to represent a typical *Nosema*-infected colony and it maintained this infection for the duration of the study (over one year). Periodically, healthy bees (queens and workers) were added to help maintain the strength of the colony. The second colony was composed of healthy bees with an

occasional individual guard at the hive entrance showing Nosema spores. Both colonies of bees were fed 50% sucrose in a Boardman feeder approximately 10 feet away from the hive so the bees were forced to fly. Pollen (or occasionally pollen substitute) was provided in petri dishes. Water was available in each hive. Both colonies had actively laying queens.

For many of the studies it was necessary to introduce marked workers into the experimental hives in order to know the ages of the various bees used and the length of time that they had been with the particular colony. This was accomplished by marking the bees which were emerging from laboratory incubated frames of sealed brood obtained from healthy free-flying colonies maintained at the bee yard. When the bees emerged, they were marked on the thorax, using a fine camel hair brush with Testor's quick-drying enamel paint[1] and held in groups. The age range for any one group was 0 to 24 hours. The marked bees were then added to the colonies in the greenhouse or to a small, free-flying colony kept under natural conditions at the bee yard. The marked bees were sampled at approximately the same time daily (between 3:30 and 4:30 P.M.) to eliminate any differences due to diurnal rhythmicity (Rensing, 1965).

Technique for Rearing Queens

Virgin queens were reared according to the following method. An actively laying queen was confined to an empty comb with a queen

[1]Testor's paint is commonly used on airplane models and can be obtained in any hobby store.

excluder for 12 hours. Then she was removed and the queen excluder replaced. When the larvae were between the ages of 12 to 18 hours old, the comb was removed from the hive and the larvae were transferred to prepared queen-cell cups with a grafting needle. Manufactured queen-cell cups were fastened to a wooden bar which was just long enough to fit between the end bars in a standard frame. There were three bars per frame. The cups were "primed" with a small amount of royal jelly which was kept frozen until thawed just before use. After the larvae were placed in the cups, a moderately high humidity was maintained by placing the cell bar on wet paper towels to prevent the larvae from drying out.

The frames of queen cups were then placed in queen nuclei which had an abundance of young nurse bees and ample stores of pollen and sugar solution. On the tenth day after grafting, the finished queen cells were removed from the colony and put in individual queen nursery cages in an incubator until the queens emerged. In this way, virgin queens were obtained. They were fed queen-cage candy and water.

The majority of the queens used in the diapause experiment were obtained from the bee yard during the process of requeening. They were fixed in modified aqueous Bouin's fluid. The age, behavior, and egg-laying history for each queen was obtained from individual records kept at the bee yard. The heads were embedded in Ester wax sectioned at 8μ and stained in aldehyde fuschin.

Histological Techniques

In most cases, the head and ventriculus of each bee were fixed together in a vial of modified aqueous Bouin's fluid under reduced pressure for 24 to 48 hours and then stored in 70% ethanol. The tissues were dehydrated and embedded in 1960 Ester wax according to the method given in Appendix I. The ventriculi were sectioned on a rotary microtome at 4μ and the heads at 8μ. The ribbons were spread on albumenized slides. The ventriculi were stained in Heidenhain's iron hematoxylin for 12 to 24 hours following a 12- to 24-hour period in 4% iron alum (Humason, 1962, p. 127-8). The serial sections of the heads were coated with celloidin and stained in paraldehyde fuschin according to the method of Ewen (1962). This method is given in Appendix II. Smears were made by grinding fresh ventriculi with a small amount of water. A drop of the homogenate was put on a slide, covered with a cover slip, and viewed by phase microscopy.

Photography

Microphotographs were taken of many of the slides in order to record and make accurate comparisons of the amounts of aldehyde-fuschin-positive material in the neurosecretory cells, axons, and corpora cardiaca. Pictures were also taken to help analyze the relative size of the corpora allata and the structure of the hypopharyngeal glands. These were taken on panatomic X ASA 40 film with equipment produced by the American Optical Company for use with the Microstar

Series 10 Microscope. This consisted of a 35 mm camera back and a separate lens and shutter assembly. The pictures were enlarged with a Simmon Omega Model D2 enlarger.

EXPERIMENTS AND RESULTS

Development of Nosema Apis

Transmission and Infection in the Host.

One of the first questions to be answered concerning the epizootiology of Nosema disease in Apis mellifera is how the parasite is transmitted and if adult bees show any age-dependent resistance or susceptibility. To do this, more than 200 newly emerged bees from a strong healthy colony were marked with blue Testor's paint on the thorax and added to a Nosema-infected colony in the greenhouse. Five bees were removed from the hive daily. Their ventriculi were ground together in a small amount of distilled water and the presence or absence of spores was determined using phase-contrast microscopy. At the same time, more than 200 newly emerged bees were marked with white paint and added to a healthy five-frame hive at the bee yard to be used as controls.

The introduced healthy bees became infected with Nosema after they were put in the infected colony. Young spores were first detected in smears of the introduced bee ventriculi on the seventh day and mature spores on the ninth day after the bees were added to the Nosema-infected colony. Differentiation between young and mature spores is made on the basis of refractility. Mature spores are more refractile

than young spores, using phase contrast microscopy. Young spores and spores which have released the polar filament are only refractile around the periphery of the spore. The vacuole can be seen in young spores. Spores which have released the polar filament appear to be devoid of internal structure.

This experiment was repeated four times using newly emerged bees and in each case Nosema spores were not detected by the smear technique until the seventh or eighth day. These results are summarized in Table 1.

The newly emerged bees added to the control colony were not infected after 16 days in the colony. This indicated that the bees remained free of the disease as adults, unless they were in a diseased colony.

On the 16th day, the remaining marked bees were removed from the control colony at the bee yard and added to the Nosema-infected colony. These were sampled and examined daily for spores. Mature Nosema spores were first observed on the fourth day when the marked bees were 20 days old. This indicated that the older bees become infected sooner or supported more rapid development of the parasite and appear more susceptible to Nosema infection than the newly emerged bees under similar conditions.

The next question was how Nosema disease is transmitted in the colony. Are spores passed from infected to non-infected workers? Is the parasite passed via the queen? To determine whether the parasite is transmitted from worker to worker, approximately 50 newly emerged

Table 1

Nosema infection in bees added to an infected colony, determined by the spore technique using five ventriculi in each sample.

Days after exposure to infected colony	Newly emerged bees				Bees 16 days old
	Blue	Pink	Green	Red	White
0	-	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	Y[a] M[b]
5	-	-	-	-	M Y
6	-	Y	-	-	Y
7	Y	-	-		Y M
8	Y	Y	Y	Y M	Y
9	Y M	Y	Y M	M	M Y
10	Y M	Y M		M	M Y
11	Y M	M		M	M
12	M	M		M	M
13	M	M		M	M
14	M	M		M	M

[a] Young spores

[b] Mature spores

bees were marked with green paint and put in a wire "push-in" cage on a center comb in the Nosema-infected colony. Under these circumstances, it was assumed that their contact with the queen would be limited but they could exchange food with other workers in the colony. The mortality rate among these bees was very high but a few survived until the eighth day and for a few days longer when young Nosema spores were detected by the smear technique. This showed that direct contact with the queen is not necessary for the transmission of Nosema. These bees may have become infected by cleaning contaminated comb or eating contaminated honey as well as from contact with infected workers.

Four single, uninfected virgin queens and infected workers (10 per queen) were placed together in queen cages used for shipping queens. They were fed bee candy and water. Histological sections of queen ventriculi revealed heavy Nosema infections by the 12th day. Under similar conditions Nosema infected queens were also able to transmit the infection to uninfected workers.

These studies demonstrate that Nosema apis can be passed between workers, from workers to queens, and from queens to workers.

One behavioral difference noted was that many of the control bees in the greenhouse were actively foraging, whereas only a few of the Nosema-infected bees of the same age were flying. This observation was confirmed when an uninfected colony was put in the greenhouse and marked bees from this colony showed active foraging behavior. This suggests that Nosema-infected bees exhibit less foraging behavior than uninfected bees when compared under similar conditions.

The conventional smear technique gives only a rough estimate of the degree of Nosema infection. It does not provide an indication of the total commitment of tissue to the parasite nor does it show clearly the non-spore forms of Nosema apis. In later experiments dealing with neurosecretion, it was necessary to know the degree of infection in individual bees. It was also necessary to detect the initial parasitization of susceptible gut cells in the host by demonstration of any forms of the parasite that might be undetectable by conventional methods. An uninfected colony was maintained in the greenhouse for daily sampling to provide a control for maturation changes in gut cell structure.

Morphology of Nosema apis.

Various stages of Nosema apis were detected by introducing newly emerged bees into healthy and Nosema-infected colonies maintained in the greenhouse. Initially one or two of the guard bees at the entrance of the hive with the healthy colony showed Nosema spores in smears. About 500 newly emerged bees from an incubated frame of healthy brood were marked with red paint for introduction into the infected colony and about 500 were marked with cream paint for introduction into the healthy colony. The marked bees were sampled daily for three weeks and then every other day for a week. Each sample contained 15 bees. Smear preparations were made from five ventriculi and the remaining ten ventriculi were prepared for histology. At the end of the experiment all of the introduced bees sampled from the infected colony were heavily

infected, but the marked bees from the healthy colony were uninfected except for one bee (out of more than 250) that was heavily infected.

The histological examinations made throughout the experiments using bees from the greenhouse proved to be an accurate procedure for detecting signs of Nosema infection since all forms of the parasite could be observed. The stages of the developmental cycle of Nosema apis are illustrated in Figure 1. In Figure 1, a, b, and c show the earliest stages (planonts) with binucleated sporoplasm; d, e, f, and g show various meront stages; h shows young spores; and i shows mature spores. Figure 2 gives a schematic diagram of the life cycle.

The incidence of infection in marked bees from the Nosema-infected colony as determined by serial sections of ventriculi showed that Nosema infection was first detected on the fourth day by the presence of intracellular meronts (Figure 3). Spores were regularly detected beginning on the seventh day (about the same time that they are detected in smears). The number of bees infected steadily increases until about the 24th day when all of the bees sampled were infected with Nosema. Even on the 24th day, however, there was considerable variation in the number of infected cells in individual bees. Some bees were lightly infected with "vegetative" stages of the parasite, whereas other bees had many cells filled with mature spores.

The foregoing experiments showed that bees become infected with Nosema apis when they are in colonies that contain many infected bees. The parasite seems to spread rapidly and the infections may be detected at an early stage of parasite development.

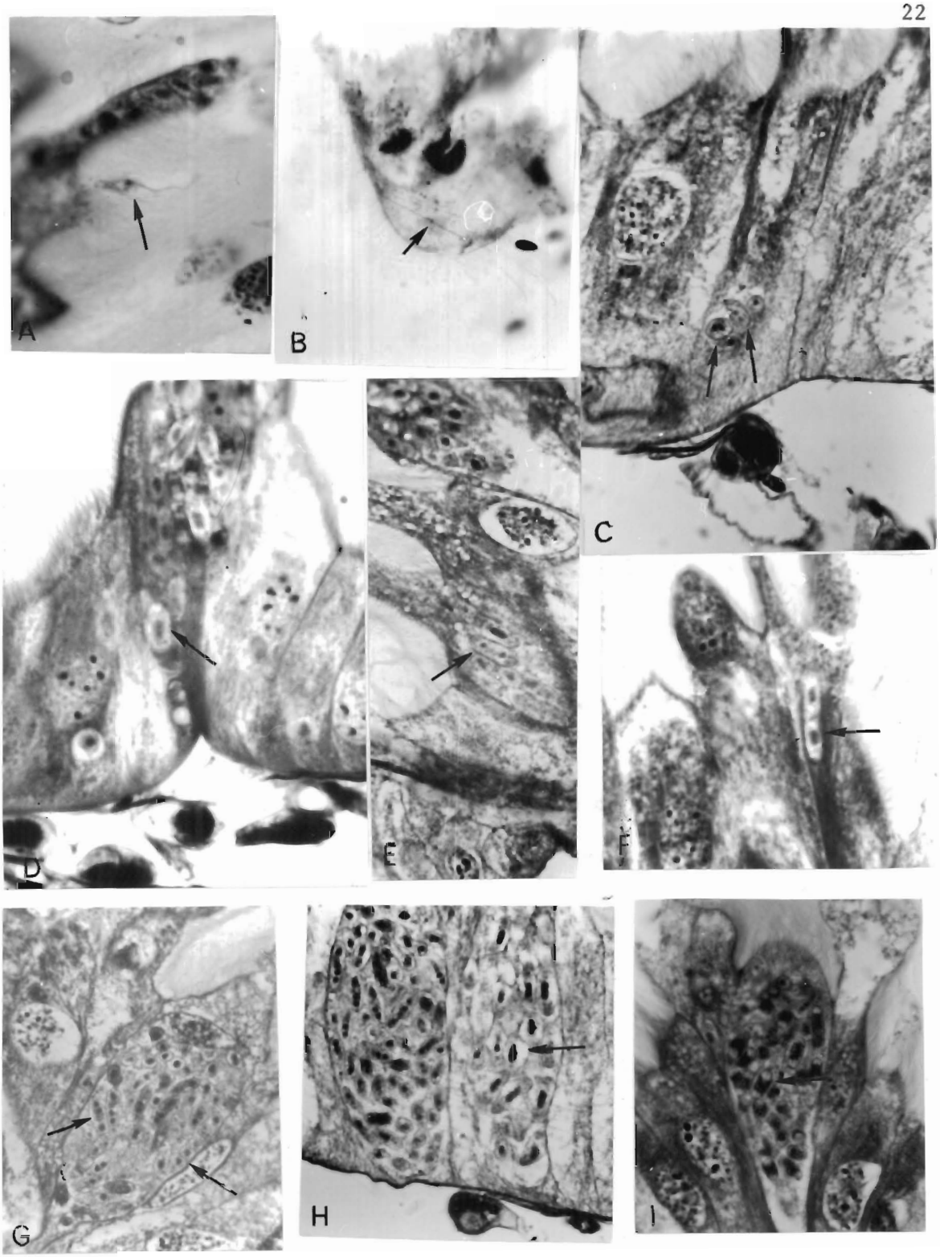


Figure 1. Developmental stages of Nosema apis Zander in honey bee ventricular cells a, b, and c, planonts; d, e, f, g, meronts; h, young spores; i, mature spores. Mag. 940x.

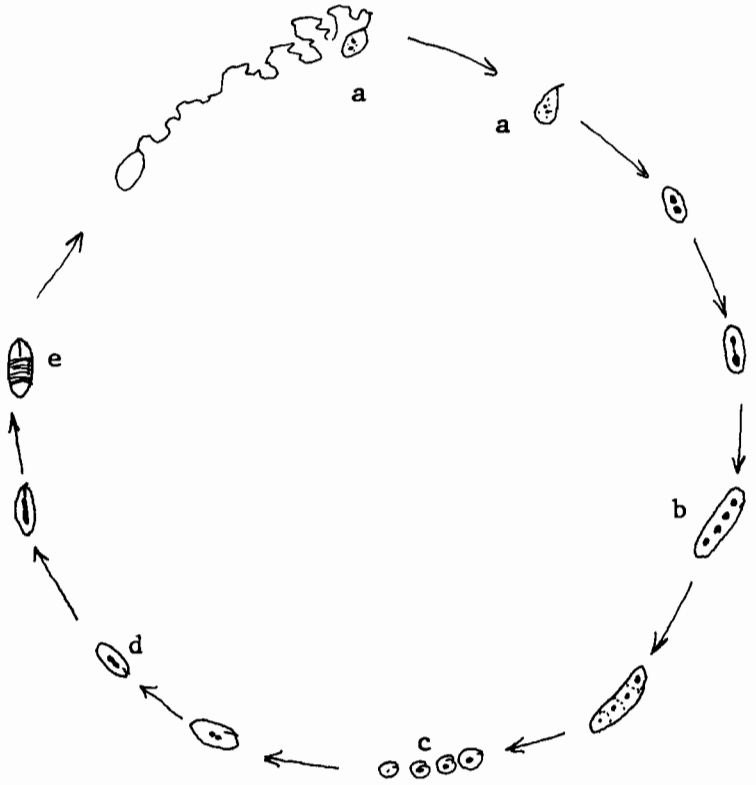


Figure 2. A diagrammitic life cycle of Nosema apis

a) planont; b) and c), meronts; d) young spore; 3) mature spore.

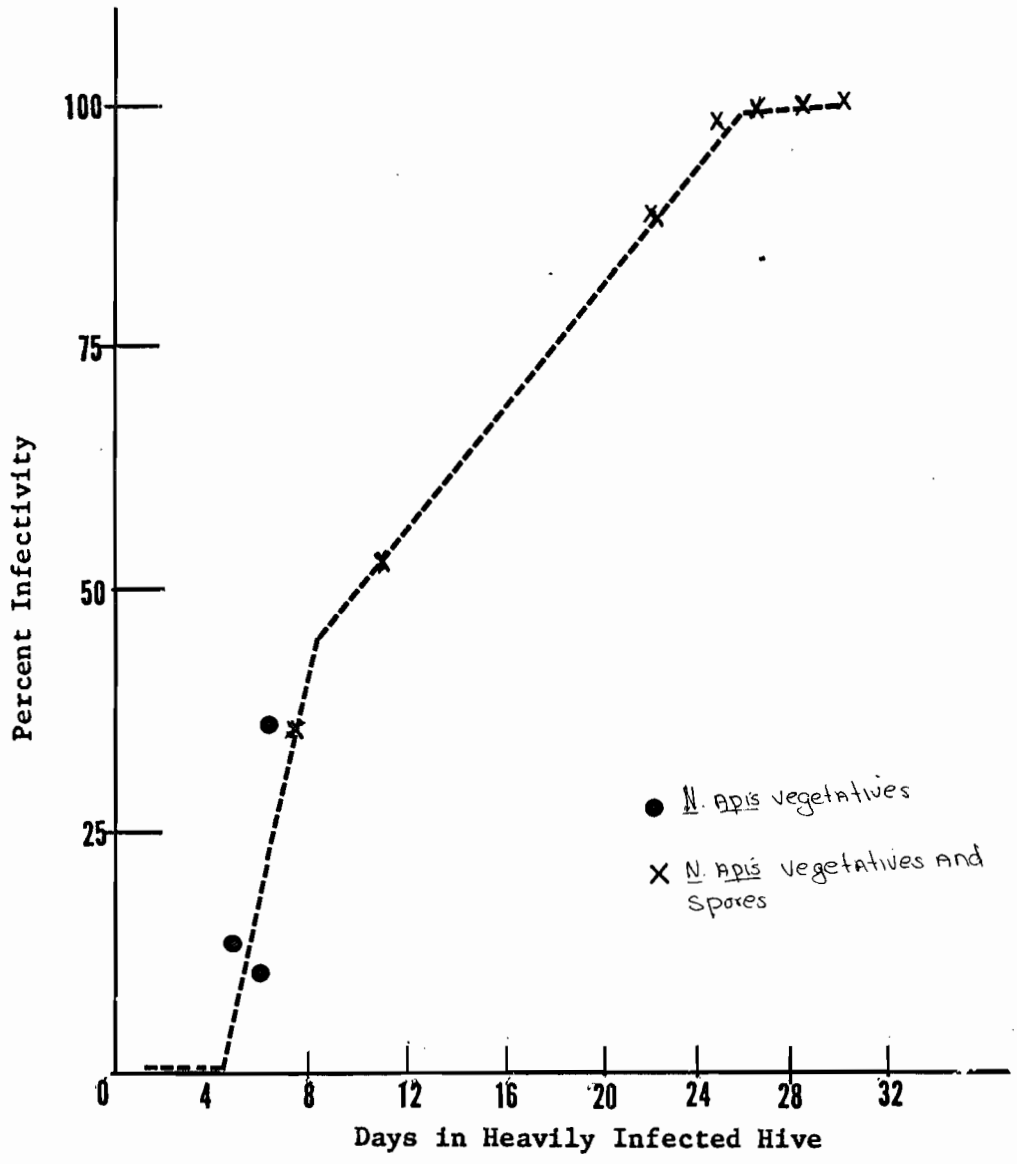


Figure 3. Percent of marked bees, added to Nosema-infected colony on Day 0, found to show Nosema apis on subsequent days.

Effects of Pollen on Parasite Development.

Several researchers have suggested that protein in the bees' diet stimulates and accelerates the development of Nosema apis (Orosi-Pal, 1966; Gontarski and Mebs, 1964). The influence of hormones on protein metabolism has been shown for several species of insect (Novak, 1966; Engelmann, 1968). It is assumed that protein metabolism in the honey bee is under hormonal control. A series of experiments was designed to help determine whether or not the feeding of pollen, the principal source of protein in a honey bee's diet, influenced neurosecretory activity and whether or not it had any effect on the development of Nosema.

Newly emerged, marked bees were put into two small observation hives, one supplied with pollen and one without, which were kept in a constant temperature incubator at 36°C. The bees in both hives were fed water and a sterile 50% sucrose solution at all times except for five hours on Day 8 when a Nosema apis spore suspension made from macerated, diseased ventriculi in 50% sucrose was fed instead. Ten bees from each hive were sampled daily. Five were used for smear preparation of their ventriculi; the other five were dissected and their heads and ventriculi were prepared for histological study. Nosema apis was detected in sections of ventriculi of both sets of bees 48 hours after feeding. N. apis appeared to develop equally well in hosts whether or not they were being fed pollen. No counts

were made of the relative numbers of spores. Examination of sections prepared from the heads of uninfected bees revealed no differences in neurosecretory activity of the same age with and without pollen.

Effects of Brood Rearing on Parasite Development.

A distinct correlation between colony infection with Nosema and colony brood-rearing was reported by Steche (1960). Since brood-rearing takes place during a specific period of the adult worker bee's life and since there is a correlation between social duties and neuro-secretory activity (Formigoni, 1956), it was of interest to see if brood-rearing influenced the development of Nosema apis.

A second set of observation hives was set up in an incubator to test whether or not the presence of a queen and brood had any effect on Nosema apis development in 8-day-old workers. At the end of the experiment (one week after feeding Nosema spores) neither colony had brood because of the low humidity in the incubator, but there was also a far lower incidence of Nosema infection than was found in the pollen/no pollen feeding experiments. Smears showed a few spores after 72 hours, but histological examination indicated that most of the ventriculi were not infected.

In an attempt to repeat this experiment under more natural conditions, two colonies were set up in the observation-hive shelters at the bee yard at the end of September. One colony (about 500 bees marked blue) had a non-laying queen, a little brood (a circle about

4 inches in diameter), and a full frame of pollen. More brood was added on the sixth day when the colony was discovered to have no brood. The second colony (about 500 bees marked red) had a laying queen, a small amount of pollen, and a large amount of brood (both sides of a frame). Both colonies were fed N. apis spores in a 50% sugar solution for 24 hours on the fourth day.

Nosema apis vegetatives were first detected 48 hours after the colony was fed spores in the bees from the colony that was rearing a lot of brood (i.e., bees marked red). The bees in the colony that had little or no brood (blue bees) did not become infected until the sixth day (the day after more brood was added). Infections in both colonies reached moderate levels and remained so for 11 days. The red colony queen was still laying at a low level and the brood in this hive was supplemented with more from another hive. Thus, the conditions when Nosema was first detected at 48 hours were similar to [illegible] found in many hives during the early spring when the incidence of Nosema is increasing rapidly. It is possible that the blue colony queen was in a state of diapause and the workers were responding to this state physiologically until more brood was added, after which they behaved like typical early spring bees. At the end of the experiment, the ventriculi from these two queens were sectioned; neither showed signs of Nosema.

These experiments were inconclusive in explaining any effects of

the amount of brood on the development of Nosema. They did suggest, however, that under situations which mimic those found in the colony in spring, Nosema apis will develop in the host ventriculi.

Neurosecretion in the Host, Apis mellifera

Normal Apis mellifera Workers.

The paraldehyde fuschin positive neurosecretory cells in Apis mellifera are situated in the anterior dorsal part of the pars intercerebralis which connects the left and right halves of the protocerebrum. Most of the cells lie close together around the median plane, virtually forming one large group. The cells form several layers with the superficial ones lying just below the neurilemma. In frontal sections, the group of neurosecretory cells tapers downward toward the bundle of axons that form the nervi corpora cardiaci I, so that more cells lie toward the surface of the brain. The axons from the two groups of neurosecretory cells cross a short distance after they leave the cell bodies and then converge to form a well-defined tract of nerve fibers. Near the posterior surface of the brain, the two neurosecretory tracts divide and innervate the paired corpora cardiaca. The corpora cardiaca are well developed organs that are closely connected with the lateral walls of the aorta. Most of the neurosecretory material collects along the surfaces of the cardiaca which are near the aorta.

In order to determine the normal appearance of the neurosecretory cells, their axons, the corpora cardiaca, the corpora allata, and the hypopharyngeal glands in worker bees of known ages, 10 marked bees were sampled from the disease-free greenhouse colony daily for four weeks.

The results are summarized in Table 2 (the data are given in Table 7 in the Appendix). Newly emerged workers have very few, fine, paraldehyde fuschin positive granules in their neurosecretory cells, and the axons appear completely devoid of material. Figure 4 shows the position in the brain of these cells, and the appearance of the granules. As the bees age, the number of granules in the cells begins to increase. By the fifth day after emergence (Table 2), the cells have accumulated a considerable amount of material and a small amount of material can be seen in the axons. The neurosecretory material continues to increase in amount so that by the 15th to the 17th day after emerging, there are more and larger granules in the cells. In addition, there is more material in the axons. These results agree completely with those found by Formigoni (1956). By the 25th day, nearly half of the bees sampled showed a reduced amount of material in the neurosecretory cells although there was still considerable material in the axons. According to Formigoni (1956), old bees show considerably reduced neurosecretory activity and the secretion disappears completely in very old bees. In the present experiment, sampling of marked bees was not continued beyond the 25th day. A few bees were sampled during

Table 2

The effect of age and season on neurosecretory activity in healthy worker bees (original data in Table 7)

Type of Bee	Age in days	No. in Sample	NSC*	Axons
Summer	1	5	+	-
"	2	3	+	-
"	3	4	+	-
"	4	2	+	-
"	5	4	+++	+
"	6	2	+++	+
"	7	4	+++	+
"	8	3	++++	++
"	9	4	+++	++
"	10	2	++++	+++
"	13	2	++++	+++
"	15	2	++++	++++
"	17	3	+++	++++
"	19	3	++++	++++
"	25	8	+++	++++
Winter (Oct)	Unknown	10	+	+
" (Nov)	"	8	+	-

*NSC = neurosecretory cells

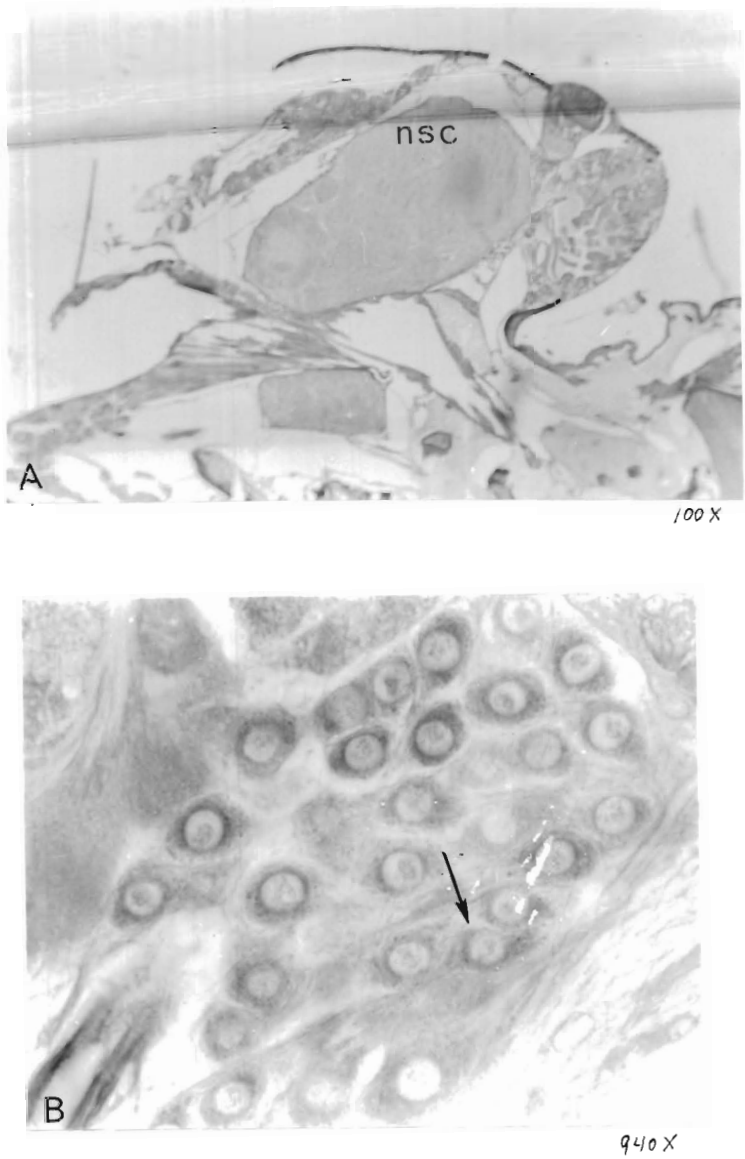


Figure 4. Neurosecretory cells in newly emerged workers.

a, position of neurosecretory cells in brain; b, granules in neurosecretory cells.

the fall and winter. These were of unknown ages but they showed greatly reduced or no neurosecretory material in the cell bodies and axons. These results are also in agreement with Formigoni's.

Since the greatest amount of neurosecretory material was seen in bees of young foraging age (15 to 20 days old), it was possible that the activity of the neurosecretory cells may increase with increased flight activity. The bees under greenhouse conditions had no opportunity for long-range flying; thus, if this was so, the neurosecretory activity should have been at a reduced level. Neurosecretory cells of 17-day-old bees from the bee yard colony were compared with those of 17-day-old bees from the uninfected greenhouse colony and were found to be similar. The disease-free greenhouse colony, therefore, was normal as far as the neurosecretory activity was concerned and was adequate control with which to compare the neurosecretory activity of the Nosema-infected colony. Apparently, neurosecretory activity is not correlated with the amount of flight activity.

Nosema-infected Apis mellifera Workers.

In order to determine if infection with Nosema apis had any observable effects on the host's hormonal balance, the neurosecretory cell activity of infected and uninfected bees of the same ages were compared. There is considerable evidence that the cerebral neurosecretory cells form an "over-all" controlling center of an insect endocrine system (Thomsen, 1952; Novak, 1966) and thus should be

a reasonable place to look for an indication of hormonal imbalance or change reflecting a pathological condition.

The heads of the same bees that were sampled daily for Nosema from the Nosema-infected colony (marked red) and the healthy colony (marked cream) in the greenhouse were sectioned and stained for neurosecretion. Thus, in each case, the amount of neurosecretory material in the cells and the degree of Nosema infection in the ventriculus of each individual bee was determined.

The results are summarized in Table 3 (data are given in Table 8 in the Appendix). In general, they show that the bees infected with Nosema have considerably less neurosecretory material in their cells and axons than do the uninfected bees from the same colony or bees from the control colonies (Table 4 , Figure 5). This is especially evident after the eighth to ninth day and less so beginning with the 25th day when some of the infected bees show increased amounts of neurosecretory product.

Neurosecretion in Apis mellifera Queens.

Virgin queens: The newly emerged queen has very little neurosecretory material in her neurosecretory cells and it is in the form of very fine granules. There is no neurosecretory material in the axons and only a small amount in the corpora cardiaca. At 4 days of age there was still little neurosecretory material in virgins kept in small cages in an incubator (Table 5). Sixteen-day-old virgins had a

Table 3

Amount of neurosecretory material in workers arranged according to the amount of *Nosema* infection from the *Nosema*-infected colony

	Very Light, Light, Moderate Infection			Heavy Infection			No Infection		
Days in Age	No. in Sample	NSC	Axons	No. in Sample	NSC	Axons	No. in Sample	NSC	Axons
5	2	++	-				1	++	-
7	3	++	-				2	+	+
8	5	++	-						
9	2	+++	+	2	+	-	2	+++	+
10	2	+++	+				1	++++	++
11	3	++	+				3	+++	+
12	2	+	-	1	++	-	2	+++	+
13				1	++	+++	4	+++	++
14				4	++	++	2	++++	+++
15				4	++	-	3	++++	++++
17				5	++	++	2	++++	+++
19				4	+	+	2	+++	
22				6	+++	++			
25				6	++	++			

Table 4

Neurosecretory activity in cells and axons of worker bees.
(Original data in Tables 3 and 8)

	Control Bees		Uninfected Bees from Nosema-infected Colony		Infected Bees from Nosema-infected Colony	
Age in Days	NSC	Axons	NSC	Axons	NSC	Axons
1 - 4	+	-	+	-		
5 - 7	+++	+	++	-	+	-
8 - 9	+++	++	+++	+	+	+
10-15	++++	+++	++++	+++	++	++
17-25	+++	++++	+++		++	+

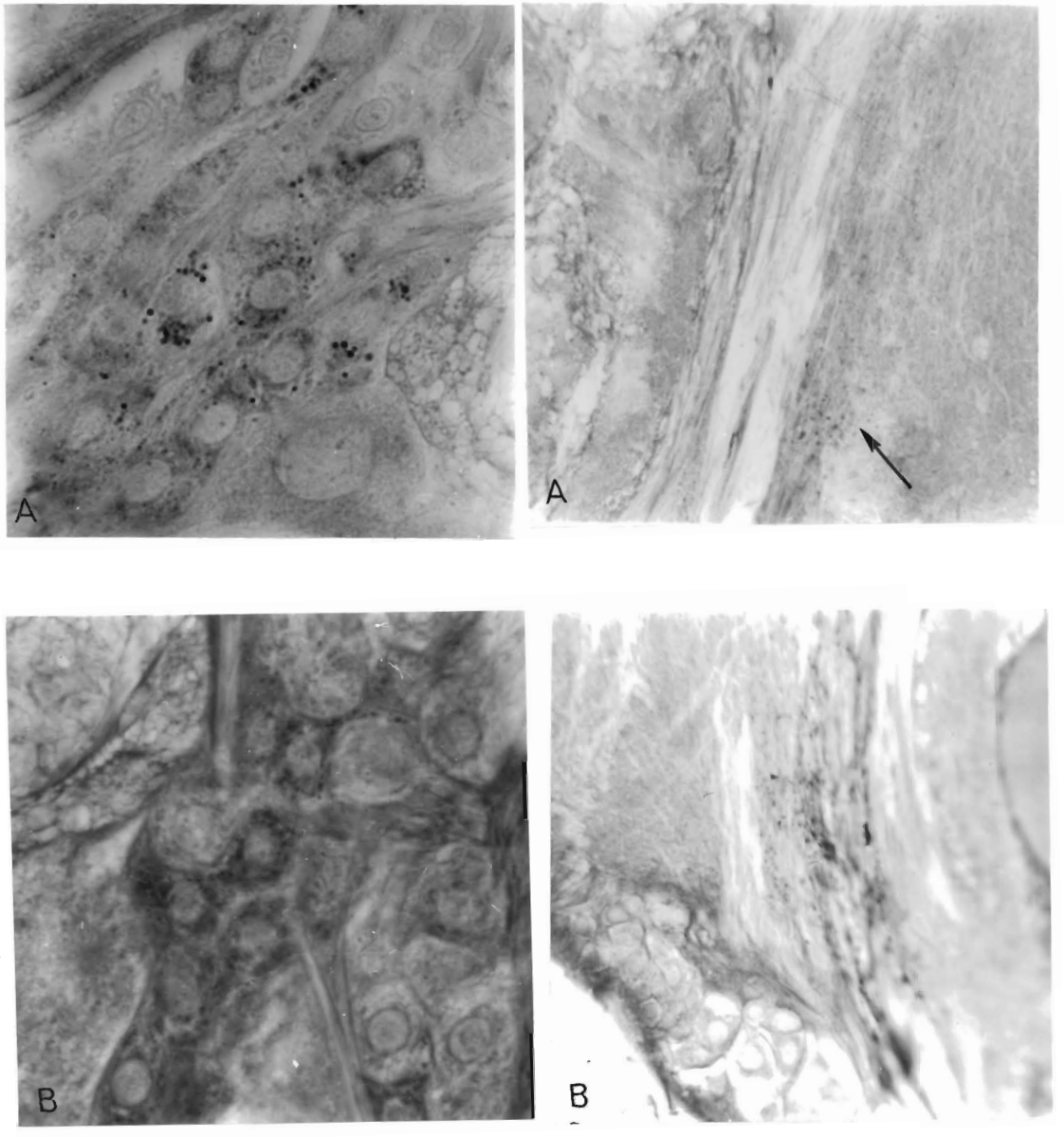


Figure 5. Neurosecretory cells and axons of Nosema-infected and -uninfected bees. Mag. 940X.

a. infected workers. b. uninfected workers.

Table 5

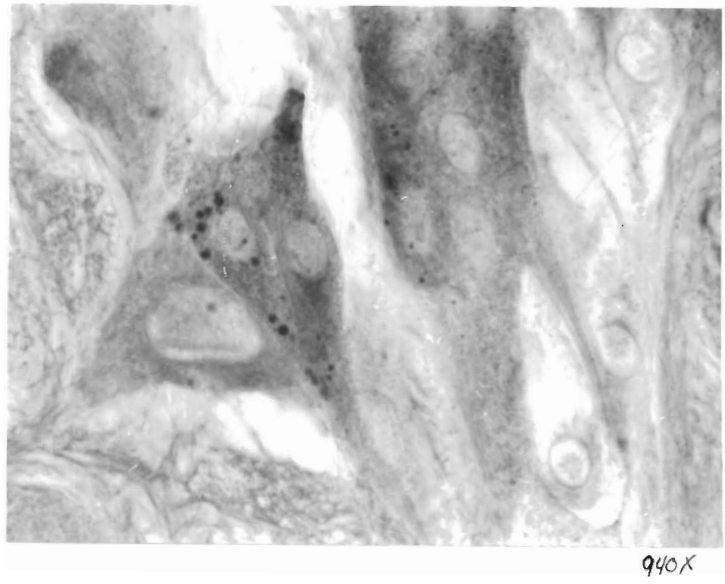
Neurosecretory activity in five virgin queens.

Age (in days)	NSC	Axons
4	+	-
16	++	++
30	-	-
30	++	++
30	+++	++

moderate amount of finely granulated material in the neurosecretory cells and in the axons. One or two cells had some larger granules. By 30 days there was a little more neurosecretory material in the cells, except in one 30-day-old virgin that had very little material and an obviously inactive corpora allata (Figure 6).

These results are different from those obtained by Biedermann (1964). His queens were kept under normal hive conditions. His results showed a sharp increase in neurosecretory material on the fifth day which correlated with the time the queens were beginning to fly. It continued to increase until after the last mating flight. It decreased just before ovipositing started on the 13th day and then increased and remained at a moderate to high level throughout the summer. Queens which were exposed by Biedermann to CO_2 on the third and fourth day, after emerging, showed a sharp increase in neurosecretory activity on the fifth day (even though they had no opportunity for flying or mating). Activity decreased on the 11th and 12th days and ovipositing started on the 14th day.

Biedermann's results suggest that the increase in neurosecretory material prior to the start of egg-laying is dependent on a certain amount of outside stimulation which the virgin queens in small cages did not receive. This stimulation is probably ordinarily provided through orientation and mating flights and the results suggest that in the place of these, CO_2 may stimulate the production of neurosecretory material necessary for the initiation and maintenance of ovipositioning behavior.



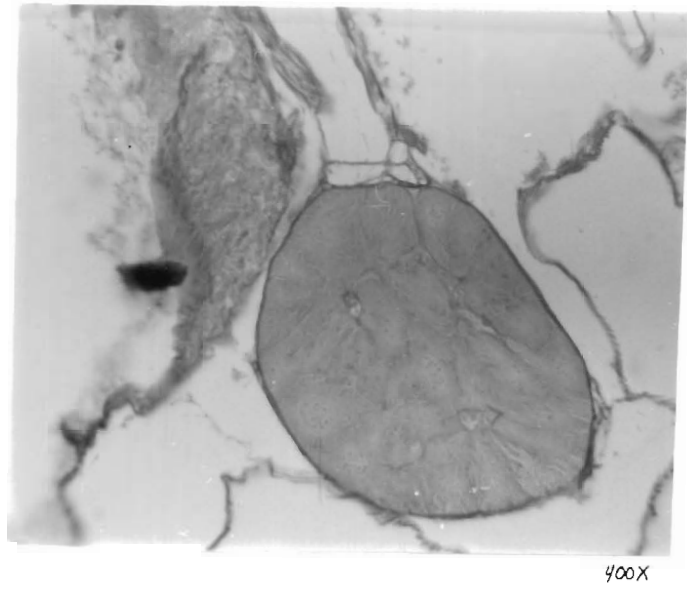


Figure 6. Neurosecretory cells and corpora allata in inactive 30-day-old virgin queen.

This is further substantiated by the fact that Biedermann also reported that CO_2 caused a rapid increase in neurosecretory material in the cells of workers at a time when it would ordinarily be increasing very gradually. Mackensen (1947) has shown that CO_2 stimulates the initial oviposition of artifically inseminated and virgin queens. When no CO_2 is given, egg-laying may not begin for 40 to 50 days. When CO_2 is given, queens start egg-laying at about 15 days which is the normal age.

Ovipositing Queens or Mated Queens.

There have been many reports in the literature concerning the positive correlation between egg-laying and protein-lipid metabolism in adult female insects (Fraenkel, 1940; Rasso and Fraenkel, 1954; Harlow, 1956; House, 1962). There are also many reports giving evidence that this metabolism is under the endocrine control of hormones from the median neurosecretory cells and the corpora allata (Hill, 1962; Sláma, 1964; Engelmann and Penny, 1966; Thomas and Nation, 1966).

In most Apis mellifera colonies, only the queen lays eggs and the workers are kept in a sexually undifferentiated state through the action of the queen substance pheromone (Butler, 1956, 1957; Butler and Fairey, 1963; Gast, 1967). In order to see if there were any detectable relationships between neurosecretion and protein-lipid metabolism in bees, it seemed reasonable to investigate the possible connections between egg-laying and the neurosecretory activity of the queen. This information was important from the standpoint of Nosema research because

Hassanein (1959) has reported that Nosema infection in queens causes distrubances in ovarian development. The anterior portion of each ovary appears to be normal but posteriorly growth ceases abruptly and the egg and nurse cells break up and disappear (Fyg, 1945). In the eggs themselves the injury is marked by the disappearance of yolk granules (Hassanein, 1959). Since the neurosecretory cell-corpora allata complex is necessary for yolk deposition in many insects, circumstantial evidence suggests that Nosema infection may cause changes in the hormonal titre.

Table 6 shows the neurosecretory cell and axon activity of queens sampled through the summer and up to the end of December. The amount of egg-laying at the time the queens were killed was obtained from individual hive records kept at the bee yard. There seems to be a fairly close correlation between the amount of egg-laying and the amount of paraldehyde-fuschin positive material in the neurosecretory cells and axons (Table 6). There were relatively few actively laying queens in the sample but this data can be supplemented by that given by Biedermann (1964) who reported that queen neurosecretory cells showed a high uniform rate of activity during the summer months. One major exception to this generalization was seen in the present experiment; summer package queens frequently showed a large amount of neurosecretory material in the cells and axons even though they had not had a chance to deposit eggs for over two weeks. One explanation for this might be that the eggs were being formed and resorbed at a constant

Table 6

Summary of neurosecretory activity in queens
(Original data in Table 9)

Characterization of Queen	NSC	Axons
From Summer Package, May	+++	+++
Artificially Inseminated, young, laying, July	+++	+++
Naturally mated young, laying July and August	+++	+++
Naturally mated, September	+	+
Naturally mated, October	++	+
Naturally mated, November	+	+
Naturally mated, not laying, December	++	-
Naturally mated, laying December	+++	+++

rate which maintained a continual stimulation for an ovigenesis-neurosecretion relationship (Flanders, 1957).

It is interesting to note that egg-laying decreases as fall and winter advance. Egg-laying is at a very low level during October (in spite of the fact that there was a surprise honey flow at this time). There is little data for November but oviposition probably comes to a complete stop during this time as most of the queens sampled during December were not laying. Table 9 in the Appendix shows that two queens, DS1 and Q-7, had just started to lay again on December 18th and 19th, respectively, and there is a noticeable increase in the amount of neurosecretory material evident (Figure 7). Queen DS1 was reported not laying on September 19, 1967. Queen G-7 was not laying on December 4, 1967, and it is probable that she stopped laying sometime in September because although the hive was checked several times at the end of the month, there is no record of any brood.

Table 9 in the Appendix indicates that the amount of secretory material in the corpora cardiaca remains at a fairly consistent high level. This suggests that changes in material in the neurosecretory cells more accurately indicate changes in the production of hormone. The amount of material in the axons seems to be an even more reliable indication of the mobilization of neurosecretory material in response to changes in the physiological state of the bee.

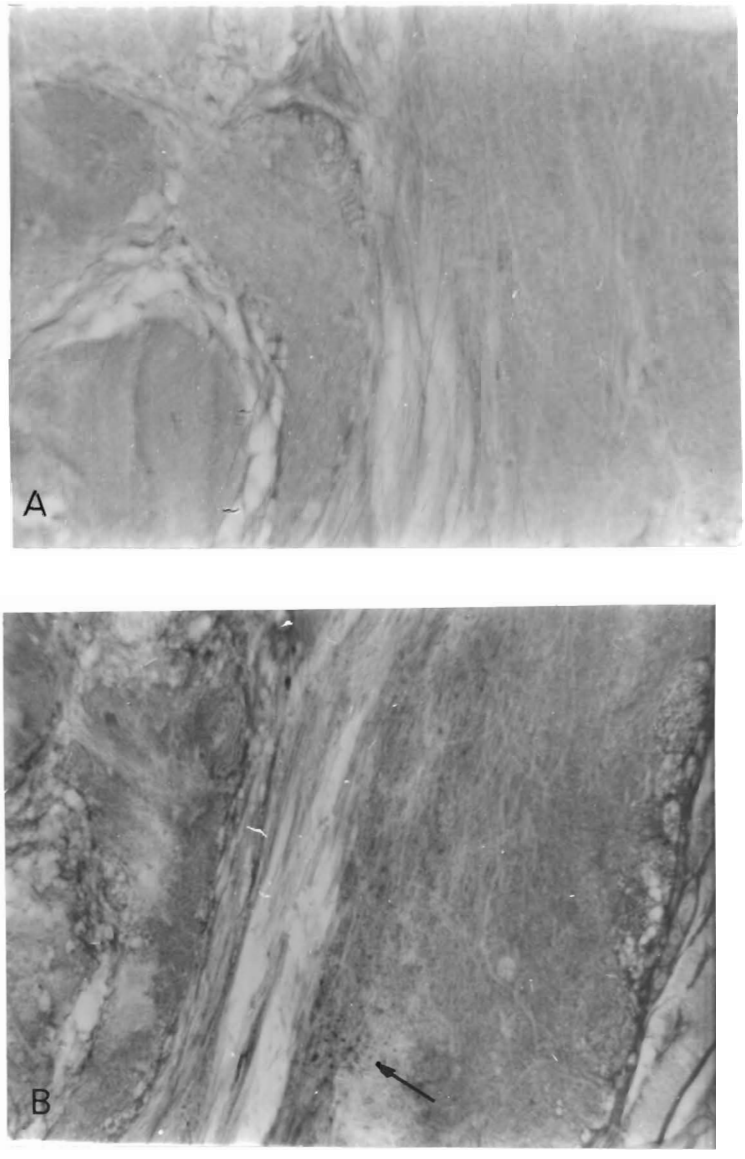


Figure 7. Neurosecretory material in axons of queens in December.

a, non-laying queen; b, laying queen. Mag. 940X.

DISCUSSION

Nosema apis is assumed to be present in some form in one or more individual adult honey bee in all colonies. When only a few workers are infected, the disease may be considered enzootic. From an apicultural point of view (e.g., pollination, honey production), the enzootic Nosema disease does not affect the economy of the hive. Ecological factors affecting the host colony and individual predispose the colony to the disease by changing the physiology of the individual bees so that an increasing number of individuals become infected. This adversely affects the growth, activity and productivity of the colony.

The results of this study suggest that the entire colony may be considered an organism composed of units, the workers and drones, whose physiological functions are interrelated with those of the queen. For the period that the queen is in reproductive diapause (i.e., not laying eggs, during the late fall and winter), the workers appear to remain in a physiological resting state also. The colonial diapause is frequently referred to as a "wintering state", characterized by the presence of long-lived workers or winter bees, and the absence of brood rearing. Once the queen begins laying in the late winter or early spring, the diapause ends and the workers that have been present during the resting stage resume an aging process similar to active foraging, short-lived "summer bees." Theoretically, since the queen

has initiated oviposition, the workers that have overwintered may be rapidly replaced by the new emerging brood. However, the break in colony diapause precedes the temperature and weather conditions outside the hive that are necessary for development of nectar and pollen sources adequate for support of a large amount of brood. In addition, low temperatures outside the colony place demands upon workers to maintain the colony temperature at a level adequate to brood rearing. Under these circumstances, the queen may oviposit at a low rate or sporadically. Consequently, the number of old Nosema susceptible bees in the colony increases. The entire population, which is physiologically prepared to propagate brood, accumulates excess protein reserves.

The pathogenesis and the epizootiology of Nosema disease may be viewed from the point of view of a relationship between protein utilization and susceptibility of individual honey bees to Nosema apis. In general, bees are most susceptible and the parasite develops at an optimum rate when more protein is available in the bees' body than is being metabolized for egg development (in the case of the queen), brood food production or flight muscle metabolism (in the case of spring and summer workers) or for the formation of the fat body (fall workers).

The highest incidences of Nosema infection among workers are found in the spring, whereas summer and fall bees have a low incidence of infection (Oertel, 1960). Starting in the fall, the majority of the

bees in the colony are young, long-lived winter bees. They emerge as adults at a time when there is very little or no brood to be nursed. They consume pollen the first two or three days as adults. Pollen is abundant in the hive in the autumn. Their hypopharyngeal glands and fat body become fully developed and their neurosecretory activity remains low. The queen stops laying and her neurosecretory activity is also low. This suggests that the metabolic rate of the bees is at a low level. As long as this physiological state is maintained, Nosema disease remains enzootic in the colony.

Late in winter or early spring neurosecretory activity increases in the queen and she resumes oviposition. This breaks the workers' diapause and their neurosecretory activity increases. The workers become physiologically prepared to rear brood. If there is only a little brood, they have an abundance of proteinaceous resources for brood food available in their bodies without the stress of brood rearing demands to match the "available resources." Under these circumstances, a few to all ventricular epithelial cells are susceptible infection sites which support rapid and complete development of N. apis. The queen may also become infected if, once reproductive diapause is broken, the conditions are not conducive to continual laying of eggs. This suggests further that protein metabolism to meet demands for oogenesis, when not carried to fruition, predisposes susceptible sites to parasite development.

As late spring and early summer advance, the environmental conditions increasingly favor the productive activities of honey bees,

the queen lays more eggs, and gradually the brood nest expands until there is an optimum and constant number of worker bees of the right physiological age to care for the young larvae. Most of the other bees in the colony are foraging. Under these conditions the protein reserves in the fat body and other tissues in the bees and additional protein intake are being used at an optimum rate and are not available for the development of the parasite. These relationships are diagrammed in Figure 8.

Beekeepers, personally contacted, recognize a "resting period" on the part of the queen in the fall and winter when egg-laying is at a reduced level and may stop completely. A few researchers have suggested that this is due to a reproductive diapause (Kauffeld, 1967, and Taber, 1967). Chauvin (1956) recorded the laying behavior of queens during the summer months and noted a sharp decrease and arrest in the fall which was not broken by the reinforcement of food and which he attributes to the onset of diapause.

In general, the seasonal changes in egg production by the queen and the subsequent rearing of brood by the workers are thought to be mainly dependent on the supply and abundance of food in the field. Laere (1966), however, has suggested that the laying behavior of the queen is controlled by a more complex series of factors than the amount of food available in the fall, or the temperature. He does not specify what these factors are. In the temperate climates oviposition may stop completely in October and does not resume until January or February even if pollen is stored in the hive. Thus, the outside temperature is

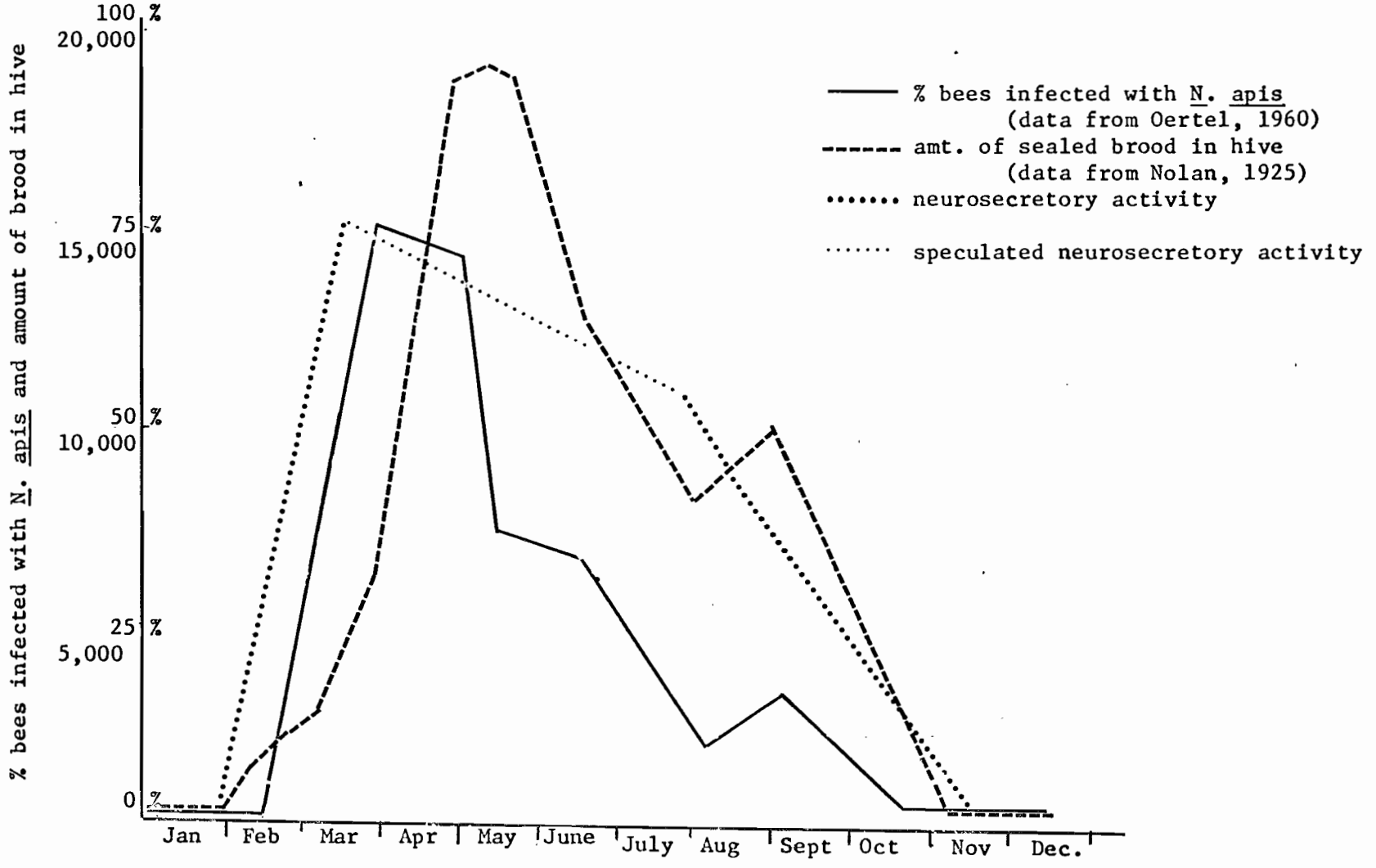


Figure 8. Theoretical correlations between Nosema infection, brood, and neurosecretory level in bee colony at various times of year.

clearly higher when egg laying ceases than when it starts again. This occurs in as widely differing climates as can be found in Louisiana and Wisconsin. In addition, the development of the fat body in fall and winter bees coincides with similar physiological changes in other species of insect going into diapause.

There has been no work until the present study to show that this suggested diapause is controlled by endocrine activity in Apis, as in other insects (Williams, 1946, 1947, 1948). According to Novak (1966) and Lee (1955, 1956), diapause is defined as a temporary interruption of development. Morphologically, in those species studied not including Apis, imaginal diapause is only evident in the interruption of growth of the ovaries and in the suppression of the functions of the accessory glands. This involves a complex syndrome of both neurosecretory cell hormone and corpora allata hormone deficiency. In queen bees there is a close correlation between the amount of neurosecretory material present in the cells and axons and what is assumed to be the degree of activity of the system. The amount of material appears to be closely related to the amount of egg production. This is further substantiated by the fact that allatectomy does not prevent the production of mature eggs in honey bee queens (Hrdy and Slama, 1963). According to Wigglesworth (1964), in insects in which the corpora allata does not seem necessary for egg maturation, the neurosecretory cells of the brain are the chief source of hormone necessary for yolk deposition. Therefore, it seems that the neurosecretory system,

especially axons containing a large amount of neurosecretory material, is the active system. The diapause situation is correlated with a small amount of material in the system. This is the opposite of the situation described by Highnam (1962) in the desert locust Schistocerea gregaria (Forskal) in which the active situation is evidenced by small amounts or the absence of neurosecretory material in the system because the hormone is released and utilized as rapidly as it is produced.

In the Egyptian grasshopper Anacridium aegyptium L., the diapausing adult neurosecretory cells contain large amounts of stainable material. When diapause is broken, the cells contain very much less material as the oocytes develop, but the material accumulates once more when the oocytes are fully grown (Geldiay, 1965, 1967).

In the adult of the beetle Nebria brevicollis (F.), there is no storage of neurosecretory material in the corpora cardiaca. During the breeding season the amount of neurosecretory material is large and constant. This uniformity is related to the continuous production of eggs. There were conspicuous amounts of neurosecretory material in the axons during the breeding season but only negligible amounts during prediapause (Ganagarajah, 1965). A similar situation is found in the chrysomelid beetle Galeruca tanaceti L. (Siew, 1965).

In queens that are actively ovipositing, the production of the neurosecretory hormone might be correlated with a variety of related physiological events. Studies in other insects have shown neurosecretory control of gut esterase activity (Thomsen and Møller, 1959,

1963; Laufer, 1960; Mordue, 1967), corpora allata activity (Strangeways-Dixon, 1961, 1962; Highnam et al., 1963; Strong, 1965), of blood protein or amino acid concentration (Hill, 1962; Engelmann, 1965a, 1956b, 1968; Wilkins, 1965, 1967; Mordue, 1965a,b). In the case of queen bees all of the processes involved with egg production and oviposition occur at a consistently high rate; it is difficult, if not impossible, to tell which ones are specifically dependent on the hormone.

The interpretation of the amount of neurosecretory material with the degree of activity is not as clear in workers as it is in queens. There is little neurosecretory material evident in newly emerged bees but the activity of the neurosecretory cells is probably initiated by the imaginal molt. According to Hagedorn and Moeller (1967), bees consume little pollen until they are about 40 hours old after which they begin to eat larger amounts. Greatest pollen consumption occurred when the bees were 2 to 3 days old (Hagedorn and Moeller, 1967). During this time there is little neurosecretory material evident. The bee then rears brood from the fourth to the fifteenth day employing active hypopharyngeal glands which develop during the period of high pollen consumption. During brood rearing the amount of neurosecretory material increases which suggests an increased rate of neurosecretory activity correlated with brood food production or protein metabolism. According to Clark and Rockstein (1964), the development of the hypopharyngeal glands and ovaries and fat body in caged bees can be induced by pollen feeding, only when such feeding is initiated before the first two weeks of adult life which indicates

a critical time-dependency of the physiological state of the worker bee. The present study suggests that this physiological state is based on an intermediate level of neurosecretory activity. The amount of neurosecretory material reaches its highest level when the bee is a young forager which suggests that the activity is now related to flight metabolism. When the worker stops brood rearing and begins foraging, the hypopharyngeal glands degenerate. This could result in an increased amount of protein in the hemolymph. This in turn stimulates neurosecretory activity which in turn stimulates corpora allata activity. The corpora allata hormone might cause the hemolymph protein to be utilized by flight muscle metabolism. This is important because according to Lotmar (1939) honey is the only nourishment of foraging bees. The average life span for a summer bee is 35 days (Clark and Rockstein, 1964) and the amount of neurosecretory material in the cells begins to decrease in some bees after the 25th day which may reflect changes due to aging.

In Leptinotarsa (potato beetle) one of the most marked effects of corpora allatectomy is the disintegration of the flight muscle sarcomeres and these giant mitochondria are regenerated very shortly after reimplantation of active corpora allata. It is known that proteid yolk formation in Leptinotarsa occurs in contact with mitochondria. It might be suggested, therefore, that the corpora allata exert part of their gonadotrophic activity by assuring the presence of mitochondria for energy metabolism associated with the synthesis of proteid yolk (Stegwee, 1963). Likewise, this suggests that the

corpora allata contain factors governing the synthesis of muscle protein.

The biosynthesis of protein is known to be RNA-dependent. The hormone from the corpora allata frequently influences protein metabolism (Slama, 1964; Telfer, 1960; Wilkens, 1967; Engelmann and Penny, 1966; Thomas and Nation, 1966; Bodenstein, 1953; Highnam et al., 1963; Coles, 1965; Ewen, 1966a; Wilkens, 1965; Pfeiffner, 1940, Wigglesworth, 1948; Doane, 1962; Strangeways-Dixon, 1962; Engelmann, 1965, 1968). L'Hélas (1953a, 1953b, 1956) noted that in corpora-allatectomized Dixippus (Order Orthoptera) the RNA content of various tissues was reduced. Likewise, Berreur (1961) has provided evidence that the RNA metabolism in Calliphora (Order Diptera) is under endocrine control.

Thus, there is abundant evidence that the corpora allata and neurosecretory cells play a part in regulating different phases of protein synthesis.

The young queen bee has a larger corpora allata than the workers (Lukoschus, 1956; Pflugfelder, 1948a) and egg-laying workers have larger corpora allata than non-laying ones (Dreischer, 1956). The corpora allata in foraging bees is much larger than in the younger nurse bees (Lukoschus, 1956) and the gland is almost twice as large in these workers as in queens during the foraging period (Habowsky and Beckel, 1964).

Gast (1967) reported that the corpora allata of adult worker bees is influenced by the queen. During the first period, growth of the corpora allata is inhibited by the queen substance pheromone, while

the second growth period occurs only in older bees when the queen is present. The present study suggests that the second growth period is correlated with increased neurosecretory activity and is related to flight muscle metabolism.

Thus, it is evident that a large corpora allata is often associated with an active neurosecretory system. This relationship has also been reported for Phormia (Order Diptera) (Orr, 1964), Schistocerca (Order Orthoptera) (Hill, 1962), and Tenebrio (Order Coleoptera) (Mordue, 1965). This situation is also frequently correlated with a high level of hemolymph protein. Since honey bees do not eat protein when they are foraging, it is reasonable to assume that the high level of hemolymph protein is due to the breakdown of brood food glands (hypopharyngeal glands).

If a male Periplaneta americana receives an injection of corpora allata extract, the neurosecretory axons develop an affinity for intense staining in approximately 6 hours. At that time, the corpora cardiaca contains more neurosecretory material than uninjected individuals. At 15 to 20 hours after injection, the axonal pathways for neurosecretion become very prominent and intensely stained. At the end of 24 hours, the neurosecretory cells become filled with material. This is interpreted as being an accumulation of neurosecretory material by delay on interference with its release (Nayar, 1962).

The evidence seems to support a relationship between protein utilization or lack of utilization by an individual bee and Nosema apis infection. Package bees are particularly susceptible to Nosema. In

1947 Farrar reported that 34 out of 39 queens from commercial sources in one year were accompanied by one or more infected attendant bees. In another year 41 out of 62 cages from commercial sources showed from one to five infected attendants, and one infected queen was found.

In one queen-mating yard all of the samples taken from 11 nuclei started with package bees without brood showed infection (Farrar, 1947). Since package bees are usually without brood for at least a week, and since the nurse bees which should be feeding brood are not able to, it is possible that protein reserves accumulate in the body predisposing the individual to infection or stimulating Nosema to develop. Protein also accumulates in queen bees that are restricted in egg laying (Flanders, 1957). Laidlaw and Eckert (1962) reported that of 2088 laying queens examined in summer, only 1% showed Nosema.

A Nosema infection of young bees is evident and the incidence is high in colonies which start brood rearing early in the spring. Incidence in summer may occur after an interruption of brood rearing (Borchert, 1930).

Morgenthaler (1949) has suggested an interpretation of the foregoing phenomena. He states that predisposition to Nosema occurs when brood rearing is initiated in winter because it stimulates heat production by workers, increases food consumption, which in turn increases the amount of material in the intestine and in general "upsets colony harmony." The situation becomes increasingly worse and the strength of the bees becomes exhausted prematurely.

To the contrary, the present study suggests that brood rearing itself is not harmful. But as soon as it starts, the resting state or diapause is broken. Once diapause is broken, the physiological process of aging is initiated in the colony and the bees become increasingly susceptible to Nosema. On the other hand, the incidence of Nosema disease in the colony remains low if, after brood rearing starts, the nurse bees can continue it at a high rate so that the proteinaceous brood food is being utilized. Bees that are not rearing brood are foraging and the replacement rate of the worker bee population is rapid. Probably the conditions most conducive to Nosema are those under which the queen starts laying, but only at a low rate, or in which brood rearing stops and starts because of unfavorable environmental conditions. This is more likely to occur the earlier in the season the queen starts laying. The relationships between N. apis disease, brood rearing, and hormonal balance are diagrammed in Figure 9 .

Moeller (1962) has stated that ". . . the first line of defense against Nosema is to provide conditions that will permit the colony to raise healthy young bees faster than the infection can spread within the colony population. Because the package colony is without emerging young bees for 3 weeks, this type of colony is especially vulnerable to Nosema." The present study shows that this is only a partial explanation of the dynamics of the interrelationships of brood-rearing, host physiology, and Nosema disease.

Steche (1965) suggested that there is a distinct correlation between the degree of infection and unfavorable weather. Equally

Explanation of Figure 9.

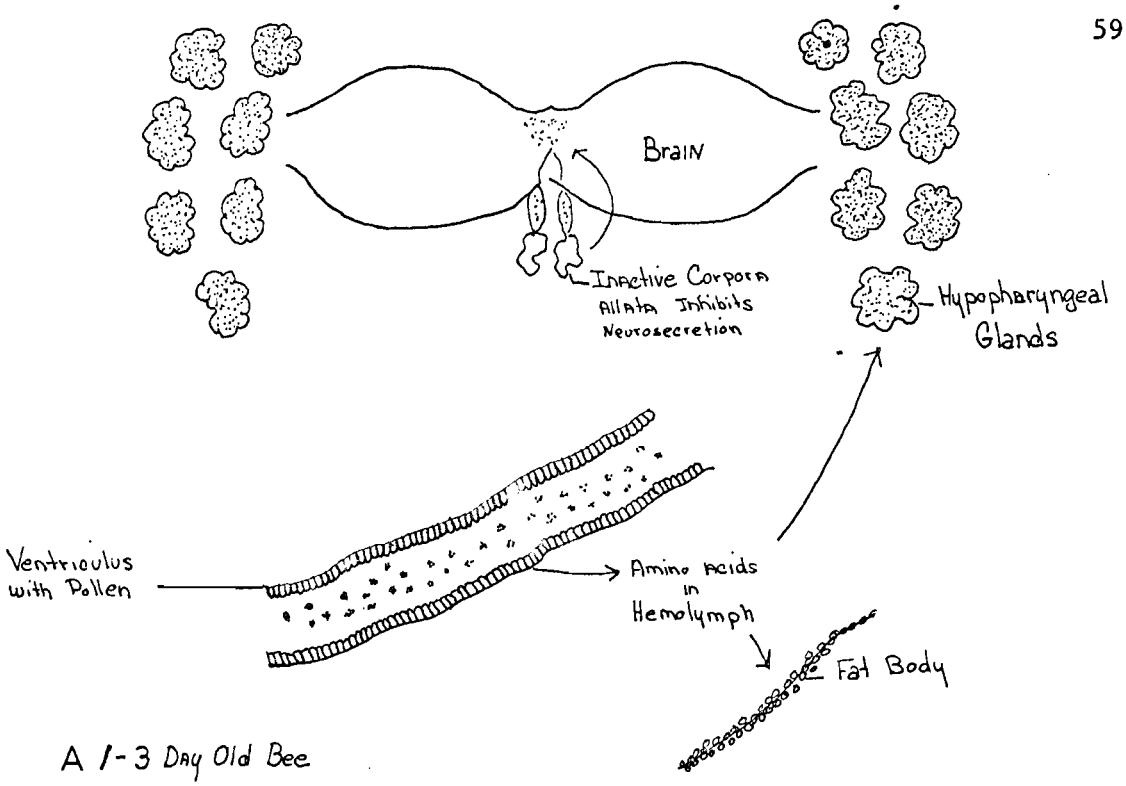
Relationships between N. apis disease, brood rearing, and hormonal balance in the bee.

A. One to three-day-old bee. There is considerable amount of pollen in the ventriculus. The amino acids from the digested protein pass by way of the hemolymph into the hypopharyngeal glands and the fat body. Neurosecretory and corpora allata activity are low.

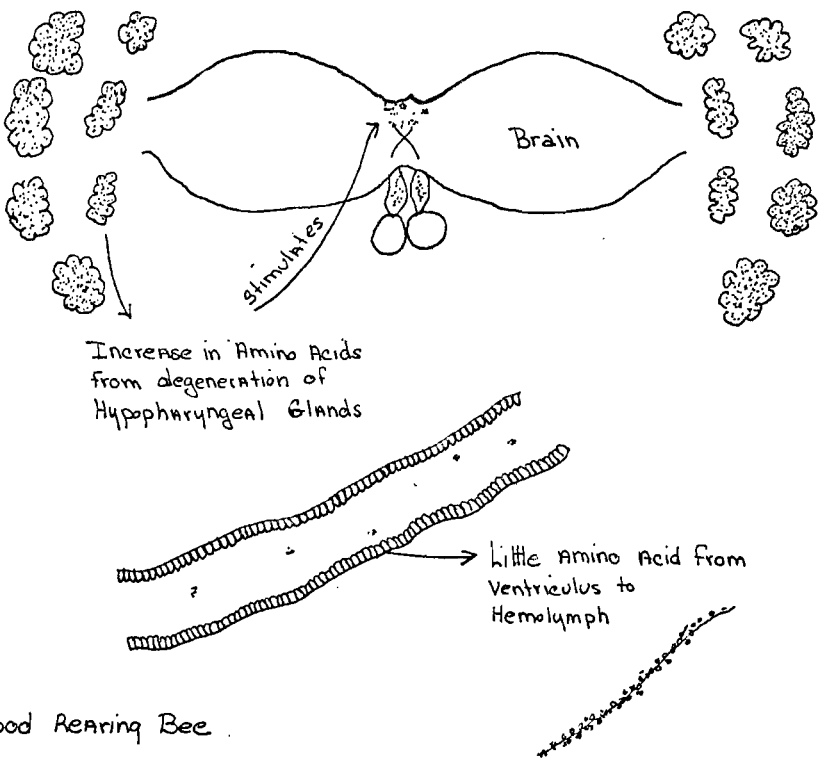
B. Brood rearing bee. There is less pollen in the ventriculus. A few of the lobes of the hypopharyngeal glands are degenerating, releasing amino acids into the hemolymph. The neurosecretory cells are becoming more active.

C. Young foraging bee. The hypopharyngeal glands are undergoing massive degeneration. The hemolymph amino acid level is high. Neurosecretory cells are stimulated which in turn stimulates corpora allata activity. The corpora allata activity stimulates RNA synthesis in the flight muscle.

D. Nosema-infected bee. The hypopharyngeal glands have undergone degeneration. The amino acids resulting from the hypopharyngeal gland degeneration have been utilized by the developing Nosema. The hemolymph amino acid content is low which inhibits neurosecretory activity.

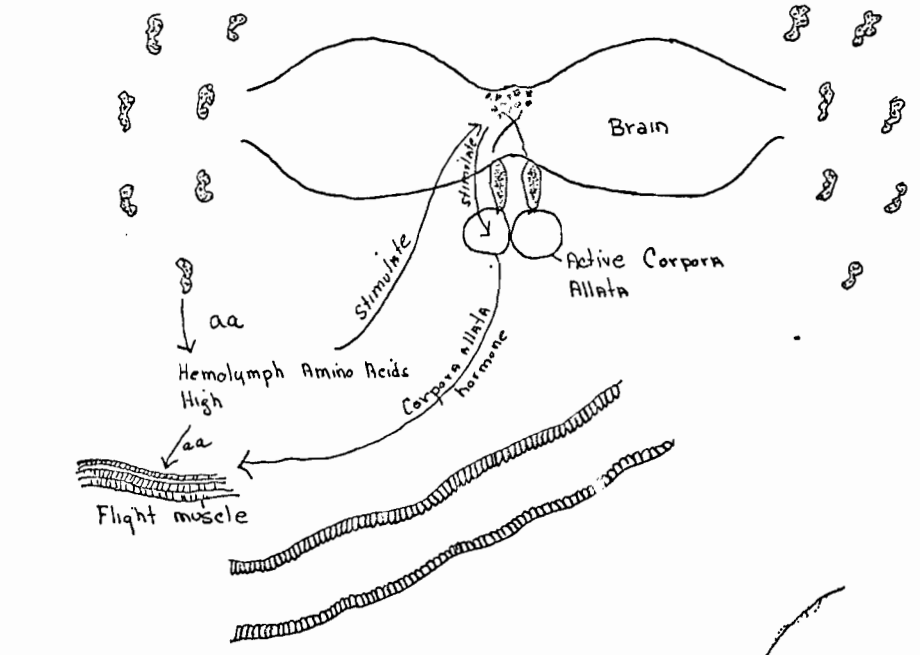


A 1-3 Day Old Bee

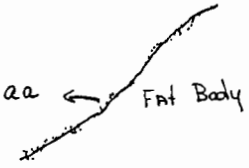


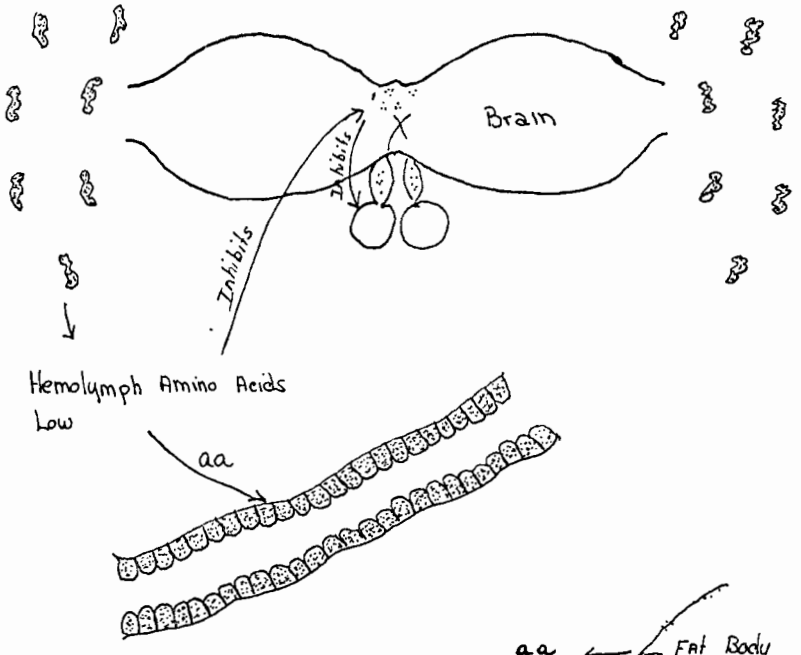
B Brood Rearing Bee

Figure 9.



C Young Foraging Bee





D *Nosema* - infected Bee

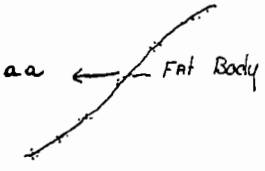


Figure 9 (Continued)

dangerous in promoting the development of Nosema apis when available brood is scarce is an increase in the supply of protein in the colony which occurs regularly if, in the spring, pollen substitutes are fed to bees, or if the surplus protein accumulated by workers cannot be used for brood rearing. Briefly stated, an interruption of brood rearing first displaces the protein budget and causes a change in the age structure of the colony (Steche, 1965). There is an increase in the average age of the "house bees" caused by lack of newly emerging bees and the susceptibility to infection is raised with increasing age (Steche, 1965).

This study shows that Nosema apis can develop in bees which have no protein in their diet. According to Haydak (1935) even bees which have had no protein in their diet are still able to rear brood for one generation. Thus, a certain amount of proteinaceous reserves are deposited during the larval stages of development. It is probable that Nosema does need a certain amount of protein for its metabolism and this may be mobilized from reserves in the bees' bodies to the cytoplasm of the ventricular cells.

The Nosema parasite interferes with the metabolism of the host cell by changing its biochemical balance. Studies have shown that the cytoplasm of ventricular cells of normally feeding worker bees have large amounts of RNA. Infection with Nosema apis caused a decrease in the amount of cellular RNA and in the rate of RNA synthesis in the host cell but increased amounts of RNA in the parasite (Przelecka and Hartwig, 1967).

The endocrine dependency of nucleaic acid metabolism is illustrated by the work of Vanderberg (1963a,b) who reported that allatectomized Rhodnius prolixus (Order Hemiptera) showed a drastic inhibition of the incorporation of uridine into RNA in the ovary, fat body, and midgut tissue.

Gontarski and Mebs (1964) reported a direct relationship between the feeding of pollen to bees and the development of Nosema. However, in further tests, they were unable to show any correlation between specific proteins or combinations of amino acids and the development of Nosema. Therefore, they concluded that other substances in pollen might influence parasite development such as vitamins and pantothenic acid. According to Bretschko (in Gontarski and Mebs, 1964), vitamin B_1 is especially favorable for the development of Nosema whereas vitamin B_2 is strongly inhibitory.

Nutritional investigations with Drosophila melanogaster and vitamin requirements revealed that these requirements varied with the protein level of the diet. These results are interpreted as confirming vitamin participation in protein metabolism (Sang, 1959). Since vitamins generally act as constituents of enzymes essential in metabolic activities, their effects eventually affect many parts and activities of an insect and probably only indirectly affect Nosema development.

One question in the Nosema-protein interrelationship is posed by the observations in the fall of the year that a worker bee's consumption

of pollen is at a high rate during the time it is accumulating protein reserves in its fat body for winter, yet at that time of year the parasite is rarely reported from routine examinations (Morgenthaler, 1939). On the other hand, feeding pollen or pollen substitutes in the spring may increase the incidence of Nosema (Steche, 1965). One major difference in the physiological state of the bees under these conditions is in the neurosecretory activity. Brood-rearing bees in the spring have a moderate amount of neurosecretory material in their cells and axons, whereas these same bees have much less material in the fall.

According to Formigoni (1956), the neurosecretory material of worker bees begins to increase in amount one week after the queen starts laying in the spring. This occurs in January or February in many hives and is the time of year generally considered by apiculturists to coincide with the noticeable increase in the incidence of Nosema according to routine observations on spores.

Nosema apis seems to develop best when the neurosecretion is at an intermediate or high level of activity. This may be why it is nearly absent in fall bees and increases when brood rearing begins again. This would also explain the age-dependent resistance of young bees under normal situations.

The present study has shown that the neurosecretory activity is lower in Nosema-infected bees than in normal bees of the same age. This can be explained in a number of ways. One is that a heavily infected bee probably cannot digest protein from pollen for absorption;

therefore, the hemolymph-protein level would remain low and the neurosecretory cells would not become activated or would be activated to a limited degree. However, protein digestion may have taken place before the Nosema develops to an extent where it can interfere with the production of the necessary enzymes needed for the breakdown of pollen. Under these circumstances the effect of the infection may be explained if the Nosema produce a corpora allata hormone-like substance which would inhibit the activation of the neurosecretory cells. The normal progression of events in an uninfected individual is probably for a high protein level in the hemolymph to activate the neurosecretory cells which in turn activate the corpora allata, causing mobilization of the protein from the hemolymph into flight muscles. If the corpora allata substance is already produced somewhere else, then the feed-back mechanism will inhibit the activation of the corpora allata, which will inhibit neurosecretory cell activity. Implantation of active corpora allata induces atrophy of the glands of the host in Pyrrhocoris (Novak and Rohdendorf, 1959). Perhaps if the Nosema produce a corpora allata hormone-like substance locally in the ventricular cells, this might channel any hemolymph protein to these cells and thus provide nutrient material for the developing Nosema. This is speculation. However, when honey bee queens are infected with Nosema, the oocytes stop developing when they reach the stage of yolk deposition (Fyg, 1945, and Hassanein, 1951). The material for yolk comes from a specific hemolymph

protein fraction which is mobilized by the corpora allata hormone (Engelmann, 1968). Thus, a Nosema-infected queen shows the same symptoms as many species of adult, egg-laying insects which have had their corpora allata removed.

CONCLUSIONS

1. Healthy adult worker bees become infected with Nosema apis when they are added to a heavily-infected colony.
2. The disease develops in the older bees faster than in younger bees.
3. Nosema apis can develop in bees which had no pollen in their diet.
4. Brood rearing may affect the development of N. apis but the mechanism of this effect remains unknown.
5. Bees which are infected with N. apis have less neurosecretory material in their cells and axons than uninfected bees of the same age.
6. There is a positive correlation between neurosecretory activity and egg laying in Apis mellifera queens.
7. Apis mellifera queens have a reproductive diapause during the winter months which is correlated with a low level of neurosecretory activity.
8. During reproductive diapause in the queen, the neurosecretory level of the worker bees in the colony is also low.
9. It is probable that a rapid increase in N. apis occurs in colonies when neurosecretory activity increases in workers following the termination of the queen's reproductive diapause.

SUMMARY

In this study, the relationships between the disease caused by Nosema apis Zander (Protozoa, Microsporidia) in the honey bee Apis mellifera (L.), and the physiology of the host as it is reflected in neurosecretory cell activity has been investigated. The life cycle of the parasite takes place in the midgut cells of the adult bee. Early stages of N. apis first appeared in newly emerged adult bees that have been added to an infected colony after they had been in the colony about four days. Spores appeared by the seventh day. The spores were shown to be passed from the workers to the queen, from queen to the workers, and between workers. Older bees were more susceptible or the parasite developed faster than in younger bees. The parasite developed in caged bees which had no protein in their diet. The effect of brood rearing on the development of the parasite was inconclusive in the present study.

Apis mellifera queens had a reproductive diapause during the fall and early winter. During diapause their neurosecretory activity was low as opposed to the high level seen during egg-laying. While the queen was in reproductive diapause, the workers were also in a resting state as suggested by the low level of their neurosecretory cell activity. Nosema apis did not appear to develop, or developed very slowly in bees that were in the resting state.

The regular sequence of maturation changes is initiated in the neurosecretory cells of workers after the queen's reproductive diapause has been broken and N. apis develops at a greatly accelerated rate. Infected bees may show a lower neurosecretory cell activity than the control bees of the same age.

These experiments suggest that the changes in neurosecretory cell activity of the bee may initiate changes in its protein metabolism and that the development of the parasite depends on the stores and accessibility of the host protein.

APPENDIX I

EMBEDDING TECHNIQUE

Ester Wax 1960 from the Gallard-Schlesinger Chemical Manufacturing Corporation, 584 Mineola Avenue, Carle Place, Long Island, New York, 11514, was used as the embedding medium. Each of the following infiltration steps was accomplished with the aid of a vacuum oven.

1. Fix in modified aqueous Bouin's fixative[1] 24 hrs to 1 week.
2. Store in 70% ethanol - indefinitely.
3. 95% ethanol - 2 hrs.
4. 100% ethanol - 1 hr.
5. 100% methanol - 1 hr.
6. 1:1 mixture of 100% methanol and methyl cellosolve - 4 hrs. to overnight.
7. 1:1 mixture of #6 and ester wax 1960 - 4 hrs. to overnight.
8. Fresh ester wax, two changes - 4 to 6 hrs. each.
9. Embed in fresh 1960 ester wax.

The tissues were embedded in paper boats of ester wax and allowed to harden in the refrigerator for 8 to 12 hrs. Then the blocks were allowed to return to room temperature and were trimmed with a single-edged razor blade.

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[1] Modified aqueous Bouin's fixative
Saturated picric acid in distilled water -575 ml.
40% formaldehyde -125 ml.
trichloroacetic acid - 4.5 gm.

APPENDIX II

Aldehyde Fuchsin Staining Technique (Ewen, 1962)

Preparation of Stain

Dissolve 3.0 gram basic fuchsin in 600 ml boiling water; boil one minute; cool, filter. To filtrate add 6 ml each of concentrated HCl and paraldehyde, leave stoppered at room temperature in the dark for 4 days. (Solution should have turned from red to deep purple.) Filter, dry the precipitate on the filter paper in a paraffin oven and store they crystals obtained in a stoppered bottle. Make up a stock solution of 0.75 grams of dry dye in 100 ml of 70% ethanol. This solution will keep for one year.

Staining Solution:

Stock solution	25 ml
70% ethanol	75 ml
Glacial acetic acid	1 ml

1. Remove paraffin in xylene - 4 changes, 2 minutes each.
2. 100% ethanol - 2 changes, 2 minutes each.
3. Dip slides in parloidin and wave dry - then put in 70% ethanol for 2 minutes (Dissolve parloidin in a 1:1 mixture of ether and absolute ethanol).
4. Water - 2 minutes.
5. Oxidize, 55 seconds in acid permanganate

$KMnO_4$	0.15 grams
Conc. H_2SO_4	0.15 ml
Distilled H_2O	50 ml

6. Rinse in distilled water.
7. Decolorize in 2% sodium bisulphite
8. Pass through rinses of distilled water, 30% ethanol and 70% ethanol to aldehyde fuchsin. Stain 3 1/2 minutes.
9. Wash in 95% ethanol
10. Differentiate, 30 seconds in acid alcohol

Absolute ethanol	100 ml
Conc. HCl	0.5 ml

11. Pass through rinses of 70% and 30% ethanol and distilled H_2O.
12. Mordant 12 - 15 minutes in phosphotungstic-phosphomolybolic acid.

Phosphotungstic acid	40 grams
Phosphomolybolic acid	1.0 grams
Distilled water	100 ml

13. Rinse in distilled water
14. Counterstain 1-3 hours:

Distilled water	100 ml
Light green SF yellowish	0.4 grams
Orange G.	1.0 grams
Chromotrope 2R	0.5 grams
Glacial acetic acid	1.0 grams.

15. Rinse very rapidly in 0.2% acetic acid in 95% ethanol.
16. Dehydrate rapidly in absolute ethanol (This also removes the paraloidin). Clear in xylene and mount in picolyte.

APPENDIX III

Table 7

Amount of neurosecretory material in control workers during the winter and summer

Type of Bee	Age in days	No. of Bee	NSC*	Axons
Summer	1	1 BHc 3/VIII	+	-
"		5 GHc 3/VIII	+	-
		7 GHc 3/VIII	-	-
		8 GHc 3/VIII	+	
		9 GHc 3/VIII	+	-
"	2	1 GHc 4/VIII		
		2 GHc 4/VIII	++	+
		9 GHc 4/VIII		
"	3	4 GHc 5/VIII	+	-
		5 GHc 5/VIII	+	-
		7 GHc 5/VIII	+	-
		8 GHc 5/VIII	++	
"	4	2 GHc 6/VIII	+	-
		9 GHc 6/VIII	+	-
"	5	2 GHc 7/VIII	+++	+
		3 GHc 7/VIII	+++	+
		5 GHc 7/VIII	+++	+
		7 GHc 7/VIII	+++	+
"	6	2 GHc 8/VIII	++	+
		4 GHc 8/VIII	++++	+
"	7	3 GHc 9/VIII	+++	
		4 GHc 9/VIII	++++	++
		8 GHc 9/VIII	+++	
		2 GHc 10/VIII	+++	++
"	8	4 GHc 10/VIII	++	
		6 GHc 10/VIII	++++	++
		7 GHc 10/VIII	++++	++
"	9	3 GHc 11/VIII	++	+
		5 GHc 11/VIII	++++	++
		6 GHc 11/VIII		
		9 GHc 11/VIII	++	
"	10	8 GHc 12/VIII	+++	++
		9 GHc 12/VIII	++++	+++
"	13	9 GHc 15/VIII	++++	++
		8 GHc 17/VIII	++++	
"	15	7 GHc 17/VIII	+++	+++
		10 GHc 17/VIII	++	++++

Table 7 (Continued)

Type of Bee	Age in days	No. of Bee	NSC	Axons
Summer	17	2 GHc 19/VIII	++++	++++
		3 GHc 19/VIII	+++	++++
		9 GHc 19/VIII	+++	++++
"	19	2 GHc 21/VIII	++++	++++
		6 GHc 21/VIII		
		7 GHc 23/VIII		
"	25	1 GHc 27/VIII	++++	++++
		2 GHc 27/VIII	++	+++
		3 GHc 27/VIII	++	
		4 GHc 27/VIII	++	++++
		5 GHc 27/VIII	++++	++++
		6 GHc		
		7 GHc		
		8 GHc 27/VIII	+++	++++
		9 GHc 27/VIII	++++	
		10 GHc 27/VIII	++	++
Winter (Oct)	Unknown	3 BL 18/X	+	+
		4 BL "	+	+
		5 BL "	+	-
		6 BL "	+	-
		7 BL "	+	+
		8 BL "	+	+
		9 BL 19/X	++	+
		15 BL "	++	++
		17 BL "	++	+
		20 BL 24/X	+	-
" (Nov)	Unknown	60 BL 23/XI	-	+
		61 BL "	-	-
		65 BL "	+	-
		66 BL "	+	-
		67 BL "	++	-
		68 BL "	+	-
		69 BL "	+	-
		77 BL "	+	-

*NSC = neurosecretory cells

Table 8

Amount of neurosecretory material in workers from Nosema-infected colony

Days Old	Worker No.	NSC	Axons	Degree of Nosema Infection
2	2GHr 4/VIII*	+	-	none
	7GHr 4/VIII	-	-	none
	8GHr 4/VIII	+	-	none
	10GHr 4/VIII	+		none
3	3GHr 5/VIII	+	-	none
	4GHr 5/VIII	+	-	none
	5GHr 5/VIII	+	-	none
	6GHr 5/VIII	+	-	none
	7GHr 5/VIII	+	-	none
	8GHr 5/VIII	+	-	none
	9GHr 5/VIII	+	-	none
4	2GHr 6/VIII	+	-	none
	3GHr 6/VIII	+	-	none
	6GHr 6/VIII	++	++	none
	7GHr 6/VIII	+	+	none
	8GHr 6/VIII	+	-	none
	9GHr 6/VIII	++	-	none
5	2GHr 7/VIII	++	-	very light
	4GHr 7/VIII	++	-	none
	9GHr 7/VIII	-	-	very light
6	2GHr 8/VIII	+	-	none
	4GHr 8/VIII	++	+	none
	6GHr 8/VIII	+	-	none
	7GHr 8/VIII	+++	-	none
	8GHr 8/VIII	++	+	none
7	1GHr 9/VIII	++	-	very light
	2GHr 9/VIII	+	+	none
	3GHr 9/VIII	+	-	none
	8GHr 9/VIII	+	-	light
	9GHR 9/VIII	++	+	very light
8	3GHr 10/VIII	+	-	moderate
	5GHr 10/VIII	+	-	light
	7GHr 10/VIII	+	-	light
	8GHr 10/VIII	++	-	light
	9GHr 10/VIII	+++	+	light

Table 8 (Continued)

Days Old	Worker No.	NSC	Axons	Degree of *Nosema* Infection
9	1GHr 11/VIII	+++	++	very light
	2GHr 11/VIII	+++	+	none
	3GHr 11/VIII	+++	+	none
	5GHr 11/VIII	++	-	moderate
	7GHr 11/VIII	+	-	heavy
	8GHr 11/VIII	+	+	heavy
10	1GHr 12/VIII	++++	++	none
	4GHr 12/VIII	+++	++	light
	10GHr 12/VIII	++	-	moderate
11	1GHr 13/VIII	++	-	none
	4GHr 13/VIII	+++	-	none
	5GHr 13/VIII	+	+	light
	6GHr 13/VIII	++	+	light
	7GHr 13/VIII	+++	+	none
	9GHr 13/VIII	++	+	light
12	3GHr 14/VIII	++	-	heavy
	4GHr 14/VIII	++++		none
	5GHr 14/VIII	++	+	none
	8GHr 14/VIII	++++	++	none
	9GHr 14/VIII	+		light
	10GHr 14/VIII	++	-	heavy
13	3GHr 15/VIII	++	-	none
	7GHr 15/VIII	++++	+++	none
	8GHr 15/VIII	++		none
	9GHr 15/VIII	++++		none
	10GHr 15/VIII	++	+++	heavy
14	1GHr 16/VIII	++	+++	heavy
	2GHr 16/VIII	++		none
	4GHr 16/VIII	++++	+++	none
	6GHr 16/VIII	++	+	heavy
	8GHr 16/VIII	++		heavy
	9GHr 16/VIII	-		heavy
15	1GHr 17/VIII	++++	+++	none
	3GHr 17/VIII	++	-	heavy
	4GHr 17/VIII	++++	++++	none
	6GHr 17/VIII	+++	++++	none
	8GHr 17/VIII	-		heavy
	9GHr 17/VIII	+++	+	heavy
	10GHr 17/VIII	+++		heavy

Table 8 (Continued)

Days Old	Worker No.	NSC	Axons	Degree of *Nosema* Infection
17	2GHr 19/VIII	-	+	heavy
	3GHr 19/VIII	+++	+++	heavy
	5GHr 19/VIII	+++		heavy
	6GHr 19/VIII	+++	+++	none
	7GHr 19/VIII	+	+	heavy
	8GHr 19/VIII	++	++	heavy
	10GHr 19/VIII	+++	++	none
19	2GHr 21/VIII		++	heavy
	3GHr 21/VIII	++		none
	5GHr 21/VIII	-	-	heavy
	7GHr 21/VIII	+++		none
	8GHr 21/VIII	+		heavy
	9GHr 21/VIII	+++	+	heavy
22	2GHr 24/VIII	++++	+++	heavy
	3GHr 24/VIII	++++	+++	heavy
	4GHr 24/VIII	++	-	heavy
	5GHr 24/VIII	-		heavy
	7GHr 24/VIII	+	+++	heavy
	8GHr 24/VIII	+++	+	heavy
	10GHr 24/VIII	++++		light
25	3GHr 27/VIII	+++	+++	heavy
	4GHr 27/VIII	-	-	heavy
	6GHr 27/VIII	+++	++	heavy
	8GHr 27/VIII	+++	+++	heavy
	9GHr 27/VIII	-		heavy
	21GHr 27/VIII	-		heavy

*All in 1967.

Table 9

Amount of neurosecretory material in queens

Queen No.	Date Killed	MNC	Axons	CC	Egg Laying Level
Q-E-1	4/IV/67	+++	+++	++	package
Q-C-2	4/IV/67	+++	++	++	package
Q-E-3	6/IV/67	++	+++	+++	package
Q-C-4	6/IV/67	+++	+++	+++	package
Q-E-5	6/IV/67	+++	+++	+++	package
Q-C-6	6/IV/67	+	++	+++	package
Q-E-7	7/IV/67	++	++	+++	package
Q-C-8	7/IV/67	+++	+++	++++	package
Q-E-9	7/IV/67	+++	++	+++	package
Q-C-10	7/IV/67	++	+	++++	package
Q-E-11	10/IV/67	++++	++++	++++	package
Q-C-12	10/IV/67	+++	++	+++	package
260	26/VI/67	++	+++	+++	moderate to high
261	26/VI/67	++	+	+++	moderate to high
262	26/VI/67	+++	+++	+++	moderate to high
TR	7/VII/67	++	+	++	
460	11/VII/67	+++	++++	+++	moderate to high
468	11/VII/67	+++	+++	++	moderate to high
BL11S	27/VII/67	++	+++	+++	moderate
1206	2/VIII/67	++	+++	++	moderate to high
501-2686	2/VIII/67	+++	++	+++	moderate to high
BL16S	4/VIII/67			+	not laying
2636	25/VIII/67			+	not laying
602-2688	28/VIII/67	+	-	+++	not laying
BL23W		++	++	+++	low
BL17S		+	-	+++	low
BL5W		+	-	+++	low
M-23	10/IX/67	+	-	+	not laying
707-2598	10/IX/67	++	++	+++	moderate
A-1	23/IX/67	++	++	+++	moderate
A-2	23/IX/67	+	++	+++	moderate to high
A-10	30/IX/67	+	+	+++	low
A-5	30/IX/67	+	-	+++	low
A-8	30/IX/67	+	-	+++	low

Table 9 (Continued)

Queen No.	Date Killed	MNC	Axons	CC	Egg Laying Level
A-6	30/IX/67	+	-	+++	low
W-2	2/X/67	+	-		low
B-4	3/X/67	+	-	+++	low
SR28	4/X/67	++	+	++	low
W-8	5/X/67	++	+	+++	moderate
B-8	9/X/67	++	+		low
B156	9/X/67	++	+	+++	low
W-17	10/X/67	+	-	++	low
701	11/X/67	+	-	+++	not laying
W-22	11/X/67	++	+	+++	low
W-20	11/X/67	+	-	++	low
SR1	12/X/67	+		++++	moderate
SR8	12/X/67	+	+	+++	low
M-2	13/X/67	++	-		low
803	16/X/67	++	+	+++	low
SR26	17/X/67	+	-	+++	low
H-7	20/X/67	+	-	+++	not laying
H-9	20/X/67	++	+	++	low
A-20	21/X/67	+	++	+++	low
A-17	21/X/67	++	+	+++	not laying
1	29/XI/66				
2	29/XI/66	++	+		
3	29/XI/66	+	-		
4	29/XI/66	-	-		
5	29/XI/66	-	-		
67-S65	13/XII/67	+	-		not laying
67-S55	13/XII/67	++	+		not laying
67-Q-2	4/XII/67	+	-	++	not laying
Steve	18/XII/67	+	-	+++	not laying
BL395	19/XII/67	+	-		not laying
DS1	18/XII/67	+++	++	++++	low
Q-7	19/XII/67	++	++	+++	low

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