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EUROPEAN FOULBROOD: ASSOCIATION WITH MICHIGAN
BLUEBERRY POLLINATION, AND CONTROL

By

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ABSTRACT

EUROPEAN FOULBROOD: ASSOCIATION WITH MICHIGAN BLUEBERRY POLLINATION, AND CONTROL

By

Gordon Ira Wardell

European foulbrood (EFB) is a bacterial disease of the larval honey bee, and is endemic to most beekeeping areas. The seasonal incidence of the disease varies, with no climatological or biological stress consistent with outbreaks of the disease. A high incidence of EFB has been observed in Michigan when colonies were taken to pollinate blueberries (Vaccinium corymbosum L.). This research studied the mechanism by which blueberries seemed to initiate the disease.

The successful cultivation of large quantities of the EFB causal organism was accomplished through culturing on anaerobic Brain Heart Infusion agar and broth, supplemented with 0.5% glucose, 0.5% yeast extract, and 0.05% cysteine HCl. A means to efficiently inoculate the organism into healthy larvae and induce high rates of EFB mortality was discovered. It was also found that larvae which were fed blueberry pollen or a near neutral (pH 6.5) diet were more

susceptible to EFB. Wide variations were found in the pH's of different pollens, with blueberry being the highest (6.4) and alfalfa being the lowest (4.4). Control of EFB was attained by the feeding of low-pH (4.4) lactic acid-acidified soy-supplement diet during blueberry pollination. The use of terramycin extender patties, either alone or in conjunction with the soy-supplement patties also provided complete protection from EFB during blueberry pollination.

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INTRODUCTION

Thousands of honey bee colonies (*Apis mellifera* L.) in Michigan are moved annually from their usual locations from fields and orchards where they are leased for pollination services. Though a grower may cultivate, fertilize, and prune his orchard or field crop, his crop may fail without adequate pollination. The pollinators, primarily honey bees, are required to transport pollen from the stamens to the pistil of the flower to assure fruit set. To adequately perform these services, the honey bee colonies should contain large, healthy populations of bees. It is during the pollination period that many colonies develop the debilitating, if not fatal, European foulbrood disease (EFB). EFB is a bacterial pathogen of the larval honey bee which weakens colonies and renders them less effective pollinators and poorer honey producers. Many beekeepers in the state of Michigan have reported, through personal communications, 50% honey production losses from colonies moved to pollinate blueberries (*Vaccinium corymbosum* L.). Several peculiar facts and stories have surrounded the occurrence of EFB. For instance:

1. EFB does not seem to follow pollination of any crop other than blueberries grown in the state, such as apples or cherries.

2. Terramycin has not always been effective in controlling EFB; but growers can almost always rely on a good honey flow to break the disease cycle.

3. Several blueberry growers in Michigan maintain their own pollinating bees; they are kept next to blueberry fields year round, but have no trouble with EFB.

4. EFB appears to wax and wane throughout the year, with no apparent cause.

5. The problem is not unique to Michigan, nor is it only found in colonies used in blueberry pollination.

Beekeepers in the eastern U.S.A. have reported high incidences of the disease following cranberry pollination as well.

An in-depth study of EFB is crucial not only to the beekeeper, whose livelihood is jeopardized; but to the entire fruit and seed industry as well, which depends on beekeepers to provide strong, healthy colonies for crop pollination.

There exists an intriguing and obviously complex set of interactions working within European foulbrood disease. This study, in an attempt to better understand the initiating factors responsible for heightened susceptibility to EFB during blueberry pollination, focused on the stresses unique to the pollination of blueberries. The one stress which makes this type of pollination unique from others is the nutritional value of the blueberry pollen itself. The effect of other potential stress factors not unique to blueberry pollination have been eliminated or controlled to minimize confusing interactions.

To understand the mechanism by which European foulbrood disease is linked to blueberry pollination, it was necessary to conduct detailed studies in the following areas:

1. Determination of the effect on the adult and brood populations of moving and reorientation in blueberry fields.

2. Estimation of the amount of blueberry pollen collected and consumed by honey bees working in blueberries.

3. Determination of the nutritional quality of blueberry pollen, in regard to both protein and micronutrients.

4. Development of a method to minimize the effect of EFB, without the use of antibiotics.

5. Investigation of the nutritional requirements of the causal organism (*Streptococcus pluton*), and development of more adequate culturing techniques.

6. Research into methods of more effective antibiotic treatment.

Subsequent discussion of the published literature, and of the methods and results of this research should clearly establish the link between EFB and blueberry pollination, and should permit more effective control of the disease through increased understanding of the causal organism.

LITERATURE REVIEW

European foulbrood disease was first described by Chesire and Cheyne (1885), who believed the causal organism to be Bacillus alvei. Howard (1900) reported a brood disease in New York which he named the New York bee disease, or "black brood." It was later found by Moore and White (1903) that the disease reported by Howard was identical to that of the infectious disease described by Chesire and Cheyne.

Many researchers, dissatisfied with earlier work on EFB, began looking further into the etiology of the disease. White (1912) published a list of five organisms associated with EFB, and also demonstrated, through bacteria free filtrates, that a virus was not the cause of the disease. White (1920) further proposed that Bacillus pluton, a lanceolate organism which he had described, was the actual causal organism. He cultivated four of the bacteria identified in the gut of the diseased larvae, but was unable to culture B. pluton. Since none of the four organisms cultivated induced the disease when inoculated into honey bee larvae, they were considered secondary invaders.

Lockhead (1928), however, believed that B. pluton and B. alvei were one and the same, but that B. alvei was

was dissociated from the B. pluton form. Sturtevant (1925) reported that the putrid meat odor and slimy consistency of larvae in the late stages of the disease were due to B. alvei. In 1934, Burnside proposed that B. pluton and Streptococcus apis were the same: and that Bacillus alvei and Bacterium eurydice were also identical.

The single disease theory proposed by White (1912) was refuted by Brochert (1935). Brochert later supported his own theories by reporting that S. apis and B. alvei produced distinct diseases when the larvae was stressed sufficiently to lower resistance and allow bacterial development (Brochert, 1936; 1937).

Tarr (1936) found that through larval starvation he could induce S. apis and B. alvei and induce a distinct disease. Later, Tarr (1937) decided that the four day period of starvation was an unnatural stress, and concluded that S. apis was now pathogenic under normal conditions. Tarr also experimented with secondary invaders, and demonstrated that the appearance, odor, and consistency of infected larvae could be altered by introducing different secondary invaders.

Pleomorphism in B. pluton, as first suggested by Lockhead in 1928 and supported by Burnside (1934), implied that this supposed causal agent transformed into a bacterium identical to B. eurydice in form. Burnside proposed the the peritropic membrane as the locale of the morphal change within the larval gut.

Currently, Bailey (1956) is credited with changing the name of *Bacillus pluton* to *Streptococcus pluton*; however, Burri (1942) had noted earlier that *B. pluton* should be transferred to the genus *Streptococcus*, due to its morphology and apparent lack of spore formation. Later, Bailey (1956) succeeded in changing the name of the organism on grounds of gram stain and morphology. The Russians, however, still use the name *Bacillus pluton*.

Successful cultivation of *S. pluton* was documented by Aleksandrova (1949), who used a medium of macerated larvae and potato extract agar; no mention was made of additional sugars incorporated into the medium. Presumably, the cultures were incubated aerobically in standard petri dishes. Two forms of isolates were observed: a small, gram-positive-staining rod form was observed on poorly enriched media, while on more highly-enriched media a typical *S. pluton* lanceolate form was reported. The rod-like forms were converted to the lancet-like forms when inoculated into healthy larvae. It was reported that these larvae soon developed the typical symptoms of European foulbrood.

Krasikova (1954) reported the presence of the organisms with EFB in healthy colonies, as well as in diseased colonies. *B. alvei*, *B. pluton*, and *S. apis* were all found in samples taken from colonies in central Russia.

In 1955, Krasikova (1954) reported that larval food inoculated in vitro inhibited B. alvei and S. apis, but not B. pluton. She also found that honey-pollen mixtures were sufficient for growth of B. alvei, S. apis, and B. pluton. She reported (1957) that healthy larvae fed a mixture of syrup and diseased, macerated larvae would develop the disease if fed the mixture anytime before the cell was sealed.

Bailey (1956) successfully cultured S. pluton on a highly enriched medium of glucose, yeast extract, and potassium phosphate. The pH was adjusted to 6.5 and incubation was at $35^{o}C$ anaerobically. Bailey reported that B. eurydice was the first to develop in the larval mid-gut, but S. pluton soon followed 24 to 48 hours later. The small, pearly-white colonies were only 1 mm at 72 hours. When the lancet-shaped S. pluton was subcultured aerobically, the organism soon took on a rod form, but would return to the cocci-lancet shape when returned to the gut.

Several confusing results are reported in Bailey's 1956 work. He found that the aerobic cultures were catalase-positive while the anaerobic cultures were catalase-negative. Generally, whether a plemorphic organism is in the aerobic or anaerobic life phase, the catalase reaction will remain consistent. If another instance, Bailey reported that when the organism was inoculated into a screw cap vial, the aerobic organism took over and developed until the oxygen was consumed; then the anaerobic form (S. pluton) predominated.

Bailey then had difficulty in recovering aerobic forms following the first aerobic transfer. This, along with the conflicting results in the catalase reaction, may indicate that there were two organisms present.

Bailey (1957a) found a medium that would support S. pluton growth consistently. The medium contained: 10g glucose, 10g yeast extract, 13.6g of KH_2PO_4, and agar, in 1000 ml of water. It was adjusted to pH 6.5 and maintained at 35°C for optimal growth. This is a very highly-enriched medium, however, being an undefined medium provided no information as to the nutritional requirements of S. pluton. Nor does it allow experimenters to delete components of the medium so as to determine the minimal nutrititonal requirements of the bacteria.

In fermentation tests, Bailey (1957b) substituted fructose, sucrose, galactose, lactose, maltose, raffinose, rhamnose, manitol, sorbitol, isositol, zylose, glycerol, and starch for glucose in the medium. Only glucose and fructose in the formula would adequately support growth.

Bailey (1957c) also reported that B. eurydice might be an integral part of the disease cycle of European foulbrood. His contention was that B. eurydice was present in large populations in the anterior end of the adult bee's alimentary tract, and was passed to the larvae during feeding by the adult nurse bees. Only through predisposition by B. eurydice, Bailey stated, would S. pluton develop sufficiently to give visible signs of disease. By his work, we might assume that EFB is a combined infection. However, Vaughn (1958) reported that larval honey bees that have been killed by other bacteria, e.g. S. apis and B. alvei both can exhibit EFB-like symptoms, if environmental stresses were correct. Reports from the Puget Sound area have indicated a major outbreak of the B. alvei form of EFB (Thurber & Pennell, 1974). These more recent forms of the disease have renewed the fires of controversy over the etiology of EFB, which appears to have been used as a collective term for a disease syndrome which may have many causes.

Bailey (1960) suggested that S. pluton slowed the growth of the larvae, and that it was only by having a high nurse bee-to-brood ratio that the infected larvae were able to survive. He viewed the infection as a starvation situation, in that the bacteria and larvae competed for the food. When the nurse bee population was great enough, the larvae received sufficient food during the larval stage; and once pupated, they could void the bacteria from the gut. The bacteria would then coat the inside of the cell and serve as a further source of infection. In this same manner, the inoculum would build in the colony with little or no sign of disease until a nectar flow began and oviposition increased, lowering the nurse bee to brood ratio. Also, when the nectar flow began, B. euridyce populations would increase on the adult bees' mouthparts and would weaken the larvae, along with S. pluton, to the point of mortality.

A second hypothesis on the cyclical nature of EFB involves antibiotic qualities of specific pollens. Individual pollens have been shown to limit growth of specific bacteria. Chauvin (1957) reported that when mice were fed diets of clover pollen and water, they produced sterile feces. An antibiotic substance was isolated in both ether and hot water pollen extracts, and was active against *Escherichia coli*, *Proteus*, and *Salmonella*, but was not active against *S. aurens*, *S. albrus*, or *B. alvei*. He also found that not all pollens contained this antibiotic; poppy (*Papaver rhoeas*); rape (*Brassica napus*); and *Prunus* spp. were without antibiotic activity. He postulated that the variable pathogenicity of EFB might be due to the antibiotic influence of these different pollens.

Another group of French authors (Rousseau, Barbier, and Uslen, 1957) studied EFB over a period of 10 years and concluded that EFB was influenced by "seasonal, regional, and climatic conditions, and in particular the assimilation of pollen by the bees." They found that by feeding pollen collected by other bees to an EFB-diseased colony, a complete remission of EFB symptomology could be achieved. Like Chauvin, the authors found wide variability in antibiotic between pollen species, and surprisingly, within species of pollen taken from different sites. In a study on another bacterial disease, American foulbrood (AFB), Rinderer, Rothenbuhler, and Gochnauer (1974) found that feeding pollen to larvae inoculated with AFB reduced mortality. The active agents responsible for this and other observed antibiotic activities have yet to be identified.

Vaughn (1962) proposed changing S. pluton and B. eurydice to the genus Corynebacteria on grounds of variable morphology, staining, and oxygen tolerance. He found the two bacteria were closely related, as detected through serological tests. His experiments utilized several of Bailey's cultures from England, which were shown to be serologically related, as were samples Vaughn had collected in Wisconsin. Vaughn agreed with Bailey and B. euridyce was less pathogenic acted as a supplemental pathogen in the EFB disease cycle.

Serological tests conducted on S. pluton samples collected from various parts of the world indicated they were very similar in agglutination; and culturally, the different strains were very similar in colony formation, bacterial morphology, and media preference (Bailey and Gibbs, 1962). The same culture medium was used as in the earlier study, with the exception of a slightly higher K:Na ratio, which was stated as essential (Bailey, 1957b).

In 1969, a group of French researchers reported that they took samples of freshly killed larvae from a colony infected with EFB, and found that when abacterial extracts were inoculated into healthy larvae, a pathogenic effect was observed (Guiffret, Vago, and Rousseau, 1969). When the inoculum was heated to 60°C the pathogenic effect declined, as it did after treatment with sacbrood anti-serum. The team also found that EFB symptoms could develop in the absence of S. pluton. By inoculating larvae with S. apis in association with sacbrood virus, EFB-like symptoms and mortality was obtained. Bailey however (1975), refuted this work, saying that the larvae died of sacbrood and that the bacteria was merely saprophytic and did not play a role in larval mortality.

Several authors (Bailey, 1968; Krasikova, 1954; Poltev and Aleksandrova, 1953) have speculated as to the means of transmission of EFB; most identify contaminated equipment and hive products, and drifting bees as chief dispersal agents. Krasikova (1954) reported finding S. pluton to be most abundant in bee bread (stored pollen), and speculated that drifting drones could be a major source of disease transmission.

Transmission of pathogens via honey bees through the floral source has been recognized in several plant disorders; but no reliable results exist concerning the transmission of honey bee pathogens through the floral source.

Very little information is available concerning the association of European foulbrood and the pollination of specific crops. Lehnert and Shimanuki (1980) acknowledged the correlation between the pollination of blueberries and cranberries and EFB, but they were unable to shed light on the cause of the association. Their discussion included antibiotic treatments and ethylene oxide fumigation as control measures. Marucci (1967) reported that many beekeepers in New Jersey were becoming reluctant to use bees in blueberry and cranberry pollination, because of the high incidence of EFB.

Subsequent to reviewing the body of literature on EFB, numerous points of controversy and areas requiring in-depth study became evident. These included the following:

1. The contribution of S. pluton to the disease cycle of EFB.
2. Development of a reliable medium for the culturing of S. pluton.
3. Understanding the roles of physiological and environmental stresses in the initiation of the disease.
4. The role of pollen in the larval diet and its relationship to the disease cycle.
5. The means of transmission of EFB.
6. The association of EFB to the pollination of specific crops.

Identification of these points established a focus for clarification of the nature of European foulbrood disease, in the context of its role in the pollination of Michigan blueberries by honey bees.

MATERIALS AND METHODS

Medium Selection

Several cultural media for _S. pluton_ reported in the literature were tested, as were a number not previously tried. The media examined included: Bailey's enriched medium (Bailey & Gibbs, 1962); potato dextrose agar (Kras-ikova, 1955); "bouillion agar" (Burnside, 1934); nutrient agar supplemented with 0.5% glucose and 0.1 starch; enriched brain heart infusion (BHI) medium with 0.5% glucose, 0.5% yeast extract and 0.05% cysteine.HCl; tomato juice agar; and blood agar. With the exception of the latter two, all of the media were formulated both aerobically and anaerob-ically.

Disease Inoculum

The source of diseased material for cultural and isola-tion purposes was taken annually from naturally occurring outbreaks of EFB in colonies which had been employed in blueberry pollination. Larval samples were either taken back to the laboratory for immediate analysis, or were frozen on dry-ice and maintained frozen until examined.

Preparation of Infected Material for Isolation

Bacterial isolations were made from combs displaying larvae with typical EFB symptoms. The larvae selected for the study were 4-5 days old, either healthy in appearance or in the first stages of the disease. Early stages of the disease were characterized by larvae of flat white color, less plump than normal, and generally out of position in the cell. In contrast, in later stages of the disease, the larvae become twisted in the cell and take on a creamy to brown color. The tracheae of these larvae appear white, making them a conspicuous feature of the brown larvae. Generally, the diseased larvae had died before the cell was sealed, and hence, were very noticeable. Dead larvae were grey to brown in color and often dried into a difficult-to-remove scale.

Specimen larvae were placed on a sterile wax dissecting dish and a ventral-longitudinal incision was made to expose the midgut. The cuticle was pinned to the wax and the midgut was isolated and rinsed three times in a sterile saline solution. Two intact midguts along with 20 ml of anaerobic brain heart infusion broth, supplemented with gluecose (BHI-G), were placed in a sterile tissue grinder and homogenized under a nitrogen atmosphere. The resulting bacterial suspension was then serially diluted in 10-fold increments, and 0.1 ml portions of the latter dilution were spread directly onto plates. All transfers for anaerobic culturing were done either in a 90% nitrogen, 10% hydrogen atmosphere glove box (Coy Mfg. Co., Ann Arbor, MI), or using the anaerobic technique per Hungate (1950).

Aerobic culture plates were sealed with parafilm and incubated at 35°C. Anaerobic plates were sealed in a GasPack jar (BBL, Cockeysville, MD) containing an activated GasPack H_2 and CO_2 generator envelope, and were then incubated at 35°C. A small amount of CO_2 gas was introduced into broth cultures through the gassing cannula at approximately 10% V/V with nitrogen. The cultures were checked every 24 hours for growth.

Isolation of Bacterial Cultures

Individual bacterial colonies were obtained from serial dilutions of the larval mid-gut contents. Streaks of these discrete bacterial colonies were used to begin stock cultures, which were subsequently maintained through periodic subcultures. Stock cultures used for inoculation were never older than the fifth subculture.

Isolates were standardized for inoculation by centrifuging broth cultures to obtain the bacterial pellet. The pellet was washed and resuspended in 2-5 ml of sterile water. One microliter of the suspension was serially diluted and plated to quantify the titre. Each inoculum vial was identified by the number of colony forming units (CFU's per microliter. Inoculations were carried out with an automatic micropipette. Control larvae were inoculated with sterile distilled water.

Analysis of Fermentation Products

Cells of S. pluton were grown under a H_2 and CO_2 (90%/10%/Vol/Vol) atmosphere in the earlier-mentioned BHI-G broth. At the end of fermentation the medium was clarified (Neish, 1952) and neutral either extracts of the clarified fermentation liquor (CLF) were assayed for organic acids by gas chromatography. A varian model 1420 gas chromatograph, equipped with a thermal conductivity detector, was used. The column was stainless steel (0.32 X 182.9cm) and contained 15% Supelco's SP-1220 and 1% H_3PO_4. The carrier gas was helium (25 ml/min) and constant temperatures were: column, 135°C; injector and detector, 165°C. A standard solution containing 20μg equivalents each of formate, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate was prepared; and a standard extract of unfermented BHI-G was used for comparison of existing acids in the medium. Non-volatile acids were first methylated and extracted into $CHCl_3$, and then injected into the same column used for the volatile acids (Holdeman and Moore, 1972).

Moving Experiments

The phenology of the blueberry bloom was mapped, and a day-by-day plan was coordinated to follow the blueberry bloom from the earliest to the latest blooming areas in the state. The experiment involved moving 30 to 40 disease-free honey bee colonies into two distinct areas. The first treatment was conducted in a commercial blueberry fields exhibiting 10% bloom. The second treatment was conducted in an area physiographically similar to the first, except no blueberries were present within 3.6 kilometers. Except for controls, all colonies were subjected to two hours of moving, twice weekly. The translocated bees were either returned to their original site and orientation, or were returned to a new blueberry site. Colonies which were moved and returned to their original site experienced the stress of moving, but not that of reorientation; while colonies moved and relocated at a new site experienced the stress of both moving and reorientation. Pollen was periodically collected from these colonies, as well as other stationary control colonies established at these blueberry sites. Cooperating beekeepers also permitted the outfitting of a number of their colonies with pollen traps. Data consisted of colony population estimates of sealed brood, corbicular pollen samples, five day-old larval samples, and observations on the general state of the colonies.

A. Pollen Analysis

Five-gram sub-samples of the pollen collected during the course of the investigation were sorted as to color and the pollen identified as to species (Olsen, 1975). Percentages of the respective pollens were recorded, and a one-gram sample of each pollen species was sent to J. Benton Jones of the University of Georgia for micronutrient analysis. The samples were analyzed for parts per million of 19 elemental micronutrients through flame photometric technique.

B. Population Estimation

Monitoring of colony population over an extended period often provides insight into the effect of different treatments on the colony. Puchta (1949) described a method to accurately estimate the sealed brood population of a colony. Brood patterns generally assume an elipsoid shape which makes it possible to apply the formula for calculation of the area of the elipse. The long axis (A) and short axis (a) of the brood pattern are measured and applied to Puchta's modified formula for the area of an eliptical brood pattern: Area = $4(\frac{A}{2})\ (\frac{a}{2})$. Puchta claims that using the number 4 in the formula, instead of π takes into account the irregularities of the brood pattern. Once the sealed brood area of each frame of the hive is calculated, a sum is determined and recorded for later comparison. This method was employed in determining sealed brood populations in all colonies utilized for the moving experiments.

C. Larval Samples

Random 4.5 to 5.0 day-old larval samples were removed from their cells, placed in marked vials, quick-frozen on dry ice, and returned to the laboratory for analysis. The larval mid-gut was checked for S. pluton presence in the experiments; later, the number of colony forming units was quantified through serial dilution on BHI agar. The pH of the mid-gut contents was ascertained by spilling each onto a shallow watchglass and testing with narrow range pH paper.

D. State of the Colony

Checking the state of the colony involved reporting the oviposition rate of the queen, field examination for diseases, and any noticeable changes in the bees.

Bacterial Transmission through Floral Source

Two eight-meter cubic flight cages were erected over blueberry bushes in bloom. The cages were fitted with one small hive of honey bees infected with EFB. All such colonies used in the experiment contained between 5000 and 8000 honey bees. The diseased colony was allowed to forage in the cage for two days. After the bees had returned to the hive on the second evening, the contaminated bees were removed from the cage and replaced by a healthy, unexposed colony. The healthy colony was allowed to forage for two days, after which it was moved to a second cage location also where diseased bees had been foraging. Halfway through the blueberry season, the healthy colony was replaced with a second healthy colony. After being moved, five larvae were sampled from each hive and either examined immediately or frozen for later analysis. By monitoring the larval mid-gut flora of the experimental hive, it was possible to quickly determine whether transmission had occurred. The test for EFB was done by anaerobic plating of the larval mid-gut contents on BHI agar. Five flowers from each cage were also sampled, frozen, and returned to the laboratory for bacterial analysis. To test the bacteria present in the nectaries of the flowers, all flowers were first washed with 5.0 ml sterile distilled water. The suspension was then serially diluted and anaerobically plated on supplemented BHI agar. These experiments were repeated on alfalfa (*Medigago sativa*) and goldenrod (*Solidago* sp.), during their respective seasons. The entire procedure was conducted for two consecutive years.

In the third year of research, the experimental method was changed from a cage experiment to an isolated field procedure. Honey bees in a cage situation are at a greater density than would be found in nature. To eliminate the possible bias of oversaturation in the cage, an isolated 0.405 hectare blueberry field was used as an experimental site. The field was 1.6 km from the nearest honey bee colony and outside of flight distance from the nearest blueberry field. Observations were made in the same manner as the first portion of the experiment. Two hives of diseased bees were used for inoculation and four uncontaminated hives as experimentals; four controls were maintained at the University apiary, approximately 1.6 km away.

Pristine Colony Establishment

According to records, until 1978 the University apiary had never experienced EFB. After the first year of this research, many colonies had been exposed to the disease. EFB research in the laboratory and in the greenhouse demanded larvae having had no previous exposure to the disease. This, then, required the establishment of pristine colonies. The bees for such colonies came from 3-pound packages of bees obtained from inspected disease-free colonies. Hives used were built of all new wood and foundation was, and located at the University apiary. To reduce drifting and robbing, the hives were outfitted with robbing screens at the entrance. These colonies were later used for pollen trapping, and larval sampling for mid-gut bacterial populations and mid-gut lumen pH.

Greenhouse Experiments

The greenhouse was utilized primarily for two types of experiments: 1) controlled dietary investigations; and 2) inoculation trials with cultivated S. pluton.

Nearly identical nucleus hives (nucs) were brought into the greenhouse in the late fall. The bees were allowed two weeks to adjust to the new surroundings, and adults were shaken from one nuc to another to equalize populations. All nucs used had no contact with either blueberries or EFB.

A. Controlled Diet Investigations

1. Pollen Diet

Due to limited greenhouse space and a limited number of available colonies, the experiment was designed to incorporate a switch-back routine originally used in cattle feeding experiments by Lucas (1956). This design seemed ideal for use with bees, since it would use the same hive over and over again and would therefore reduce interhive variance. The procedure was as follows:

Queenright nucs, identical in strength, were fed blueberry pollen for two weeks, and were then switched to a standardized 1:1 mix of clover and alfalfa pollen. After two weeks, the nuc was switched back to blueberry pollen, and so on, for ten weeks--or five complete switches. This procedure was conducted with four nucs: two on a blueberry pollen diet, and two on the clover-alfalfa mixture. The pollen was fed by pouring sorted corbicular pollen into the edges of the brood combs and lighting spraying it with a 1:1 sugar:water syrup solution. Larval samples were taken twice weekly from the four-day-old larval population. The larval samples were either taken to the laboratory for immediate analysis or were quick-frozen to examine later. In the laboratory, the mid-gut pH was tested and recorded. The bacterial populations were quantified through serial dilution. Initially, the pH of the mid-gut was tested by spilling the lumen contents into a shallow watch glass, where narrow-range pH litmus paper and indicator dyes were used to determine pH. Later, mid-gut pH was checked with a micro-pH-probe (M1-401, Microelectrodes, Inc., Londonderry, NH), having the element tip mounted in a 12-gauge needle, and attached to a Sargent (model #12) pH meter. This ultra-small probe provided a means to check the gut lumen pH without tissue interference, and with a higher degree of reproducibility.

2. Soy-supplement diet

The second set of dietary experiments involved feeding bees with pH-adjusted soy flour supplement cakes. The procedure was identical to the first experiment, except that alfalfa and clover pollens were replaced with soy flour supplement cakes adjusted to pH 4.4; and blueberry pollen was replaced by pH 6.5 soy supplement. Lactic acid was chosen to lower the pH, as it is the constituent responsible for lowering the pH of bee bread (stored pollen) (Pain and Maugenet, 1966). The soy supplement was formulated according to Hydak's (1945) proportions of soy flour, brewer's yeast, sugar, and water. Ten ml of lactic acid was added to each

454 g of diet, resulting in a 4.4 pH. As a check, soy-supplement pH was tested in the same manner as corbicular pollen; that is, one gram of supplement was suspended in 10 ml of distilled water and subjected to pH analysis using a Sargent pH meter (Pain and Maugenet, 1966).

B. Inoculation Trials

Nucleus colonies used in this experiment had never been exposed to European foulbrood, and had not received antibiotic treatments to control either S. pluton or normal gut microbes. The bees were allowed to self-feed on sugar syrup and pans of dried and sifted blueberry pollen. To further increase the stress on the larval population, the nucs underwent high diurnal temperature variation (night 10^{o}C, day 25^{o}C), a low nurse bee to brood ratio (by adding unsealed brood), and high adult mortality due to greenhouse flight conditions.

Frames of brood containing fifty or more two-day-old larvae were chosen for inoculation. The inoculated larvae were deliniated from the brood pattern with fine wire stretched over the frame. All adult bees were removed from the frames to be inoculated to prevent the removal of inoculated brood food. Between 50 and 100 larvae were inoculated from each of the 7 nucs treated. The inoculated larvae received approximately 10^4 viable cells of S. pluton bacteria in 0.5 μl of suspension. An equal number of larvae were inoculated with distilled water to act as controls. Where possible control larvae and treated larvae were located on opposite sides of the same frame; when this was not possible, two frames of larvae were used, with treated and control larvae positioned to face each other. The frames of inoculated larvae were then placed in a 35°C incubator for 6 hours to allow the larvae to consume the inoculum. The larvae were returned to their original hives and their condition was checked every twenty-four hours. Inspection continued until the larvae began pupation, or showed obvious signs of the disease.

Supplement and Drug Feeding During Blueberry Pollination

In an attempt to lower the mid-gut pH of the larval honey bee during blueberry pollination, Hydak's supplemental cakes were adjusted to pH 4.4 with lactic acid. In the first treatment, patties of the material (each weighing 454 g) were placed between the brood chambers of ten commercial pollination units at different field locations. A continuous supply of the supplement was made available to these colonies during pollination, and for two weeks after being moved from the blueberry fields to their home apiaries.

The second treatment involved prophylactic drug treatment with terramycin extender patties. The patty is a mixture of terramycin, powdered sugar, and vegetable shortening in the following proportions:

Mix: 112 g sifted powdered sugar
10 g animal formula terramycin (TM-25)
Blend in: 112 g shortening

The final mixture was divided into 220 g portions and pressed onto waxed paper for ease of handling. These extender patties were then placed between the brood chambers of ten commercial colonies, and replaced when depleted.

A third treatment incorporated both the extender patty and the pH-adjusted soy-supplement cake into a single colony. Ten commercial pollination colonies were treated in this manner during blueberry pollination. Methods and quantities of application were identical to the earlier treatments. Ten control colonies, which received no antibiotics or supplement, were designated and located beside the treated colonies.

Five 5-day old larval samples from all three treatments were collected twice weekly. The samples were immediately frozen and returned to the laboratory for analysis. The midgut pH was tested and the contents examined microscopically. In all three experiments, colonies used were donated by commercial beekeepers who had moved them to blueberry fields for pollination. Treatments began as soon as these bees arrived in the blueberry fields, and were continued for 2 weeks after their return from blueberry pollination.

Soy Supplement Acidification Preference Test

In an attempt to determine whether different acids used to lower the pH of soy supplement could affect the rate of its consumption by honey bees, 3 kg of Hydak's supplement formula was divided into five equal portions. Each portion received either hydrochloric, succinic, tartaric, or lactic acid in sufficient quantities to lower the pH of each formulation to 4.4. The fifth portion of formula was used as a control and remained at its original pH of approximately 6.5. From each of the five formulations, individual 100 g patties were weighed, and placed on labelled waxed paper indicating the type of acid present in the patty. On June 6, 1981, one patty of each formula was placed on the top bars of selected colonies from the University apiary in East Lansing. The patties were laid in a circular formation, around the edges of the brood nest. After both 24 and 48 hours, each patty was scraped from the top bars of the colony, weighed, and returned to the same position. It was hoped that from these recorded measurements, preference for a particular formulation would be seen, as evidenced by more-rapid consumption of one patty or another.

RESULTS

Medium Selection

The best growth of *S. pluton* was obtained on Brain Heart Infusion agar and broth (Difco) prepared anaerobically, and supplemented with 0.3% glucose, 0.5% yeast extract, and 0.5% cysteine hydrochloride. Optimal growth was found to occur at 35^{o}C, pH 6.5-7.0, and in approximately 10% CO_2 atmosphere. Coupling this medium with the strict anaerobic sample transferal and serial dilution technique of Hungate (1950), luxuriant growth of *S. pluton* was consistently obtained.

Very slow growth of S. pluton was observed on Bailey's enriched medium and on potato dextrose agar, both prepared anaerobically. Colonies on Bailey's medium generally required 5 to 7 days to become 0.5mm in diameter. No anaerobic or aerobic growth of *S. pluton* was observed on Burnside 's bouillion agar, nutrient agar, tomato juice agar, or blood agar.

Isolate Characteristics

The isolates of *S. pluton* grown on supplemented BHI agar formed convex, translucent colonies 1.5 to 2.0 mm in diameter, and were greyish-white in color, with even margins.

Generally, the colonies were at their maximum size after 72 hours. Young cultures were gram-positive, but turned gram-variable when 4 to 5 days old. The cells were lanceolate or oval in shape, 0.5 X 1.5 μ in size. When smears were made, the cells were usually arranged in pairs, chains, or mats of large numbers. Cells were also found to be non-motile.

Early studies on the fermentation of various carbohydrates added to Bailey's medium found only fructose and glucose as suitable energy sources. No growth was observed when the medium was supplemented with sucrose, xylose, inositol, mannitol, sorbitol, maltose, galactose, or raffinose (Table 1). Discoloration of phenol-red indicator dye revealed acid formation with a final pH of between 5.0 and 5.5, with no gas produced. Lactic and acetic acids were produced during the fermentation of glucose, as determined by gas chromatographic analysis.

Moving Experiments

Even though sample sizes from the two site comparisons (bees moved into blueberries, and bees moved outside of blueberries) were different, statistical analysis of the data was accomplished through the use of a fourfold contingency test (Mainland and Murray, 1952). To make the comparison, the larger sample was reduced to the size of the smaller sample (with the original porportions of the larger

Table 1. Biochemical reaction of Michigan strains of S. pluton (M1) and Bailey's standard (B1).

Test or Substrate	Reaction of Strain[a] M1	B1
Catalase reaction	-	-
Litmus Milk	-	-
Red Blood Cell Haemolysis	-	-
BHI-G Medium at pH 5.0[b]	-	-
BHI-G Medium at pH 5.5	-	-
BHI-G Medium at pH 6.0	±	±
BHI-G Medium at pH 6.5	+	+
BHI-G Medium at pH 7.0	+	±
BHI-G Medium at pH 7.5	-	-
Bailey's Medium with Glucose	+	+
Bailey's Medium with Fructose	+	+
Bailey's Medium with Sucrose[c]	-	-
Bailey's Medium with Xylose[c]	-	-
Bailey's Medium with Inositol	-	-
Bailey's Medium with Mannitol	-	-
Bailey's Medium with Sorbitol	-	-
Bailey's Medium with Maltose	-	-
Bailey's Medium with Galactose	-	-
Bailey's Medium with Raffinose	-	-

[a]Reaction + indicates a positive reaction or growth; ± reaction indicates a week positive reaction; and - indicates a negative reaction.

[b]BHI-G indicates Brain Heart Infusion medium supplemented with glucose.

[c]Sugar which was sterile filtered and later added to autoclaved medium.

Table 2. Results of the moving experiment, in which some bees were moved into blueberry fields, and others into a similar area without blueberries.

Bees Moved into Blueberries

	Healthy	EFB	Chalk	Sac	♀-less	TOTAL
Stationary	1	5	0	0	2	8
Moved & Re-oriented	1	6	1	0	0	8
Moved only	2	6	0	0	0	8
TOTAL:	4	17	1	0	2	24

Bees Moved Outside of Blueberries

	Healthy	EFB	Chalk	Sac	♀	TOTAL
Stationary	3	0	1	0	0	4
Moved & Re-oriented	1	0	2	0	1	4
Moved only	1	0	1	1	1	4
TOTAL:	5	0	4	1	2	12

Note: Tables indicate incidence of European foulbrood (EFB), chalkbrood (Chalk), sacbrood (Sac), and queenlessness (♀-less), in groups of colonies subjected to different stresses, either in blueberry pollination or in similar areas without blueberries.

Table 3: Statistical analysis of moving experiment results.

Adjusted [1] Results of the Moving Experiments

Site	Healthy	EFB	Chalk	Sac	♀-less	TOTAL
In Blueberries	2	8½	½	1	1	12
Outside Blueberries	5	0	4	1	2	12

Minimum ratios required in fourfold contingency table for significance at the 5% level[2]:

n = 12: 0/5, 1/7, 2/8, 3/9, 4/10, 5/11, 6/12

[1]Sample size of bees moved into blueberries reduced to match sample size outside blueberries; all proportions are constant.

[2]Taken from Mainland and Murray, 1952; Table 1.

and the smaller samples unchanged); and the two numbers from corresponding hive condition columns were compared against the contingency table ($P \leq .05$) to determine whether the ratio was significantly different. The value n (number of colonies sampled) was equal to the smaller sample (n=12).

The results of the moving experiments are presented in Table 2; statistical comparisons using a fourfold contingency test can be seen in Table 3. The only colonies which developed EFB symptoms were those treatments moved into the blueberry fields. The bees moved to the non-blueberry area expressed a high incidence (above 10% of the brood affected) of other stress-related disorders; but no EFB was found through either visual observation of the mid-gut contents, or through anaerobic culturing on BHI agar. Using the contingency table, where n=12 (Table 3), a comparison with zero (0) as one data point requires at least 5 from the other set (ratio 0/5) to give significance at the .05 level. EFB data from the moving experiment shows a ratio of 0/8.5, indicating significance. Chalk brood results yield 0.5 as the numerator figure in the ratio; by interpolating between the ratios of 0/5 and 1/7 from the contingency table, the denominator value would have to be 6 or greater in order to give significance. The result 0.5/4 therefore lacks significance. By similar analysis, the ratios of colonies which expressed sacbrood, queenlessness, and healthy conditions also lacked significance.

Through design, it was hoped that this experiment would reveal the effect of moving and re-orientation on the incidence of EFB and other stress-related diseases. However, no clear differences can be seen between treatments within sites. Bees which were stationary within blueberry fields showed no difference in health when compared to either bees which were only moved, or those moved and re-oriented. Similarly, bee hives treated in the same manner outside of blueberries showed no difference between treatments.

Pollen

Pollen trapped from colonies moved into blueberry fields at 10% bloom showed considerable variability in the proportion of blueberry pollen collected (Table 4). Colonies which were moved and re-oriented regularly (MR1, MR2) collected significantly more blueberry pollen than did colonies which were moved but were no re-oriented (M1, M2). The least amount of blueberry pollen was collected by the grower-owned colonies (L1, L2), which were situated beside the blueberry fields during the entire year. When samples of like treatments were pooled (Table 5), statistical separation of means revealed that colonies which were moved and reoriented collected significantly more ($P \leq 0.05$) blueberry pollen than did any other treatment group.

There was no difference in blueberry pollen collection between colonies which were moved in at 10% bloom, and those moved regularly but not re-oriented. Samples taken from the stationary, grower-owned colonies (L1, L2) were found to contain significantly ($P \leq 0.05$) less blueberry pollen than those collected by any other group (Table 5).

Table 4. Statistical separation of mean percentages of blueberry pollination.

Treatment	Code[a]	Mean Percentage[b]	SD[c]
Moved & Re-oriented 1	MR1	70.8	18.8
Moved & Re-oriented 2	MR2	41.4	18.4
Control 4	C4	41.4	10.9
Moved 2	M2	31.2	27.2
Control 2	C2	30.6	18.4
Moved 1	M1	22.4	7.8
Control 1	C1	17.3	12.5
Control 3	C3	10.2	11.5
Laduc 1	L1	3.8	3.4
Laduc 2	L2	2.8	1.9

[a]C1 through C4: moved to blueberry field at 10% bloom.
MR1 through MR2: moved and re-oriented twice weekly.
M1 through M2: moved twice weekly and returned to original orientation.
L1 through 2: stationary in blueberries all year.

[b]Mean percentage of blueberry pollen for five samples. Means connected by solid lines do not differ significantly ($P = .05$).

[c]Standard deviation from individual means.

concentrations five to ten times higher in blueberry, than in other pollens. No elements were found to be significantly lower in blueberry pollen than in other pollen sources tested. When comparing non-blueberry to spring and summer pollens, only phosphorous and potassium were significantly different. Micronutrient comparisons between spring and summer pollens collected at the University apiary showed chromium to be significantly lower in spring samples than in summer samples; this was the only micronutrient difference between the two.

B. Pollen pH

Corbicular pollen pellets were separated by color and were then classified. The pH of individual pollen types varied widely, as can be seen in Table 7. Three samples which showed the greatest deviation from their means were: blueberry (Paw Paw 1); summer mixture (MSU Apiary); and an unsorted mixture from Laduc's blueberry fields, numbers 1 and 2 (Paw Paw). These three had a standard deviation of $\pm$ .444; all other samples had relatively smaller standard deviations, from $\pm$ .055 to $\pm$ .251. Several patterns can be observed in this data: 1) the consistently highest pH was from blueberry samples (pH 6.0 to 6.4); 2) non-blueberry samples collected at the University apiary at the same time were considerably lower (pH 4.6); 3) other fruit pollens and summer pollens also had lower pH's (5.3 to 5.7); 4) alfalfa had the lowest pollen pH observed (4.4); 5) cranberry

Table 5. Statistical separation of means from combined like treatments representing the percentage of blueberry pollen collected.

Site Code	Number	Mean	SD
MR1 - MR 2	10	56.1	23.4
M1 - M2	10	26.8	19.4
C1 - C2	20	24.9	17.6
L1 - L4	10	3.3	2.7

Means connected by a line are not significantly different at the 0.05 level as determined through Duncan's Multiple Range Test.

A. Pollen Micronutrient Concentrations

Four major groups of pollen were analyzed for micronutrient concentrations. Pollen trap collections from blueberry fields were separated into blueberry and non-blueberry fractions, and trap mixtures of spring and summer pollens were tested to determine whether major micronutrient differences existed between blueberry, non-blueberry, and known nutritious pollen sources. Statistical separation of elemental means revealed eight elements which had significantly higher ($P \leq 0.05$) concentrations in blueberry pollen than in non-blueberry pollen collected from the same vicinity (P, Fe, Mn, Al, Si, Co, Sr, Ba) (Table 6). Manganese showed the most striking difference, with

Table 6. Statistical separation[1] of mean micronutrient concentrations of four different pollen groups, reported in parts per million.

Element	Blueberry pollen		Nonblueberry[2] pollen		Spring mixtures		Summer mixtures	
	mean[3]	SD	mean	SD	mean	SD	mean	SD
P	0.43	± 0.04 a	0.26	± 0.16 b	0.39	± 0.10 a	0.46	± 0.17 a
K	0.54	± 0.02ab	0.41	± 0.15 a	0.52	± 0.04ab	0.63	± 0.04 b
Ca	0.19	± 0.04ab	0.15	± 0.03 a	0.17	± 0.06 a	0.14	± 0.01 a
Mg	0.19	± 0.02 a	0.11	± 0.06 a	0.18	± 0.06 a	0.41	± 0.04 b
Fe	124.0	±41.2 a	65.6	±18.9 b	74.50	±27.51 b	94.9	± 5.26 b
Mn	253.0	±15.9 a	59.09	±47.4 b	29.95	± 4.35 b	26.44	± 0.6 b
B	16.33	± 9.5 a	29.68	±24.4 a	22.07	± 4.2 a	13.47	± 7.3 a
Cu	5.45	± 4.1 a	7.25	± 2.3 a	6.5	± 1.6 a	9.6	± 0.42 a
Zn	51.07	± 5.5 a	43.57	±12.5 a	59.16	±11.0 a	58.09	±14.8 a
Na	42.62	± 17.6 a	38.95	±10.9 a	37.42	±10.0 a	56.21	±37.9 a
Al	77.77	± 29.4 a	35.43	±10.7 b	17.74	±14.6 b	26.26	± 2.9 b
Si	79.83	± 25.4 a	47.55	± 5.4 b	41.05	±13.9 b	36.93	± 4.2 b
Co	0.72	± 0.25 a	0.32	± 0.1 b	0.20	± 0.05 b	0.38	± 0.06
Cr	0.52	± 0.10 a	0.46	± 0.13 a	0.28	± 0.13 b	0.49	± 0.03 a
Ni	0.12	± 0.08 a	0.08	± 0.04 a	0.04	± 0.08 a	0.13	± 0.10 a
Pb	5.2	± 1.7 a	4.10	± 1.5 a	4.20	± 0.46 a	5.4	± 0.48 a
Cd	0.23	± 0.04 a	0.33	± 0.14 a	0.08	± 0.08 a	0.18	± 0.05 a
Sr	2.44	± 1.3 a	0.99	± 0.38 b	1.6	± 0.6 b	0.62	± 0.23 b
Ba	12.38	± 7.6 a	3.03	± 1.4 b	2.21	± 1.2 b	0.97	± 0.19 b

[1] Elemental concentrations followed by the same letter are not significantly different at the 0.05 level, as determined through Student's t-test.

[2] Represents pollen found in blueberry field samples after blueberry pollen portion has been removed.

[3] Each mean represents six samples analyzed, and the standard deviation for each mean (SD).

Table 7. Mean pH and standard deviation of 1 gram of corbicular pollen suspended in 10 ml of distilled water.

Pollen type	Site	Mean pH[1]	SD
Blueberry	Paw Paw	6.4	±.444
Blueberry	Allendale	6.2	±.152
Blueberry	Paw Paw	6.2	±.164
Blueberry	Covert	6.1	±.084
Blueberry	Agnew	6.0	±.251
Unsorted Blueberry[2]	Allendale	5.9	±.148
Unsorted Blueberry	Paw Paw	5.7	±.148
Unsorted from L1,2	Paw Paw	5.4	±.444
Cranberry	Wisconsin	6.2	±.152
Spring Mixture	MSU Apiary	4.6	±.055
Summer Mixture	MSU Apiary	5.1	±.444
Fall Mixture	MSU Apiary	4.5	±.192
Dandelion	Paw Paw	4.9	±.114
Dandelion	MSU Apiary	5.7	±.130
Alfalfa	Agnew	4.7	±.110
Alfalfa	MSU Apiary	4.4	±.114
Clover	Covert	5.3	±.130
Clover	MSU Apiary	4.8	±.227
Goldenrod	MSU Apiary	4.5	±.114
Apple	MSU Hort. Farm	5.3	±.114
Cherry	Traverse City	5.5	±.110

[1] $n = 5$

[2] Unsorted blueberry represents a mixture of all pollens coming into the hive during blueberry pollination.

pollen collected from Wisconsin had a pH similar to Michigan blueberry pollen (pH 6.2). Considerable differences were observed in the pH of specific pollens from different sites; for example, dandelion pollen from a blueberry field had a pH of 4.9, while the same pollen from the University apiary had a pH of 5.7. Site differences were observed in all pollens examined.

Colony Population Estimation

Results of the population monitoring experiment on colonies which were moved into blueberry pollination were less satisfactory. Resultant populations, in many cases, were in excess of the colonies' brood rearing capabilities. Correction factors could not be implemented because the degree of error between large and small colonies was not consistent. The inconsistency was related to the number of brood cycles present in the frame. Frames with two and three brood cycles had a large number of open and unsealed cells in the middle of the eliptical brood pattern, which made accurate axis estimation difficult. Small colonies did not have as many holes in the brood patterns, resulting in a lower degree of error and rendering it impossible to develop a correction factor to salvage the data. Due to these reasons, the data was not reported, and no substantial information was gained on the effect of moving and re-orientation, as well as EFB infection, on colony populations.

Bacterial Transmission Through Floral Source

During the first two years of the experiment, all four healthy colonies caged on blueberry bushes upon which EFB-diseased colonies had been foraging, contracted EFB. However, none of the four colonies caged on alfalfa were afflicted with the disease, despite the fact that diseased colonies had recently been foraging at the same site. Goldenrod showed mixed results: the first year, two healthy colonies placed in the cage contracted EFB; but the second year, neither of the colonies showed evidence of it, either through typically-diseased larvae in the colony, or _S. pluton_ bacterial presence in the mid-gut. In the third year of experimentation, four healthy colonies were allowed to forage freely within an isolated blueberry field, which had previously been foraged upon by two EFB-infected colonies. Three of the four healthy colonies contracted the disease. A similar experiment involving both goldenrod and alfalfa was not carried out during the third year, due to inadequate field conditions.

It was felt that no statistically-supported conclusions could be drawn from this set of experiments due to the biased nature of two main elements. First, the caged situations presented the bees with an unnaturally high population density, and thus introduced an uncharacteristic element of stress. Second, the natural variation in the egg-laying rate of the queen during the field season heavily influenced the experiment's results, through the corresponding nurse-bee-to-brood ratio. Additionally, data from the third year's experiments in blueberries was insufficient to allow statistical analysis, due to limitations on the availability of strong, healthy colonies during the short field season.

Attempts to isolate S. pluton from blueberry flowers visited by contaminated bees failed to show any connection between the disease-causing organism and the floral nectaries. One of the major problems in the experiment was a rapid-growing, motile bacterium which would consistently overrun the agar plate before S. pluton colonies could be identified.

Pristine Colony Establishment

The pristine colony established at the MSU apiary was found to have considerable bacterial populations in the larval mid-guts sampled. Four healthy, five-day-old larvae were sampled, and the total number of colony forming units (CFU's) per mid-gut was estimated through the plating of serial dilutions on supplemented BHI agar, incubated anaerobically. The number of viable bacteria per mid-gut fluctuated between 9×10^3 and 2×10^8 CFU's over the summer period (Figure 1). Three peaks were seen in the bacterial populations: 1) late June; 2) mid-August; 3) late September and early October. All of these coincided with dearth periods in the Michigan nectar flow.

The pH of mid-gut contents of the same larvae from the

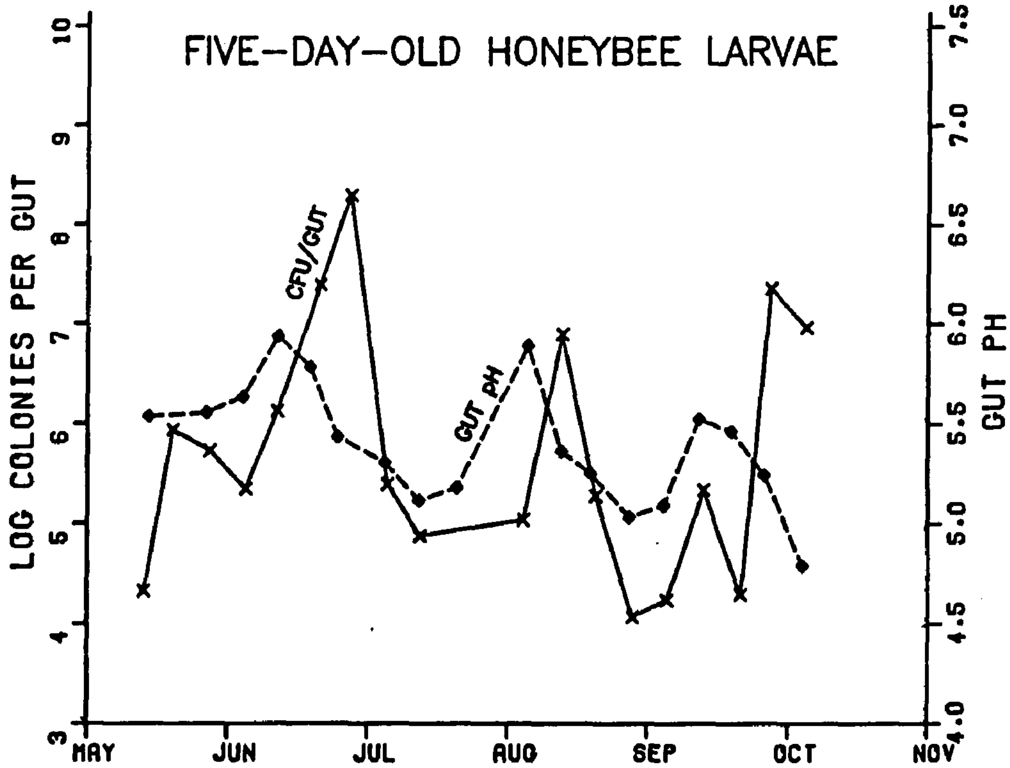

Figure 1. Graph of five-day-old larval gut pH from samples taken from healthy honey bee colonies and the number of bacterial colony forming units per larval gut

pristine colony were found to be highly variable. Each data point (Figure 1) represents the mean of four five-day-old larval mid-guts tested with a pH microprobe. When mid-gut pH was superimposed graphically over CFU's per mid-gut, the result was similar to the peaks and valleys seen in the microfloral populations. However, the CFU's were observed to lag behind the change in gut pH, in a consistent manner, with the lag interval being approximately a two-week period.

Greenhouse Experiments

A. Controlled Diet Investigations

1. Pollen Diet

Larvae from colonies which were fed pure diets of blueberry pollen had mid-gut pH's consistently ranging between 6.0 and 6.5. When the diet was switched to the 1:1 clover-alfalfa mixture, the mid-gut pH dropped to a pH range between 5.3 and 5.7, after a short lag period (Table 8). Bacterial populations in the mid-gut showed corresponding fluctuations, but accurate quantification was not possible, since larvae of the same age were not always available in the greenhouse.

2. Soy-Supplement Diet

Results of the switchback routine using pH-buffered soy-supplement cakes can be seen in Table 9. The results show the ability of the pH 4.4 diet to lower the larval mid-gut pH, and that of the pH 6.5 diet to raise the mid-gut pH. The differences between the two diets were not clearly distinct in all nucs treated, since the adult bees had a definite preference for the acidified supplement, which they were found to store in the comb. The range of larval mid-gut pH's determined after the larvae were fed pH 4.4 supplement was from 5.2 to 5.9; the 6.5 supplement produced larval mid-gut pH's of 5.7 to 6.2.

B. Inoculation Trials

The inoculation trials resulted in a consistently high mortality, due to symptoms of European foulbrood (Fig. 2). In some cases, larval disappearance was usually high in both the inoculated and control treatments (5A and 3A); but all inoculation trials developed between 42% and 82% EFB-infected larvae. Chalkbrood was common in most of the nucs, though it rarely accounted for more than 8% of the inoculated or control larvae.

Supplement and Drug Feeding During Blueberry Pollination

Table 10 illustrates the statistical separation of the mean larval gut pH of four dietary and antibiotic treatments. At the 5% confidence level, the pH-adjusted (pH 4.4) soy flour cake significantly lowered the treated colonies' gut pH below those of the control, terramycin extender-fed, and terramycin extender-plus-supplement-fed colonies.

No significant difference was noted in gut pH between the terramycin extender patty treatment and the control group. When supplement and extender patties were placed on a

Figure 2.
Inoculation trials with S. pluton

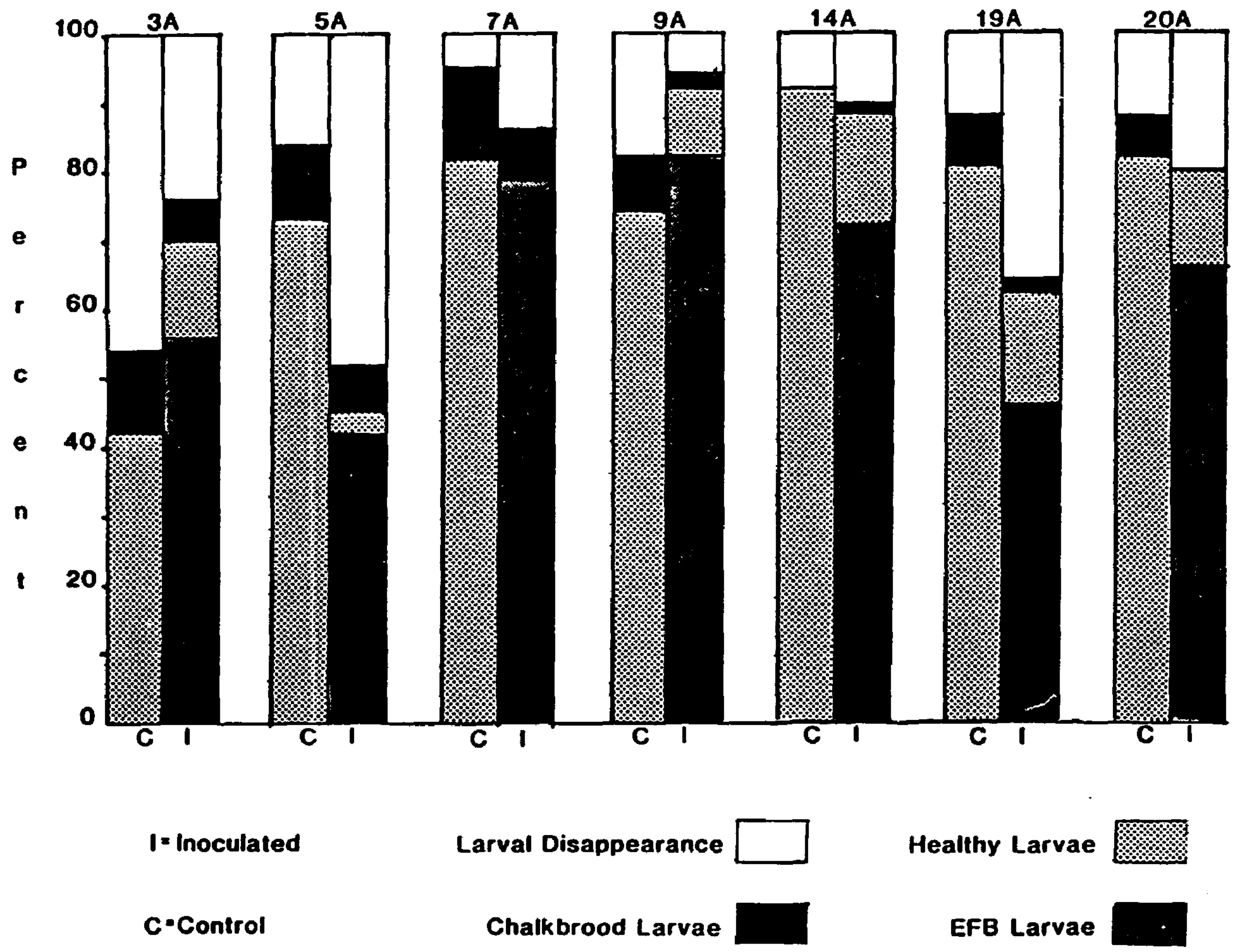

Table 8. Mean mid-gut pH of larvae alternately fed blueberry pollen and a 1:1 mixture of clover and alfalfa pollen, in a switchback routine.

NUC 4A

Pollen type	BB'		C/A		BB		C/A		BB	
Week No.	1	2	3	4	5	6	7	8	9	10
Early[3]	5.8	6.0	6.5	5.8	5.9	6.0	6.5	5.7	NB[6]	6.5
Late[4]	6.0	6.6	6.5	5.6	5.6	6.4	6.4	5.3	5.9	6.7
Weekly $\bar{X}$[5]	5.9	6.3	6.5	5.7	5.7	6.2	6.4	5.5	5.9	6.6

NUC 7A

Pollen type Week No.	1	2	3	4	5	6	7	8	9	10
Early	5.6	6.7	6.7	5.9	5.5	NB	6.3	5.4	5.3	NB
Late	6.3	6.6	6.6	6.0	NB	NB	6.0	6.1	5.9	NB
Weekly X	5.9	6.6	6.6	6.0	5.5	NB	6.2	5.7	5.6	NB

NUC 9A

Pollen type Week No.	1	2	3	4	5	6	7	8	9	10
Early	5.5	6.2	6.0	5.9	5.8	NB	5.9	6.0	6.2	NB
Late	5.8	6.1	6.0	5.8	5.9	5.2	5.7	6.1	6.0	6.0
Weekly X	5.7	6.1	6.0	5.9	5.9	5.2	5.8	6.1	6.1	6.0

Cont. Table 8.

NUC 14A

Pollen type Week No.	1	2	3	4	5	6	7	8	9	10
Early	5.6	6.0	5.5	NB	NB	5.7	5.7	6.4	6.5	6.0
Late	6.1	5.7	NB	NB	NB	5.7	5.8	6.7	6.2	5.9
Weekly $\bar{X}$	5.8	5.9	5.5	NB	NB	5.7	5.8	6.5	6.3	6.0

[1]BB - blueberry pollen

[2]C/A - 1:1 mixture of clover and alfalfa pollen

[3]Early - indicates mean of 3 samples taken early in the week

[4]Late - indicates mean of 3 samples taken late in the week.

[5]Weekly - indicates mean of 6 samples taken during the week

[6]NB - no brood available for analysis

Table 9. Mean mid-gut pH of larvae alternately fed supplement diets of pH 4.4 and 6.6 in a switchback routine.

NUC 3A

Supplement pH	4.4		6.5		4.4
Week No.	1	2	3	4	5
Early	5.9	5.9	5.8	6.0	6.0
Late	5.6	5.7	6.1	6.2	5.8
Weekly $\bar{X}$	5.7	5.8	5.9	6.1	5.9

NUC 4A

Supplement pH	4.4		6.5		4.4
Week No.	1	2	3	4	5
Early	NB[4]	5.4	5.7	5.7	5.8
Late	5.2	5.5	5.8	5.7	NB
Weekly $\bar{X}$	5.2	5.5	5.7	5.7	5.8

NUC 9A

Supplement pH	6.5		4.4		6.5
Week No.	1	2	3	4	5
Early	6.0	6.3	NB	5.6	5.4
Late	6.2	NB	5.3	5.7	5.6
Weekly $\bar{X}$	6.1	6.3	5.3	5.6	5.5

NUC 14A

Supplement pH	6.5		4.4		6.5
Week No.	1	2	3	4	5
Early	6.2	6.8	6.8	5.9	NB
Late	7.0	6.4	6.0	5.8	NB
Weekly $\bar{X}$	6.6	6.6	6.4	5.9	NB

1 EW - indicates mean of 3 samples taken early in the week

2 LW - indicates mean of 3 samples taken late in the week

3 $\bar{X}$ - indicates weekly mean of 6 samples taken during the week

4 NB - no brood available for analysis

Table 10. Statistical separation of means and standard deviation of larval mid-gut pH from colonies fed supplement and/or extender patty.

Treatment	Number	Mean[1]	S.D.
Control	30	6.74	± 0.18
Extender	30	6.61	± 0.18
Supplement & Extender	30	6.40	± 0.24
Supplement	30	6.18	± 0.25
Supplement + Pollen	9	5.80	± 0.12

[1]Means connected by a solid line are not significantly different at the 0.5 level as determined through Duncan's multiple range test.

Table 11. Analysis of variance and statistical separation of pooled treatments: Mid-gut pH of supplement fed colonies not fed supplement

	Analysis of Variance			
Source	SS	DF	MS	F
Treatment	7.11	1	7.11	10.87**
Error	39.24	60	0.654	
Total	46.35	61		

t = 197.8**

** Denotes a highly significant differences between samples.

colony, the resulting larval mid-gut pH was significantly lower than the two treatments which did not receive the pH-adjusted supplement.

An extra colony at one site was outfitted with a pollen trap and fed supplement simultaneously. As a result of feeding the pH 4.4 supplement and removing a portion of the incoming pollen, the average larval mid-gut pH was significantly lower than all other types of treatments.

Table 11 shows the analysis of variance and t-test results of the pooled treatments fed supplement, and pooled treatments not fed supplement, during blueberry pollination. With a 1% level of confidence, highly-significant differences were noted between the two pooled treatments, indicating that the low pH supplement significantly lowered the mid-gut pH in treated colonies.

None of the honey bee colonies which were given the pH 4.4 supplement developed symptoms of EFB. All of the colonies had extensive brood nests with large, healthy larval populations. S. pluton, though found in many of the larvae, was at extremely low titres (less than 10^3 CFU/mid-gut). The two other treatments, supplement plus antibiotic extender patty, and the antibiotic extender patty alone, were also free of any EFB symptoms; and no S. pluton was found in serial dilutions of midgut contents.

The control colonies experienced 70% infection with EFB. The remaining 30% of the control group had sizable populations of S. pluton present in the larvae (greater than 10^7 CFU/mid-gut), although the disease was not expressed. The presence of S. pluton was determined in all cases through the anaerobic culturing of midgut contents on supplemented BHI medium

Soy-Supplement Acidification Preference Test

Four honey bee colonies were simultaneously exposed to soy-supplements acidified with four different acids, and to one control with unchanged pH (Table 12). Statistical analysis of the means failed to show any significant differences between the treatments. Similarly, comparisons of individual means with Student's t-test revealed no differences between any pairs, either after 24-hour consumption or 48-hour consumption. Statistically, no acidified supplement was preferred by the four colonies tested; but trends in consumption showed the lactic acid and hydrochoric acid to be preferred over the tartaric, succinic, and control treatments.

Table 12. Test results of honey bee preference for soy-supplements acidified with different acids.

Patty[1] weight after 28 hours

	Acid				
Colony No.	Control	Hydro-chloric	Succinic	Tart-aric	Lactic
3	44.5	38.5	61.0	50.0	51.0
9	61.5	59.0	72.0	61.5	55.5
17	78.0	70.0	75.0	69.0	65.0
45	59.0	59.5	61.0	70.0	62.5
Mean Weight	60.8	56.7	67.2	62.6	58.5
SD[2]	13.72	13.18	7.32	9.23	6.42

Patty Weight after 48 hours

	Acid				
Colony No.	Control	Hydro-chloric	Succinic	Tart-aric	Lactic
3	0.0	6.0	25.0	4.0	0.0
9	19.0	20.0	35.0	14.5	9.5
17	50.0	36.0	39.0	34.5	31.0
45	11.0	16.0	20.0	35.5	23.0
Mean Weight	20.0	19.5	29.7	21.6	15.8
SD	4.5	12.5	8.8	14.9	13.8

[1]Initial patty weight was 100g.

[2]SD, indicates the standard deviation from the mean.

DISCUSSION

Since 1920, tremendous amounts of time and energy have been expended in the quest to understand the etiology of European foulbrood disease. The bacterium responsible for this disorder has been isolated, and disease treatments and antibiotic prophylactic measures have been described to cope with the problem. However, very little effort has been directed toward an understanding of predisposing factors which initiate the disease symptoms. As previously mentioned, unlike other forms of fruit pollination, honey bees moved to blueberry pollination often develop an unusually-high incidence of EFB. This author believed that if the factor or factors which initiate the disease following blueberry pollination could be understood, perhaps a comprehensive understanding of the disease cycle would emerge.

Though the focal point of this study concerns the high incidence of EFB following blueberry pollination, peripheral questions concerning the culturing of the bacteria, its successful inoculation into the larvae, and the normal state of the larval gut demanded careful investigation. Techniques which have been described in this thesis have overcome not only previous shortcomings in research methods and results, but have answered many critical questions concerning the conditional pathogenicity of European foulbrood, and its heightened incidence following blueberry pollination.

Medium Selection and Isolate Characteristics

The luxuriant growth of S. pluton hinges on several factors: An enriched medium; proper carbohydrates; low oxygen tension; suitable pH of the medium; and an environmental temperature which approximates that found in the hive. The results show that if any of these factors were not closely observed, growth of the isolates was slow and cell morphology was altered, resulting in thin, short rods.

Supplemented Brain Heart Infusion medium provided for the best growth of S. pluton when isolating the bacterium from the larval gut. Bailey's enriched medium was the only other formulation which supported any sizable amount of S. pluton development. However, comparing BHI with Bailey's medium: the BHI would display fully-developed, 2 mm colonies to develop. Cells taken from Bailey's medium were smaller in size, and were less likely to show the lanceolate morphology seen in the larval mid-gut or on the anaerobic BHI medium.

One of the unique qualities of S. pluton is its ability to ferment only glucose and fructose. An organism commonly confused with S. pluton is Streptococcus faecalis (Bailey and Gibbs, 1962). *S. faecalis*, however, is capable of fermenting mannitol and sorbitol, as well as glucose and fructose; and this peculiarity provides an easily-distinguishable characteristic for determining *S. pluton* from *S. faecalis*. Since none of the isolates taken from outbreaks of EFB in Michigan demonstrated the ability to ferment mannitol or sorbitol, it is unlikely that *S. faecalis* would be causing larval mortality, as seen by Vaughn (1962) in isolated EFB-like outbreaks in Wisconsin.

Dr. L. Bailey (personal communication) recently indicated his intention of transferring the generic epithet of *S. pluton* to *Mellisococcus*, thereby creating a new genus specifically for this organism. This proposal is based on the cells' DNA base composition. Since it was not the intention of this paper to become entangled in the taxonomy of the causal organism, detailed studies of its biochemistry, serology, and other taxonomic characters were not undertaken. However, sufficient information was obtained from the cultures to ascertain that the isolates were indeed the same as *S. pluton* described by Bailey and others as the etiological agent of EFB.

Moving Experiments

The moving and re-orientation experiments clearly demonstrated the association between blueberry pollination and EFB. Seventy percent of the colonies moved into blueberry fields developed EFB, but none of the bees in a physiographically-similar area without blueberries showed symptoms of EFB, or S. pluton bacteria in mid-gut cultures.

No significant differences were detected in disease incidence between colonies which were moved, and those which were moved and re-oriented. The additional stress of re-orientation did not increase susceptibility to the disease, but rather, hastened its onset. Colonies which were moved and re-oriented developed EFB before all other treatments. Early disease onset in this circumstance was most likely due to the continual loss of field bees, due to drifting during repeated re-orientation.

The colonies in both the moving, and moving and re-orientation treatments outside of the blueberry fields suffered from an ususually-high incidence of sacbrood, chalkbrood, and queenlessness. The increased incidence of these disorders was indicative of the stress to which the colonies were exposed through these repeated manuevers. The treatment in which colonies of bees were moved to blueberry fields at 10% bloom and then remained stationary, showed no significant difference in disease incidence from all other blueberry treatments. These data indicate that the original move to blueberries was sufficient to predispose colonies to develop EFB. Hence, moving stress was a contributing factor. This may in part explain why moving colonies to pollinate other fruit crops does not predispose these colonies to develop EFB; periodic moving stress apparently only accentuates the disease. Blueberry pollination was therefore viewed as the major cause of EFB in colonies associated with this crop. For this reason, blueberry pollen was investigated as a possible link between blueberry pollination and outbreaks of EFB.

Pollen

Considerable variation in the collection of blueberry pollen was observed within the control, and the moving and re-orientation treatments. Differences such as these might be expected, considering the variation in colony preference and the intense competition existing in forced pollination. Preferential collection of specific pollens has been well-documented in the past; Shimanuki et al. (1967) reported highly significant differences in the percentage of cranberry pollen collected by colonies employed for pollination services. Colonies with a preference for alfalfa pollen were isolated by Nye and Mackenson (1965). The authors also found that the trait was inheritable, and proposed breeding lines of specific pollinators. McLellan (1977) supported these findings, and added that differences in the relative amounts of the same pollen harvested by different colonies varied to a greater degree within sites than between sites. The differences seen in the relative percentages of blueberry pollen collected within treatments is likely a reflection of competitive and environmental forces, in conjunction with biological preferences for particular pollens.

Colonies which were moved and re-oriented regularly collected significantly more blueberry pollen than did the other treatments. This indicates that re-orientation tended to focus the colonies' pollen foragers in the blueberry field, and that moving alone had little or no effect on pollen foraging, unless it was coupled with re-orientation. Lesaffre et al. (1977) reported finding the dispersion around a beehive related to flower density, flower competition in and near the blueberry field, and re-orientation of the colonies. Honey bees moved into a blueberry field during bloom concentrated on the blueberries, since they were the most abundant and the nearest source of pollen and nectar. The data indicate that a colony of bees initially worked closest to the hive, collecting mainly blueberry pollen; subsequently, the bees began to search out other pollen sources, as the flight patterns extended further from the hive. The grower-owned colonies (L1 and L2) which remained near the blueberry field year-round had no history of EFB, and collected the least amount of blueberry pollen. This low blueberry pollen collection can be attributed to the flight patterns established before the blueberries came into bloom.

Since blueberries only bloom for a three-week period, the blueberry fields are non-productive the remainder of the year, forcing the honey bees to forage elsewhere. When blueberries come into bloom, without forced re-orientation these bees retain their pollen foraging patterns outside of blueberries. Ramifications of the collection differences will be discussed at a later point.

A. Pollen Micronutrient Concentrations

Differences found in micronutrient concentrations between samples revealed that manganese levels were approximately five to ten times higher in blueberry pollen than in other pollens sampled. The significance of these findings has not yet been fully understood; however, Bailey (1959) noted that a high phosphate and divalent ion concentration was necessary for maximum growth of *S. pluton*. Unknown factors other than soil acidity were believed responsible for the high manganese levels, since none of the pollens in the non-blueberry fraction showed high levels of manganese, even though they were grown on the same soil as the blueberry plants. McLellan (1977) found similar results in the entire family of Ericaceae, with manganese in pollen reading 250 ppm. In holding with these findings, pollen from cranberry (also a member of the family Ericaceae) had a comparably high manganese content.

Recently, reports in the literature have indicated that certain individuals in plant communities have been found to act as nutrient pumps, bringing specific minerals from deep in the soil, and incorporating them into leaf and stem tissue, where they are made available to other plants through leaching and decay, when the nutrient-packed leaves and stems fall to the surface soil. High amounts of calcium and aluminum were reported to be incorporated into dogwood (*Cornus florida*) (Thomas, 1969), and ironwood (*Carpinus caroliniana*) (Kruckeburg, 1969), respectively. Thereby, high manganese incorporation may be a hold-over from a period when members of Ericaceae fulfilled a functional niche in the biotic community as manganese pumps.

Very little is known about the role micronutrients play in larval nutrition. Further research is needed to determine what effect, if any, the micronutrient differences observed between blueberry pollen and other species may have on the development of EFB disease.

B. Pollen pH

Gut pH is a very intricate and sensitive component of digestion; changes in pH can alter enzyme activity, membrane permeability, and many other important processes. Pollen is the one factor in the larval diet which changes over time. For these reasons, pollen pH became an enlightening section of the study. A wide variety of pH's were found in the pollen types examined; interestingly, all pollen species demonstrated characteristic pH ranges. Alfalfa pollen pH consistently fell within the range of 4.4 to 4.7, and clover between 4.8 and 5.2. Blueberry and cranberry pollen induced pH readings of 6.0 to 6.4, and were the highest pollen pH's recorded. To find such variation among pollens was surprising; never before have differences such as these been reported. Using similar techniques, Paine and Mougenet (1966) found slight variation in pollen pH, between 4.4 and 5.0. They attributed much of the differences observed to site variation in soil pH and the possible effect of agricultural chemicals added to the soil. Results from this study found considerable site variation within species as well. For instance, dandelion pollen from blueberry fields had a pH of 5.7. Although differences between sites existed, little variance was found within species from a site.

The significance of these findings lies not in the pollen pH alone, but the effect it has on larval gut pH when fed to older larvae. Worker larvae generally receive pollen in their diet after the third day of feeding; pollen is maintained in the diet for three days until the cells are closed for pupation. The effect of pH change on the metabolic activity of the mid-gut is unknown; but a pH change such as blueberry pollen (up to 6.4) would bring the mid-gut pH into the optimal growth range pH of S. pluton.

A diet of clover or alfalfa pollen would conceivably induce a mid-gut pH of between 4.8 and 5.2. The effect of pollen on larval mid-gut pH will be discussed at greater length in a later section.

Colony Population Estimation

Three major drawbacks were found in estimating population through the quantification of sealed brood using the Puchta method. They were: 1) total disruption of the colony through frame-by-frame examination, thereby an increased risk of queen supersedure; 2) the amount of time required when observing a large number of colonies; 3) most importantly, the lack of accuracy in estimation, which is primarily a product of the inability to quickly and accurately determine the number of missing cells in a brood pattern, usually resulting in gross over-estimation. It was this third factor which was most troublesome in this particular experiment, since larger colonies often had two or three brood cycles with large sections in the middle of the brood patterns containing empty cells or unsealed brood. Smaller colonies did not always have this feature, eliminating the possibility of introducing correction factors to salvage the data. Findings suggest the need for a less disruptive and more accurate method of determining colony population. Perhaps the Jeffree (1951) method of visual estimation of adult population, or a flight trap quantification of foragers would be a simpler and perhaps more accurate method of population estimation.

Bacterial Transmission through Floral Source

Despite the lack of analyzable data, several points became clear during the course of the experiment. First, the use of small cages for confinement on the target crop may exert unusual stress on the colony through overcrowding and may bias the experiment's outcome. If the disease is contracted by the experimental colonies, there is no certainty that it was passed through the flowers' nectaries. Second, a more direct method to determine whether the disease is passed through the floral source is needed; perhaps the use of scanning electron microscopy to search for the bacterium in the nectaries and on the adult mouthparts would be a more satisfactory approach. Finally, in the isolation of *S. pluton* on bacterial agar medium, methods should be employed to limit motile bacteria and other forms which may over-run the plate before the slower-growing *S. pluton* can be identified.

Pristine Colony Establishment

In 1971, Gilliam reported finding no bacterial growth in the healthy larval mid-gut. She sampled larvae for both anaerobic and aerobic bacterial inhabitants, but found none, and concluded that the mid-gut was maintained as a sterile environment. Contrary to Gilliam's findings, this study's pristine colony experiment demonstrated sizable populations of bacteria in the mid-gut which fluctuated through time, in apparent response to the gut pH of the larvae. As the mid-gut pH increased, the viable bacterial count increased; and conversely, as the mid-gut pH decreased, the bacterial count decreased correspondingly. The highest pH and CFU quantitifications were observed in the months of June and early August, the two periods of the year in Michigan in which EFB is known to be most severe.

The waxing and waning nature of EFB has perplexed researchers for many years and, at least in part, appears due to changes in the mid-gut pH (or to factors of the pollen corresponding with these changes in mid-gut pH). The population response of the bacteria to the change in pH was not immediate; a short lag was seen in bacterial increase following the increase of pH. Decreasing trends in mid-gut pH also preceded microbial decline by one to two weeks; lag responses such as these could be attributed to high titres of bacteria present inside the cell of the young larvae. The bacteria are deposited in the cell by previous larvae through the expulsion of the meconium, and are then ingested by the next generation of larvae during feeding. Each successive larva acts as a "growth chamber," allowing higher titres of bacteria to build, providing that mid-gut growth factors are favorable. When the mid-gut environment becomes less favorable, the bacterial population may continue to increase due to the sheer numbers of the inoculum and their modifying effect on the gut. As the mid-gut becomes even less favorable through generations of larvae, the growth rates of the bacteria decline, and a lower titre results. Graphically, the changes in pH and bacterial populations appear to follow local floristic patterns. A major downward trend of total gut bacteria in early July corresponds with the primary honey and pollen flow of clover and alfalfa. When these flows end in late July, the upward trend of pH and total bacteria returns, only to drop drastically in the early fall.

There exist two current hypotheses explaining this rise and fall of microbial populations in honey been larvae. Bailey (1968) believes the cyclic partial starvation and allows *S. pluton* to abound. Later, when a honey flow begins, cell space is critical and sick larvae are not tolerated; the colony becomes more hygenic. Consequently, sick larvae are removed before they can expel the meconium loaded with bacteria. Brood rearing also is reduced at this time, raising the nurse bee-to-brood ratio; larvae are therefore better fed, and thus more likely to survive infection. The second hypothesis involves the antibiotic qualities of specific pollens (Chauvin, 1957). Individual pollen types have been shown to limit the growth of specific bacteria, indicating that the waxing and waning nature of EFB may be a result of *S. pluton*'s sensitivity to particular pollens.

Both of these hypotheses address methods which could influence microbial mid-gut populations; however, data from this paper contributes information on another control mechanism, the mid-gut pH. The mid-gut pH of larvae old enough to be fed pollen is potentially influenced by the type of pollen fed to the larvae. The following discussion will illustrate the relationship between pollen in the larval diet and mid-gut pH.

Greenhouse Experiments

A. Controlled Diet Investigations

The fact that specific pollens fed to a colony can alter the mid-gut pH of older larvae is interesting, from the standpoint of possible changes in permiability of the larval gut membrane and enzyme activity. Also, because many bacteria grow in a very narrow pH range, the changing of gut pH by feeding specific pollens could be a useful tool in disease control for honey bees, and possibly even with other pollen-feeding insects. In switch-back experiments on greenhouse colonies, clover and alfalfa pollen consistently lowered larval gut pH, while blueberry pollen raised larval gut pH. As gut pH was raised and lowered, the gut bacterial populations changed accordingly. Presumably, pH is the regulating factor in this case, since similar results were obtained when the same feeding experiment was conducted with soy-supplement cakes adjusted to pH 6.5 or 4.4. Correspondingly, if in the greenhouse it is possible to lower the mid-gut pH below optimal growth range of S. pluton, it can also be done in the blueberry field, providing the colony will accept the supplement in the presence of actual field-collected pollen.

B. Inoculation Trials

The ability to consistently infect 70% of the inoculated larvae with EFB may open new avenues of disease research. Until now, high levels of mortality could only be produced by the use of whole mid-gut macerations or meconial washings from brood cells. None of these methods, however, used pure S. pluton cultures. But by using a specific set of stresses (low nurse bee-to-brood ratio, high diurnal variation in temperature, a diet near pH 6.5, and forced ingestion of the inoculum), a consistent degree of mortality can be achieved. Now that mortality can be produced more regularly or predictably, in-depth studies can be undertaken into the etiology of European foulbrood disease and its association with nutrition and stress.

The high degree of larval disappearance seen in some treatments, but not consistenly in all, may reflect differences in hygenic behavior between colonies. The inconsistency also may reflect differences in the position of the larvae in the hives; since not all treatments could be placed on opposite sides of a single frame, some treatments were put on different frames facing each other in the broodnest. Further research needs to be conducted in this area;

perhaps through the use of identical lines of artificially-inseminated queens genetic differences could be minimized, allowing a more accurate assessment of the reason for larval disappearance.

Supplement and Drug Feeding During Blueberry Pollination

Honey bee colonies fed pH-adjusted soy-supplement consumed the product at a surprising rate. Allotments were increased to three pounds per week to insure adequate supply. Traditionally, the acceptance of soy-supplement has been poor. Evidently, the addition of lactic acid to the soy-supplement cakes changed that bias. Paine and Maugenet (1966) explained that in attempts to artificially ensilage pollen with cultures of Lactobacillus spp., the end product was of low pH but was distasteful to honey bees. However, when cultures of Lactobacillus and yeast were inoculated into pollen, the honey bees accepted it as enthusiastically as their own stored pollen. This leads to one possible explanation for the acceptance of the formula used in the blueberry field experiments--that is, lactic acid and brewer's yeast in the soy-supplement mixture combined to closely mimic actual bee bread.

The ability of the pH-adjusted soy-supplement to successfully lower gut pH and prevent EFB symptoms was most dramatic. Although several of the colonies contained low levels of S. pluton, none developed EFB; but controls suffered a 70% incidence of EFB. The ability to control EFB through a non-pharmacological means is important in light of reports of terramycin resistance (Arro, 1967; Machado and Lemos, 1974), and a growing concern over terramycin contamination of honey. The results indicate the feasibility of EFB control by feeding pH-adjusted soy-supplement cakes during critical periods of the year when colonies appear most susceptible.

Terramycin extender patties gave complete control over S. pluton populations when either fed to the colony alone, or in conjunction with soy-supplement. Although it did not lower gut pH, the action of the antibiotic was responsible for bacterial suppression. When the patties were fed in conjunction with the pH 4.4 soy-supplement cakes, larval midgut was significantly higher than in the treatment of supplement alone. The higher pH readings were most likely due to the lipids (in the form of vegetable shortening) in the extender patty.

The successful control over bacteria associated with EFB can be attributed to the slow release of terramycin into the colony through the extender patty. The shortening in the mixture slowed consumption, and its hydrophobic nature extended the activity of the moisture-sensitive terramycin. Most beekeepers treat colonies with drugs infrequently during the spring with dry mixtures of terramycin and powdered sugar. But because terramycin used in this manner oxidizes rapidly in the presence of moisture, the period of disease protection is brief. Many colonies are regularly treated with dry terramycin before they go into blueberry pollination; but in the dampness of the hive, they may lose their protection soon after arriving. Shortening added to the mixture, however, provides disease control for the desired period of time. Recently, Gilliam and Argauer (1975) reported similar success with extender patties in providing excellent disease control. They also found that terramycin in patty form did not appear in the stored honey, but only in the broodnest. Until the extender patty form of drug treatment is registered in the USA for commercial use, only syrup and dry terramycin can be recommended.

A dramatic demonstration of the ability of the pH-adjusted soy-supplement cakes to lower larval mid-gut pH was seen in field colonies which received soy-supplement along with a reduction of incoming pollen through pollen trapping. Four-and-a-half to five-day-old larvae from this treatment possessed mid-gut pH's similar to larvae of colonies from non-blueberry areas. Reduction of the incoming pollen resulted in greater percentages of supplement and stored pollen being fed to the larvae, permitting the retention of low mid-gut pH.

Soy-Supplement Acidification Preference Test

Despite the lack of statistically significant differences in the rate of consumption of acidified soy-supplements, trends in the data indicate that when supplement was lowered to pH 4.4 with either lactic or hydrochloric acids, these formulations were preferred over the control, tartaric acid and succinic acid formulations. The different rates of consumption indicate that the honey bees are not strictly attracted to low-pH supplement, but to soy-supplement acidified with particular acids.

More in-depth research is needed in the area of supplement acidification; different acids and pH's should be investigated, as well as consumption rates at various times of the year. Tracing the use of the acidified supplement in the colony would also be of great benefit, including the gathering of information on what proportion is fed to the larvae, and what proportion is consumed by adults. Such studies might provide better insight into the needs of the colony, leading subsequently to improved methods of management.

Flow Diagram of Major Factors Influencing European Foulbrood Disease

Figure 3 depicts major interacting stress factors previously discussed in this thesis which are believed responsible for the unusually high incidence of EFB in association with blueberry pollination. It should be noted that each factor is capable of exerting its own influence upon the larvae, and that the environment is capable of modifying all factors, either individually or in a series of a more complex nature, as indicated in the diagram.

The process of moving and re-orientation, for example, can affect the type and quantity of pollen and nectar entering the colony. This research has already shown that the nutritional quality of pollen is subject to wide variance; and that in the case of blueberry pollen, larvae fed large quantities of this relatively-high pH substance become susceptible to S. pluton proliferation. The nurse bee-to-brood ratio can also be affected by the nutritional value of pollen and nectar, mainly by insufficient physiological and hypopharangeal glandular development, as a result of poor diet (Free, 1961; Maurizio, 1954). Other diseases may result in a lowered nurse bee-to-brood ratio, as well. In such cases, S. pluton is given the opportunity to accelerate its development and cause the death of increasing numbers of larvae.

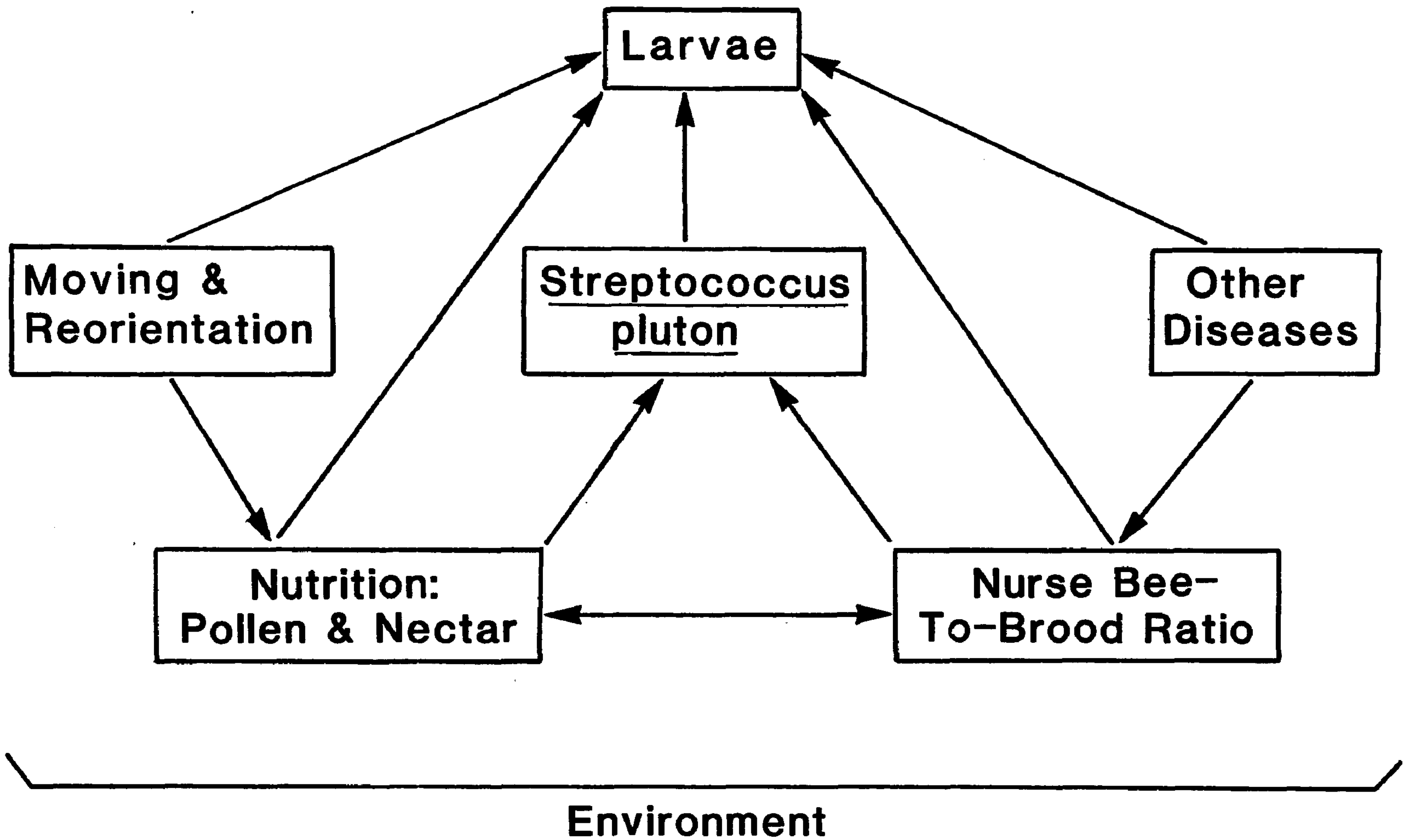

Figure 3. Flow diagram of major factors influencing European foulbrood disease

SUMMARY

This investigation has dealt specifically with the occurrence of European foulbrood disease following blueberry pollination. Having arrived at a solid understanding of the initiating mechanism of this disease in blueberries more comprehensive investigations of the disease's variability in other problem areas and in other seasons may now be more fruitfully undertaken.

As can be seen in the flow diagram depicting the interaction of stress factors in EFB (Fig. 3), this study enables future researchers to control factors which were previously not fully understood. For example, the effect of re-orientation on foraging and the influence of various pollens on the mid-gut pH can be accounted for, and their contribution in disease initiation in various situations can be ascertained.

During the course of the investigation a number of contributions, both directly and indirectly related to EFB in blueberry pollination, were made. The following list outlines the major findings and accomplishments of this study:

1. Consistent cultivation of large populations of Streptococcus pluton on enriched media.

2. Identification of a simple inoculation technique and reproducible stress conditions which, in conjunction with

S. pluton cultures, result in consistent larval mortality due to EFB.

3. The heightened EFB susceptibility of colonies moved to blueberries for pollination, due to an increase in mid-gut pH, in conjunction with a combination of stresses. The pH levels were raised to the optimal growth range of S. pluton (pH 6.5) by the feeding of blueberry pollen to larvae old enough to receive pollen in their diet.

4. The pH of different pollen species varies greatly. The highest mean was blueberry at 6.2, and the lowest mean was in alfalfa at pH 3.9. Variation within species was noted between sites, indicating that local conditions play a role in the pH of pollen.

5. Blueberry pollen contains manganese in concentrations five to ten times greater than that of other pollens tested; but its relationship to EFB, if any, is unknown.

6. Great fluctuations of bacterial mid-gut populations occurred within colonies throughout the year. The fluctuations appear to be associated, in part, to pollen pH.

7. EFB can be effectively controlled through feeding the colony pH-adjusted soy-supplement during critical periods of the year. At present, lactic acid appears to be the best acidifying agent.

8. Honey bee colonies moved to blueberries for pollination at approximately 10% bloom spend more time foraging

among blueberries after re-orientation than do previously established colonies; pollination colonies collect unusually high amounts of blueberry pollen.

9. Terramycin extender patties are very effective in controlling EFB during blueberry pollination, because their formulation prolongs the presence of active terramycin in the colony.

The following are recommendations for further research, based on the findings of this study:

1. Determination of the effect of blueberry nectar on the larval gut pH.
2. Investigation of the influence of soil parameters such as pH, mineral levels, and moisture content, on pollen's nutritional quality.
3. A better understanding of the acceptance of acidified pollen cakes by colonies actively foraging field pollen.
4. Further research into the interactions between *S. pluton* and the early larval stage.
5. Monitoring of the pH of corbicular pollens in as many regions as possible, in part, due to the fact that variation in pH may occur, even within a single plant species.

6. The planting of bee forage, which is being recommended by many apiculturists, taking into account the pH of the pollen source. Not only are supplemental forage crops needed; but those crops must produce pollen that does not alter the normal gut pH.

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