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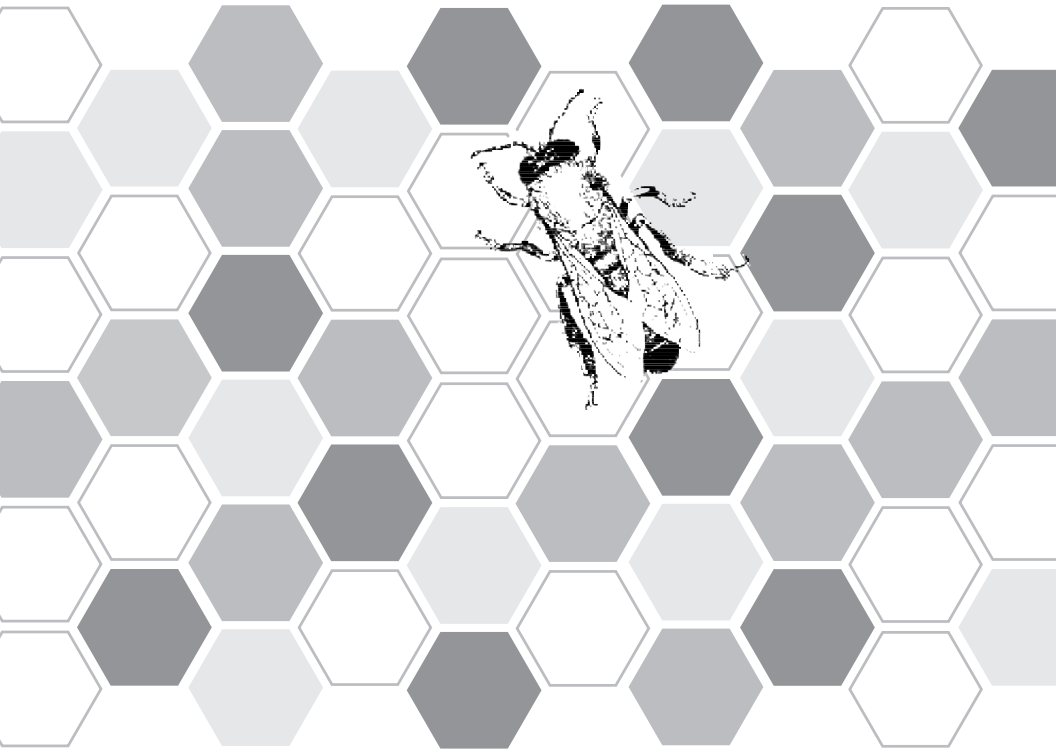
Handbook
Number 690

Diagnosis of Honey Bee Diseases



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Diagnosis of Honey Bee Diseases

Hachiro Shimanuki and David A. Knox

Abstract

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Apiary inspectors and beekeepers must be able to recognize bee diseases and parasites and to differentiate the serious diseases from the less important ones. This handbook describes laboratory techniques used to diagnose diseases and other abnormalities of the honey bee and to identify parasites and pests. Emphasis is placed on the techniques used by the U.S. Department of Agriculture Bee Research Laboratory. Included are directions for submitting, through APHIS-PPQ or state regulators, samples of suspected Africanized honey bees for identification of subspecies. Also included are directions for sending diseased brood and adult honey bees for diagnosis of bee disease.

Keywords: Africanized honey bee, honey bee disease, honey bee disorder, honey bee parasite, honey bee pest

While supplies last, single copies of this publication may be obtained at no cost from Bee Research Laboratory, Building 476, BARC-East, Beltsville, MD 20705.

Copies of this publication may be purchased from the National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161; telephone (703) 605–6000.

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Contents

Tables

Figures

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Introduction

Inspection for bee diseases is an important part of beekeeping. Apiary inspectors and beekeepers must be able to recognize bee diseases and parasites and to differentiate the serious diseases from the less important ones. The purpose of this publication is to identify parasites, pests, and other abnormalities of the honey bee and to acquaint readers with the laboratory techniques used to diagnose diseases. We realize that different laboratory methods are used by others; where possible, those methods are described. However, we emphasize the techniques used at the Bee Research Laboratory, of the Agricultural Research Service, U.S. Department of Agriculture.

Symptoms

Brood combs of healthy colonies typically have a solid and compact brood pattern. Almost every cell from the center of the comb outward contains an egg, larva, or pupa. The cappings are uniform in color and are convex (higher in the center than at the margins). The unfinished cappings of healthy brood may appear to have punctures, but since cells are always capped from the outer edges to the middle, the holes are always centered and have smooth edges.

By comparison, brood combs of diseased colonies usually have a spotty brood pattern (pepperbox appearance), and the cappings tend to be darker, concave (sunken), and punctured. The combs may contain the dried remains of larvae or pupae (called *scales*), which are found lying lengthwise on the bottom sides of brood cells. Sometimes scales are difficult to locate because of the condition of the comb. In such cases, scale material can easily be located using longwave ultraviolet or near-ultraviolet light. Exposure to wavelengths of 3,100 to 4,000 angstroms causes scale material to fluoresce. Some discretion must be used with this technique

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Table 1. Comparative symptoms of various brood diseases of honey bees

Symptom	American foulbrood	European foulbrood	Sacbrood	Chalkbrood
Appearance of brood comb	Sealed brood. Discolored, sunken, or punctured cappings.	Unsealed brood. Some sealed brood in advanced cases with discolored, sunken, or punctured cappings.	Sealed brood. Scattered cells with punctured cappings.	Sealed and unsealed brood.
Age of dead brood	***Usually older sealed larvae or young pupae.****	***Usually young unsealed larvae***; occasionally older sealed larvae. Typically in coiled stage.	Usually older sealed larvae; occasionally young unsealed larvae. Upright in cells.	Usually older larvae. Upright in cells.
Color of dead brood	Dull white, becoming light brown, coffee brown to dark brown, or almost black.	Dull white, becoming yellowish white to brown, dark brown, or almost black.	Grayish or straw-colored, becoming brown, grayish black, or black. Head end darker.	***Chalk white.*** Sometimes mottledwith black spots.
Consistency of dead brood	***Soft, becoming sticky to ropy.***	Watery; rarely sticky or ropy. ***Granular.***	***Watery and granular; tough skin forms a sac.***	Watery to pastelike.
Odor of dead brood	Slight to pronounced odor.	Slightly sour to penetratingly sour.	None to slightly sour.	Slight, non-objectionable.
Scale characteristics	Uniformly lies flat on lower side of cell. Adheres tightly to cell wall. ***Fine, threadlike tongue of dead pupae maybe present.*** Head lies flat. Brittle. Black.	Usually twisted in cell. Does not adhere tightly to cell wall. Rubbery. Black.	Head prominently curled toward center of cell. Does not adhere tightly to cell wall. Rough texture. Brittle. Black.	***Does not adhere to cell wall.*** Brittle. Chalky white, mottled, or even black.

* Bold italics indicate the most useful field characteristics.

because honey and pollen may also fluoresce. Symptoms of various brood diseases are summarized in table 1.

By contrast, the gross symptoms of most adult bee diseases are not unique. The inability to fly, unhooked wings, and dysentery, for instance, are general symptoms associated with many disorders. Symptoms of a contagious disease are sometimes mimicked because of unrelated factors. For example, a brood neglected because of a shortage of nurse bees will often die from either chilling or starvation. Disease symptoms can also be the result of a failing queen, laying workers, toxic chemicals, or poisonous plants (see Noninfectious Disorders, p. 37.).

Bacterial Diseases

American Foulbrood

Paenibacillus (formerly *Bacillus*) *larvae* subsp. *larvae*, hereafter referred to as *P. larvae*, (see appendix E) is the bacterium that causes American foulbrood disease (AFB). *P. larvae* is a slender rod with slightly rounded ends and a tendency to grow in chains (fig. 1). The rod varies greatly in length, from

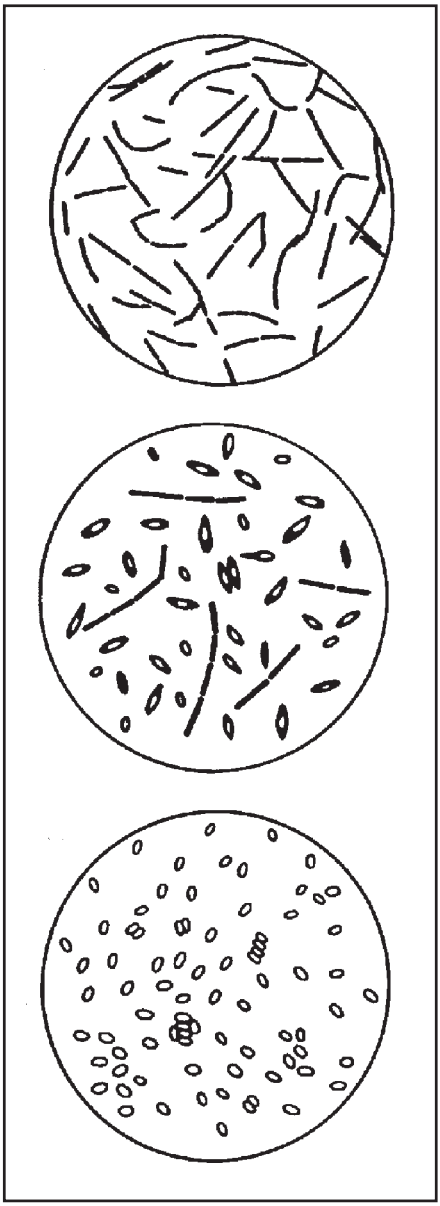


Figure 1. Bacterium that causes American foulbrood disease (not to scale). Top, *Paenibacillus larvae* vegetative cells. Middle, forming *P. larvae* spores. Bottom, *P. larvae* spores.

about 2.5 to 5 micrometers (μm); it is about 0.5 μm wide. The spore is oval and about twice as long as wide, about 0.6 × 1.3 μm. When stained with carbol fuchsin, the spores have reddish-purple walls and clear centers. They may form clusters and appear to be stacked. About 2.5 billion spores are produced in each infected larva. If the larva has been infected for less than 10 days, the vegetative cells are present and some newly formed spores may be seen. Adult bees are not susceptible to AFB.

The modified hanging drop technique (appendix A) can be very useful for differentiating American foulbrood from other brood diseases. In areas of the smear where pockets of water are formed in the oil, *P. larvae* spores exhibit Brownian movement. This movement is an extremely valuable diagnostic technique because the spores of other species associated with known bee diseases usually remain fixed (see table 2). It is important to note that Brownian movement can be affected by slide preparation, and debris and other bacteria can exhibit this motion. Therefore, Brownian movement must not be the sole diagnostic criterion but should be considered along with the characteristic morphology of the spores and the gross larval symptoms. If microscopic examination is not conclusive, cultural tests can be made using the same suspension.

Cultivation of *Paenibacillus larvae*

Thiamine (vitamin B_1) and some amino acids are required for the growth of *P. larvae*. Routine culture media such as nutrient agar will not support the growth of this organism. Good vegetative growth (but not sporulation) occurs on Difco brain-heart infusion fortified with 0.1 mg thiamine hydrochloride per liter of medium (BHIT) and adjusted to a pH of 6.6 with

Table 2. Differentiation of spore-forming bacteria in honey bees

Species	Brownian movement*	Catalase production	Nitrate reduction	Growth on nutrient agar
Paenibacillus larvae	+	–	+	–
Paenibacillus alvei	–	+	–	+
Brevibacillus laterosporus	–	+	+	+
Paenibacillus pulvifaciens	–	–	+	+

*In modified hanging drop technique.

hydrochloric acid. Satisfactory growth and sporulation occur on the yeast extract, soluble starch, and glucose media recommended by Bailey and Lee (1962). The medium can be liquid, semisolid (0.3 percent agar), or solid (2 percent agar). *P. larvae* spores also reproduce in the hemolymph of honey bee larvae, pupae, and adults when artificially introduced by injection (Michael 1960, Wilson and Rothenbuhler 1968, Wilson 1970). For more information on sporulation, see Dingman and Stahly (1983).

To culture *P. larvae*, we prepare spore suspensions by mixing diseased material (3 to 5 scales) with 9 ml sterile water in screw-cap tubes. (We use moist cotton swabs to remove and transfer the scales from the comb to the tubes.) The suspension is heat shocked at 80° C for 10 minutes (effective time) to kill non-spore-forming bacteria. A sterile cotton swab or L-shaped spreader is used to evenly spread about 0.2 ml of the suspension over the surface of BHIT agar plates, which are then incubated for 72 hours at 34° C. Individual colonies are small (1–2 mm) and opaque; however, if large numbers of viable *P. larvae* spores are inoculated, a solid layer of growth will cover the plate.

There are no reliable methods for making plate counts of *P. larvae* because fewer than 10 percent of the spores produce visible growth on the presently available media (Shimanuki 1963). By calibrating our methods using spore and plate counts, we determined that a minimum of 100 *P. larvae* spores are required to produce visible growth on BHIT.

Diagnostic tests for *Paenibacillus larvae*

Holst milk test. The Holst milk test (Holst 1946) is a simple test based on the high level of proteolytic enzymes produced by sporulating *P. larvae*. The test is conducted by suspending a suspect scale or a smear of a diseased larva in a tube containing 3 to 4 ml of 1-percent powdered skim milk in water. The tube is then incubated at 37° C. If AFB is present, the suspension should clear in 10 to 20 minutes. It should be noted that this test is not always reliable.

Nitrate reduction. *P. larvae* reduces nitrate to nitrite (Lochhead 1937). The nitrate reduction test can be performed on a medium such as BHIT, with potassium nitrate added (1–2 mg/L of medium). After growth has occurred, adding a drop of sulfanilic acid-alpha-naphthol reagent produces a red color if nitrate has been reduced to nitrite. Diagnosis should not be based on this test alone but, rather, on the test along with larval gross symptoms, bacterial morphology, and growth characteristics of the bacterial colony.

Catalase production. A drop of 3-percent hydrogen peroxide is placed on an actively growing culture on a solid medium. Most aerobic bacteria break down the peroxide to water and oxygen and produce a bubbly foam, but *P. larvae* is almost always negative for this reaction (Haynes 1972).

Fluorescent antibody. The fluorescent antibody technique requires preparation of specific antibodies stained with a fluorescent dye. Rabbits are injected with pure cultures of *P. larvae*, and the active antiserum is collected and stained with a fluorescent dye. This fluorescent antiserum is mixed with a bacterial smear on a slide and allowed to react. The excess antiserum is washed off the slide, and the slide is then examined with a fluorescence microscope (see appendix A). *P. larvae* appears as brightly fluorescing bodies on a dark background (Toschkov et al. 1970, Zhavnenko 1971, Otte 1973, Peng and Peng 1979).

Viability test for *Paenibacillus larvae*

American foulbrood is transmitted by the spores of *P. larvae*. These spores have been shown to remain viable for over 70 years (Shimanuki and Knox 1994). One method of controlling AFB is to destroy the viability of the spores in contaminated bee equipment. This can be accomplished by gamma or electron beam irradiation or by fumigation with a sterilant gas such as ethylene oxide. Assessment of the efficacy of these methods should be based on the number of spores that remain viable in a test sample of brood comb containing at least 10 AFB scales.

To test viability, prepare a spore suspension from the sample comb by mixing 10 scales in 10 ml sterile water. Since each scale contains about 2.5 billion spores (Sturtevant 1932), 1 ml of the suspension should contain 2.5 billion spores. Spread a portion of the suspension (0.2 ml = 500 million spores) onto solid BHIT plates as previously described (p. 5) and incubate for 72 to 96 hours. The results of the viability test are reported as the approximate number of viable spores per scale, as follows:

- 0 colonies form on the medium = <100 viable spores per scale
- 1–9 colonies = <1,000 viable spores per scale
- 10–99 colonies = <10,000 viable spores per scale
- over 100 colonies = >10,000 viable spores per scale
- plate is completely overgrown with colonies = no detectable reduction of viable spores.

Terramycin (Oxytetracycline hydrochloride)/*Paenibacillus larvae* resistance tests

Isolates of *P. larvae* can be screened for sensitivity to oxytetracycline hydrochloride (OTC) based on the size of inhibition zones on agar plates. A spore suspension of the *P. larvae* isolate to be tested is spread on solid BHIT as described previously. A disk (BBL Sensi-Disc) containing 5 μg tetracycline[2] is then placed on the plate, and the plate is incubated at 34° C for 72 hours. The zones formed by sensitive strains usually are greater than 50 mm in diameter. Alternatively, OTC incorporated into liquid BHIT inhibits the growth of sensitive isolates of *P. larvae* at concentrations as low as 12 μg/L of medium (Gochnauer 1954). Care is necessary in doing these tests, and adequate controls should be included.

Any substantial reduction of the zone size or an increase in the concentration of OTC required to prevent growth of *P. larvae* is evidence of the development of resistance (Gochnauer 1954). However, when interpreting test results, the effects of growth rates should be considered. Because isolates of *P. larvae* grow at different rates, one may falsely conclude that an isolate is resistant.

Detection of *Paenibacillus larvae* spores in hive products

Honey. Occasionally it may be necessary to examine honey for the presence of *P. larvae*. Due to the high concentration of carbohydrate and other natural bacteriostatic substance(s) in honey, the examination of honey requires special considerations.

Dilution. The classical method (Sturtevant 1932, 1936) is to dilute the honey 1:9 with water, centrifuge the mixture to concentrate the spores in the sediment, and then microscopically examine the sediment for the presence of spores. However, conclusive demonstration of the presence of *P. larvae* spores in honey requires cultural techniques. Hornitzky and Clark (1991) report the following technique:

- Dilute a 125-ml honey sample with 250 ml phosphate-buffered saline (pH 7.2) and centrifuge in 500-ml bottles for 45 minutes at 3,000 G.
- Pour the supernatant off, leaving approximately 3 ml of fluid, and mix with the sediment in the bottle.

[2] Tetracycline discs were substituted for oxytetracycline discs; the inhibition zones of the tetracyclines are similar (Gochnauer 1970).

- Place a 0.5-ml aliquot in a 5-ml glass bottle and heat it in a water bath at 80° C for 15 minutes. After heating, streak the suspension on an agar plate containing an appropriate media.
- Incubate the agar plate at 37° C for 72 hours and examine it for colonies of *P. larvae*.

Direct inoculation (Hansen 1984a,b). Place a 5-g aliquot of the honey in 50-ml sterile beakers and set in a water bath for 5 minutes (effective time) at 88° to 92° C. After heating, consecutively streak three agar plates that contain appropriate media, using an inoculating loop. Incubate the plates at 37° C for 72 hours and examine for colonies of *P. larvae*. This method can detect *P. larvae* when more than 2,000 spores are present in 1 g honey. While quick and easy, the direct inoculation of honey onto agar plates is limited by the amount of honey that can be sampled and the likelihood of contaminants.

Dialysis (Shimanuki and Knox 1988). Heat the honey to 45° C to permit easier handling and to decrease viscosity, allowing for more uniform distribution of any spores. Place 25 ml of honey in a 50-ml beaker and dilute with 10 ml of sterile water. The diluted honey is then transferred into a 1.75-inch (44-mm) dialysis tube. Tie the open end after the tube is filled, and submerge the tube in running water for 7 to 8 hours or in a water bath, changing the water three or four times during the period. Following dialysis, the contents of the tube are centrifuged at about 20,000 G for 20 minutes. The supernatant is carefully removed with a pipet to leave approximately 1 ml of residue. This residue is resuspended in 9 ml of water in a screw-cap vial and heat shocked at 80° C for 10 minutes to kill non-spore-forming bacteria. Next, spread 0.4 ml of the suspension onto a plate of BHIT agar. Incubate the plate at 34° C for 72 hours and examine for colonies of *P. larvae*.

Since about 100 *P. larvae* spores are required to produce visible growth on BHIT, this technique can demonstrate the presence of *P. larvae* spores in samples that contain a minimum of 80 spores per ml of undiluted honey (25 ml honey × 100 spores/ml = 2,500 spores/dialysis = 2,500 spores in 10 ml or 250 spores/ml; 0.4 ml = a 100-spore inoculum). Lower spore levels can possibly be detected by the use of larger honey samples or a second centrifugation to further concentrate the spores.

Difficulties can sometimes occur when honey samples contain *Paenibacillus alvei* or other spore-forming bacteria that may completely cover the surface of the plate. Hornitzky and Clark (1991) overcame this problem by supplementing their media with 3 μg/nalidixic acid/ml of media.

Pollen. *Paenibacillus larvae* spores can also be recovered from bee-collected pollen pellets by physically removing bits of AFB scale. A series of sieves of different sizes is helpful. If scales are not detected, one may pass a water-pollen suspension through No. 2 filter paper, centrifuge the filtrate, and culture the pellet as described in the dialysis method (Gochnauer and Corner 1974).

Beeswax. We have had some success in recovering spores which are morphologically similar to those of *P. larvae* by melting beeswax in boiling water, removing the beeswax cake after cooling, and centrifuging the water at 5,000 G for 20 minutes. The sediment is then examined microscopically for the presence of spores. Spores have also been recovered from contaminated beeswax by chloroform extraction (Kostecki 1969). However, in both cases, positive identification of the spores is not possible because the recovery techniques render the spores nonviable.

European Foulbrood

The bacterium *Melissococcus pluton* (= *Streptococcus pluton*) causes the brood disease European foulbrood (EFB). *Streptococcus pluton* was reclassified into the new genus *Melissococcus* by Bailey and Collins (1982a,b). *M. pluton* is generally observed early in the infection cycle before the appearance of the varied microflora associated with this disease. The *M. pluton* cell is short, non-spore-forming, and lancet shaped. The cell measures 0.5–0.7 $\times$ 1.0 μm and occurs singly, in pairs, or in chains (fig. 2). When stained with carbol fuchsin, the organism appears dark purple against a lighter background. Some distortion occurs during the fixing and staining process; this can be reduced by negative staining. *M. pluton* can also be detected using enzyme-linked immunosorbent assays (Pinnock and Featherstone 1984), polyclonal antisera (Allen and Ball 1993), or polymerase chain reaction (Govan et al. 1998).

Cultivation of *Melissococcus pluton*

M. pluton can be isolated on a medium developed by Bailey (1959). The medium consists of 1 g yeast extract (Difco), 1 g glucose, 1.35 g potassium dihydrogen phosphate (KH_2PO_4), 1 g soluble starch, 2 g agar, and distilled water to make 100 ml. The pH is adjusted to 6.6 with potassium hydroxide (KOH), and the mixture is autoclaved at 10 lb per inch^2 (116^{o}C) for 20 minutes. It has been found that the addition of cysteine (0.1 g/100 ml) improves the multiplication of *M. pluton* (Bailey and Collins 1982b).

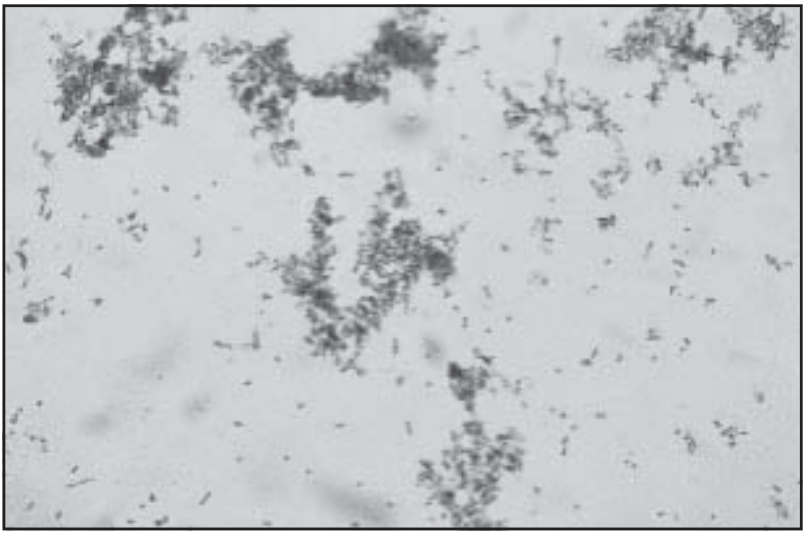


Figure 2. *Melissococcus pluton* and *Paenibacillus alvei* (×1,000).

It is difficult to isolate *M. pluton* on artificial media because of its growth requirements and competition from other bacteria. Once isolated, identification of *M. pluton* is also difficult due to its pleomorphic nature. *M. pluton* is best isolated when few, if any, other organisms are present. According to Bailey (1959), it is best to dry smears of diseased larval midguts on a slide. A water suspension of this material or a suspension prepared from larvae (apparently healthy, infected, or dead), cappings, and so forth can be streaked on freshly prepared Bailey's agar medium. Decimal dilutions of these suspensions can also be inoculated into molten Bailey's agar medium (45° C) and poured into plates (Bailey and Ball 1991). The plates are incubated anaerobically at 34° C. [We use the "Gas Pak" (BBL) Anaerobic System to obtain anaerobic conditions.] Small white colonies of *M. pluton* should appear after 4 days. See Allen and Ball (1993) for additional information.

Organisms associated with European foulbrood

Some organisms do not cause European foulbrood, but they influence the odor and consistency of the dead brood and can be helpful in diagnosis. These secondary invaders include the following:

Paenibacillus alvei (=*Bacillus alvei*). The bacterium *Paenibacillus alvei* is frequently present in cases of EFB. It is a rod 0.5–0.8 μm wide × 2.0–

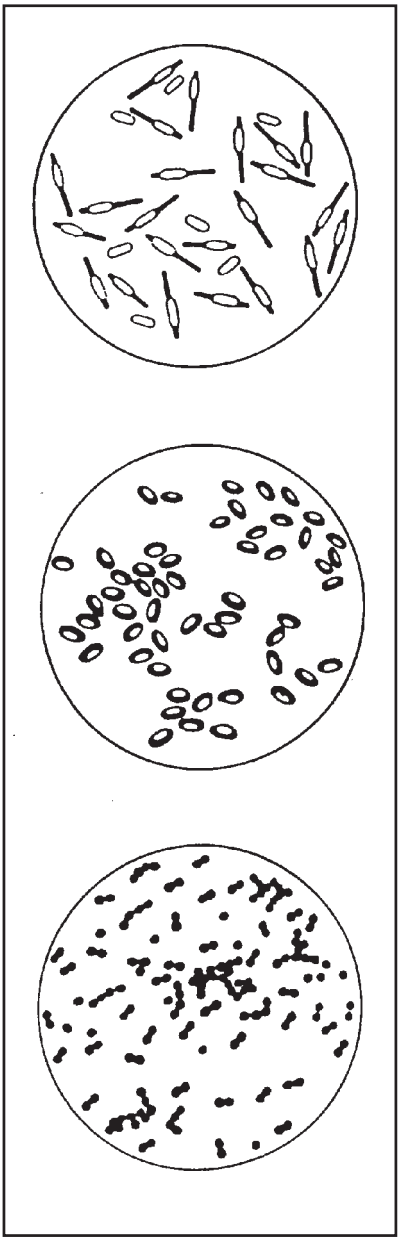


Figure 3. Bacteria associated with European foulbrood disease (not to scale). Top, *Paenibacillus alvei.* Middle, *Brevibacillus laterosporus.* Bottom, *Enterococcus faecalis.*

5.0 μm long (figs. 2 and 3). Spores measure 0.8 × 1.8–2.2 μm. Like *P. larvae*, *P. alvei* spores may be clumped and appear stacked. The sporangium may be observed attached to the spore. Typical cultures of *P. alvei* spread vigorously on nutrient agar and may show motile colonies. The bacterium produces a sour odor as it grows.

Brevibacillus laterosporus (= *Bacillus laterosporus* = *Bacillus orpheus*). Rods of *Brevibacillus laterosporus* measure 0.5–0.8 × 2.0–5.0 μm, and the spores measure 1.0–1.3 × 1.2–1.5 μm (fig. 3). An important diagnostic feature is the production of a canoe-shaped parasporal body that stains very heavily along one side and the two ends and remains firmly adhered to the spore after lysis of the sporangium. The clear portion with the finely outlined wall is the spore. *B. laterosporus* grows moderately on nutrient agar, becoming dull and opaque, and spreads actively if the agar surface is moist. Growth on nutrient agar with 1-percent glucose added (glucose agar) is more luxuriant and may become wrinkled.

Enterococcus faecalis (= *Streptococcus faecalis* = *S. apis* = *S. liquefaciens*). Ovoid cells (elongated in the direction of chain) are 0.5–1.0 μm in diameter and are usually in pairs or short chains (fig. 3). This organism

resembles *Melissococcus pluton* and may exhibit Brownian movement when the modified hanging drop technique is used. Growth occurs on nutrient agar usually within 1 day. Colonies are generally smaller than 2 mm; they are smooth and convex, with a well-defined border. When magnified, the colonies appear light brown and granular.

Bacterium eurydice (= *Achromobacter eurydice*). There is no standard description of *Bacterium eurydice*. White (1912) described this organism as a small, slender, gram-negative rod with slightly rounded ends, occurring singly or in pairs, and measuring 0.5–1.4 μm long × 0.4–0.7 μm wide. According to White (1920), *Bact. eurydice* is best isolated by plating the midgut contents of infected larvae on glucose agar and incubating at room temperature. Growth is slow and never luxuriant, and colonies are convex, smooth, and glistening. However, later researchers, who were unable to isolate *Bact. eurydice* as described by White, used the name *Bact. eurydice* for a gram-positive bacterium isolated from diseased larvae. Therefore, mention of the name in the literature causes confusion.

Paenibacillus apiarius (= Bacillus apiarius). The bacterium *Paenibacillus apiarius* is rarely encountered and may or may not be legitimately associated with EFB. Rods are 0.6–0.8 μm in diameter and often smaller at the poles. Special diagnostic features include the ridged, thick, rectangular spore coat and the stainable remnants of the sporangium, which remain attached for a considerable time. Growth can occur on Sabouraud dextrose medium.

Powdery Scale

Paenibacillus larvae subsp. *pulvifaciens* (= *Bacillus pulvifaciens*) (see appendix E) is the bacterium suspected of causing powdery scale disease. This disease is seldom reported because the incidence is low or, perhaps, because the average beekeeper is unable to identify it. A useful diagnostic characteristic is the scale that results from the dead larva. The scale is light brown to yellow and extends from the base to the top of the cell. The scale is powdery; when handled it crumbles into a dust.

P. pulvifaciens vegetative cells measure 0.3–0.6 × 1.5–3.0 μm. The spores are 1.0 × 1.3–1.5 μm. The bacterium can be isolated on nutrient agar, but growth is more luxuriant on glucose agar. When first isolated, the organism produces a reddish-brown pigment that can be lost by subculturing. *P. pulvifaciens* closely resembles *P. larvae*, but the spores do not exhibit Brownian movement in the modified hanging drop technique. Also,

P. pulvifaciens is distinguished by its ability to grow at 20° C on nutrient agar (see table 2).

Septicemia

Pseudomonas aeruginosa (= *Pseudomonas apiseptica*) is the bacterium that causes septicemia in adult honey bees. This disease results in the destruction of connective tissues of the thorax, legs, wings, and antennae. Consequently, the affected bees fall apart when handled. Dead or dying bees may have a putrid odor.

P. aeruginosa rods measure 0.5–0.8 × 1.5–3.0 μm. They are gram-negative and occur singly, in pairs, or in short chains. A bacterial smear and Gram stain (appendix A) can easily be prepared after removing a wing from the thorax and dipping the wing base in a drop of water on a microscope slide. To isolate this organism, streak the base of a wing across Difco *Pseudomonas* isolation agar or *Pseudomonas* agar F. The optimum temperature for growth is 37° C. *P. aeruginosa* in culture is characterized by the excretion of diffusible yellow-green pigments that fluoresce in ultraviolet light (wavelength below 260 nanometers).

Septicemia can also be diagnosed by reproducing the disease symptoms in healthy, caged bees. This is accomplished by preparing a water extract (macerate the equivalent of one suspect bee per ml of water) and inoculating healthy bees in the thorax (see appendix B) or dipping them in the water extract. Bees with septicemia die within 24 hours; they exhibit the typical odor and "break apart" symptom after about 48 hours.

Spiroplasmosis

Spiroplasma species is the bacterium that causes spiroplasmosis in adult honey bees. *Spiroplasma* is a helical, motile, cell-wall-free prokaryote that is found in the hemolymph of infected adult honey bees. The organism is a tiny, coiled, and sometimes branched filament 0.7–1.2 μm in diameter. Its length increases with age and ranges from 2 μm to more than 10 μm (Clark 1977, 1978a).

Spiroplasma can best be seen in the hemolymph, using dark-field microscopy (appendix A). They can also be seen by using the oil-immersion objective of a phase-contrast microscope. Hemolymph can be taken from adult bees by puncturing the intersegmental membrane directly behind the first coxae, using a fine capillary tube made from the tip of a Pasteur pipet. This organism can be cultured in standard mycoplasma broth medium

(GIBCO) and in Singh's mosquito tissue culture medium with 20-percent fetal calf serum.

Fungal Diseases

Chalkbrood

The fungus *Ascosphaera apis* causes chalkbrood disease. It is a heterothallic organism and develops a characteristic spore cyst when opposite thalli (+ and –) fuse (fig. 4). Spore cysts measure 47–140 μm in diameter. Spore balls enclosed within the cyst are 9–19 μm in diameter, and individual spores are 3.0–4.0 × 1.4–2.0 μm.

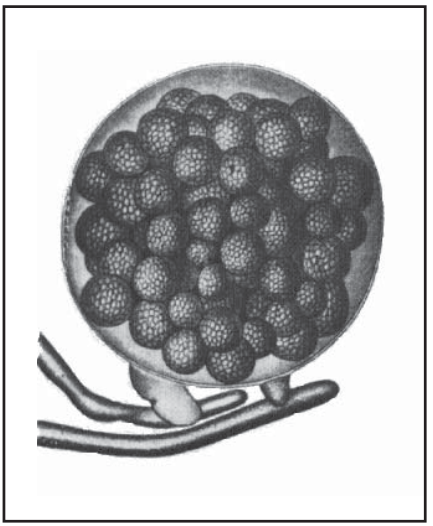


Figure 4. Spore cyst of *Ascosphaera apis* containing spore balls, which in turn contain spores.

Chalkbrood disease can be easily identified by its gross symptoms. An affected larva becomes overgrown by fluffy, cottonlike mycelia and swells to the size of the brood cell. If only one thallic strain (+ or –) is present, the larva dries into a hard, shrunken, white, chalklike mummy—thus the name *chalkbrood*. When the + and – thalli are present in a larva, spore cysts can form, and the resulting mummies appear either mottled (black on white) or completely black. In hives with large numbers of infected larvae, mummies can be found at the hive entrances or on the bottom boards. Mummies can sometimes be removed from brood cells by tapping the comb against a solid surface. This easy removal of larval remains also differentiates chalkbrood from other brood diseases.

Ascosphaera apis grows luxuriantly on potato dextrose agar fortified with 4 g yeast extract per liter. Growth and sporulation also occur on malt agar but less profusely and with no aerial hyphae; these characteristics facilitate subculturing and microscopic examination. Cultures have a characteristic fruity odor similar to that of fermenting peaches. The optimum temperature for growth is 30° C.

A. apis can be easily isolated from newly infected larvae or "soft" mummies. Place the larvae or mummies directly on the medium and incubate them. New mycelial growth is usually visible within 24 hours. Small blocks of agar containing mycelia can be transferred to new plates to obtain pure cultures and isolates of the + and – thalli. *A. apis* can be isolated from old mummies by placing the mummies on water agar (agar with no added nutrients), incubating them, and transferring the new mycelial growth to a nutrient medium. Difficulties sometimes occur because *A. apis* may fail to grow or may be overgrown by other fungi, which can contaminate old mummies.

If only one thallus (+ or –) is isolated, a fluffy cottonlike growth will eventually cover the plate. When both the + and – thalli are present, fruiting bodies form throughout the culture. The + and – thalli are morphologically identical. They can be distinguished by inoculating isolates on opposing sides of a plate. When opposite thalli grow together, a line of spore cysts forms at the juncture.

Stonebrood

Stonebrood is a larval disease usually caused by *Aspergillus flavus. Aspergillus fumigatus, A. niger*, and sometimes other *Aspergillus* species are associated with the disease. These fungi are common soil inhabitants that are also pathogenic to adult bees, other insects, mammals, and birds. The disease is difficult to identify in its early stages of infection. The fungus grows rapidly and forms a characteristic whitish-yellow collarlike ring near the head end of the infected larva. A wet mount prepared from the larva shows mycelia penetrating throughout the insect. After death, the infected larva becomes hardened and quite difficult to crush—hence the name *stonebrood*. Eventually, the fungus erupts from the integument of the insect and forms a false skin. At this stage, the larva may be covered with green, powdery fungal spores. The spores of *A. flavus* are yellow green, those of *A. fumigatus* are gray green, and those of *A. niger*, black. These spores can become so numerous that they fill the comb cells containing the affected larvae.

Stonebrood can usually be diagnosed from gross symptoms, but positive identification of the fungus requires its cultivation in the laboratory and subsequent examination of its conidial heads (fig. 5). *Aspergillus* spp. can be grown on potato dextrose or Sabouraud dextrose agars. **Caution**: These fungi can cause respiratory diseases in humans and other animals.

Protozoan Diseases

Protozoa are predominately microscopic and usually occur as single cells. No protozoa are commonly found in association with the brood of honey bees.

Nosema Disease

The protozoan *Nosema apis* causes nosema disease in adult honey bees. *N. apis* spores are large, oval bodies, 4–6 μm long × 2–4 μm wide (fig. 6). The spores develop exclusively within the epithelial cells of the ventriculus of the adult honey bee. The disease usually manifests itself in bees that are confined, so the heaviest infections are found in winter bees, package bees, bees from hives used for pollination in greenhouses, and so on.

No single symptom typifies the disease. Differences between healthy bees and heavily infected bees can be seen by removing the digestive tract and examining the ventriculus. The ventriculus of a healthy bee is straw brown, and the individual circular constrictions can be clearly seen (fig. 7). In a heavily

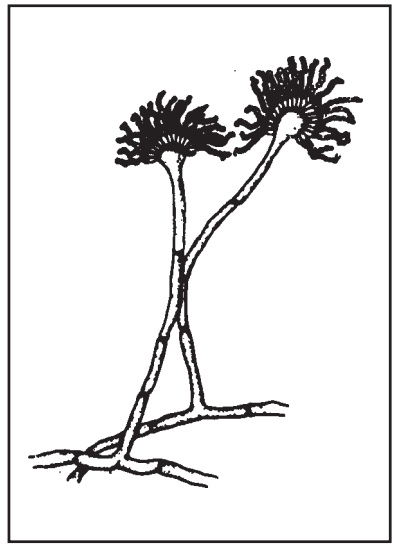


Figure 5. Conidial heads of *Aspergillus flavus.*

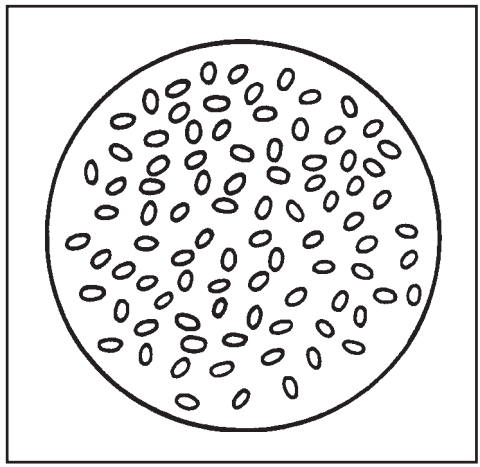


Figure 6. *Nosema* spores as they appear in a wet mount (×400).

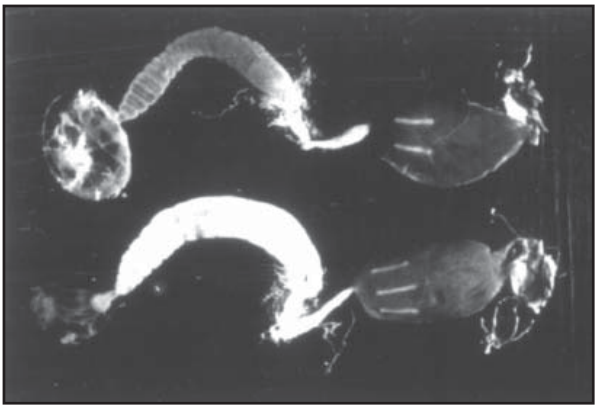


Figure 7. Honey bee digestive tracts. Top, from a healthy honey bee. Note the individual circular constrictions on the ventriculus. Bottom, from a honey bee with nosema disease.

infected bee, the ventriculus is white, soft, and swollen, and the constrictions are obscured (White 1918). However, positive diagnosis can be made only by microscopic examination of suspect bees or their fecal material for the presence of *N. apis* spores (appendix A). Samples of bees to be examined can be dried or preserved in alcohol. In a partially decomposed sample, the presence of yeasts and molds resembling *N. apis* may make accurate diagnosis difficult.

For quick, routine examinations, the abdomens from 10 or more bees are removed, placed in a dish with 1.0 ml water per bee abdomen, and ground with a pestle or the rounded end of a clean test tube. A cleaner preparation can be obtained by grinding digestive tracts removed from the abdomens. A wet mount is prepared from the resulting suspension and examined under the high dry objective of a compound microscope. Alternatively, individual bees can be examined to obtain the approximate percentage of infected bees in a colony.

Coprological examination

By examining fecal material, *Nosema* can also be detected without sacrificing workers or queens. On glass plates collect feces of worker bees near the hive entrance, scrape off a deposit, mix it with water, and prepare a wet mount from the resulting suspension (Wilson and Ellis 1966). Suspect queens can be confined in small petri dishes or in glass tubes and allowed to walk freely. They usually defecate within 1 hour. Queen feces appear as drops of clear, colorless liquid, which can then be transferred to a microscope slide with a pipet or capillary tube. Place a cover glass over the feces before examination with a high, dry objective (L'Arrivee and Hrytsak 1964).

Counting Nosema spores

A quantitative measure of levels of *Nosema* can be obtained using a hemocytometer, an instrument used to count human blood cells. It consists of a special cover glass and a ruled chamber that holds a specific volume of fluid. These chambers cost about $100 and are available from scientific supply houses. Remove a sample of the spore suspension made from the abdomens, preferably with a loop, and place it under the cover glass. When the droplet touches the chamber and cover glass, it will flow under the cover glass, thereby filling the chamber and ensuring the correct volume. Allow about 3 minutes for the spores to settle, but count them before the sample begins to dry in the chamber.

To count, find the ruled area and focus the microscope on the spores so they are sharply defined. The squares are arranged in groups of 16, with each group bounded by double lines. Count all the spores in the block bounded by the double lines. Score the spores that cross the double lines if they are on the upper or right side but not if they are on the lower or left side. A hand tally counter is convenient to count the cells within a square. To obtain a good average, count five blocks of 16 small squares.

Since each small square in the chamber has dimensions of $0.05 \times 0.05 \times 0.1$ mm, the total volume is 0.00025 mm^3, that is, 1/4000 of 1 mm^3. Find the average number of spores per square and multiply this figure by 4,000 to obtain the number of spores per cubic millimeter. To determine the number per cubic centimeter (milliliter), multiply the number per cubic millimeter by 1,000. If you started with the equivalent of 1 ml of water for each ground-up bee, you can use the following equation to determine the number of spores per bee, which is equal to the number of spores per cubic centimeter:

(total number of spores counted) (4×10^6)/number of squares counted = number of spores per bee.

The suspension must be diluted if the spores are so numerous that accurate counts cannot be made. Usually, accurate counts can be obtained with a dilution of 1 part spore suspension to either 1 or 9 parts water (which gives a dilution factor of 2 or 10, respectively). This factor must then be incorporated into the equation. For more detail see Cantwell (1970).

Removal of digestive tract

Intact digestive tracts that have been removed from adult honey bees are very useful for the detection of protozoan diseases (refer to fig. 7). The digestive tracts can be obtained easily by removing the bee's head to free the digestive tract, grasping as much of the stinger as possible with a pair of fine tweezers, and then with a steady, gentle pull withdrawing the entire digestive tract.

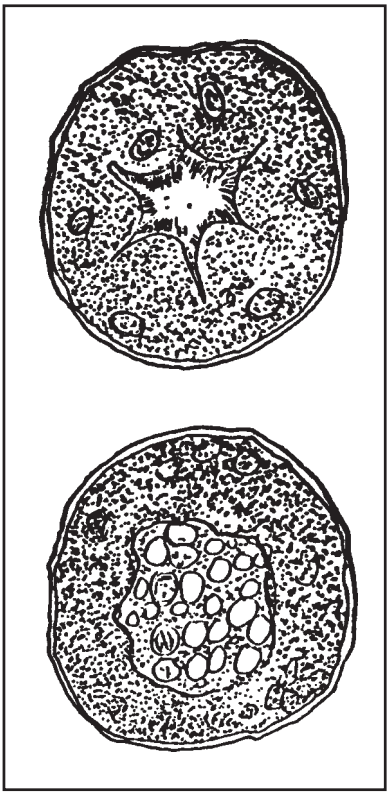


Figure 8. Cross sections of Malpighian tubules. Top, healthy tubule. Bottom, tubule containing cysts of *Malpighamoeba mellificae.*

Amoeba Disease

Malpighamoeba mellificae causes amoeba disease. Since this protozoan is found in the Malpighian tubules of adult bees, diagnosis can be made only by removing and microscopically examining the tubules for the presence of amoeba cysts. The cysts measure 5–8 μm in diameter (fig. 8).

Malpighian tubules are long, threadlike projections originating at the junction of the midgut and the hindgut. They can be teased away from the digestive tract with a pair of fine tweezers. Once this is done, place them in a drop of water on a microscope slide and position a cover glass over them, applying uniform pressure to obtain a flat surface for microscopic examination. *M. mellificae* can be discerned using a high, dry objective and then changing to the oil immersion objective for more detail.

Other Protozoa

Gregarines

Four gregarines (protozoans of the order Gregarinida) are associated with honey bees: *Monoica apis*, *Apigregarina stammeri*, *Acuta rousseaui*, and

Leidyana apis. The immature stages, or cephalonts, average about 16 × 44 μm. Cephalonts are oval and consist of two distinct body segments; the posterior segment is larger. The mature stages, or sporonts, average about 35 × 85 μm and have a reduced anterior segment.

Gregarines are found attached to the epithelium of the midgut of adult honey bees. To view gregarines, gently remove the midgut from the digestive tract of a suspect bee and place it on a microscope slide in a drop of water. The midgut can be separated from the digestive tract at the point of attachment with the proventriculus (honey stomach) and hindgut, using fine tweezers and a scalpel. Gently break open the midgut with fine tweezers and a probe, and place a cover glass over the resulting suspension. Gregarines can be seen using the low-power objective of a compound microscope.

Flagellates

The flagellates associated with honey bees are *Crithidia* (= *Leptomonas*) species. Flagellates have been found either free in the lumen or attached to the epithelium of the hindgut and rectum of adult honey bees (Fyg 1954). Flagellates vary in size from 5 to 30 μm. Some appear as pearlike bodies with flagella; others are long threadlike forms or are round without flagellae (Lotmar 1946).

To look for flagellates, remove the digestive tract of a suspect bee as described and place it in a drop of water on a microscope slide. Then, using fine tweezers and a scalpel, separate the hindgut and rectum at the point of attachment with the midgut. Macerate the hindgut and rectum, using the tweezers and a probe. Place a cover glass on the resulting suspension and observe under the high, dry objective of the microscope.

Viral Diseases

Sacbrood

Sacbrood is the only common brood disease caused by a virus. Since sacbrood-diseased larvae are relatively free from bacteria, laboratory verification is usually based on gross symptoms and the absence of bacteria. Positive diagnosis requires the use of a special antiserum or molecular techniques. Affected larvae change from pearly white to gray and finally black. Death occurs when the larvae are upright, just before pupation. Consequently, affected larvae are usually found in capped cells. Head development of diseased larvae is typically retarded. The head

region is usually darker than the rest of the body and may lean toward the center of the cell. When affected larvae are carefully removed from their cells, they appear to be a sac filled with water. Typically the scales are brittle but easy to remove. Sacbrood-diseased larvae have no characteristic odor.

Chronic Bee Paralysis

Adult bees affected by chronic bee paralysis are usually found on the top bars of the combs. They appear to tremble uncontrollably and are unable to fly. In severe cases, large numbers of bees are found crawling out the hive entrance. Individual bees are frequently black, hairless, and shiny. In some cases, paralysislike symptoms can be caused by toxic chemicals.

Ideally, the diagnosis of this disease is made using serological techniques. Since this is beyond the capability of most laboratories, diagnosis is usually made by observing symptoms in individual bees and, when possible, colony behavior. Chronic paralysis can be diagnosed by reproducing the disease symptoms in caged bees. This can be done by spraying, feeding, or injecting a water extract made from suspect bees. The extract is prepared by macerating the equivalent of one suspect bee in 1 ml water. This is then centrifuged to eliminate large suspended matter and passed through a 0.45-μm filter to remove bacteria. To feed up to 20 caged bees, mix 2 ml of the extract with an equal volume of sugar syrup. For inoculation, each bee receives 1.0 μl of the extract through a dorsal abdominal intersegmental membrane (see appendix B). The symptoms of paralysis should be visible after 6 days. Control bees should be treated with extracts made from healthy bees.

Filamentous Virus

Filamentous virus is also known as F-virus and bee rickettsiosis. This disease, previously thought to be of rickettsial origin, can be diagnosed by examining the hemolymph of infected adult bees using dark-field or phase-contrast microscopy. The hemolymph of infected honey bees is milky white and contains many spherical to rod-shaped viral particles of a size close to the limit of resolution for light microscopy. The viral particles consist of a folded nucleocapsid within a viral envelope and are 0.4×0.1 μm (Clark 1978b).

Acute Paralysis Bee Virus and Kashmir Bee Virus

Acute paralysis bee virus (APBV) and Kashmir bee virus (KBV) are two serlogically related viruses, and the antiserum produced from one virus will cross-react with the other virus (Hung et al. 1996). These viruses commonly occur in apparently healthy adult bees. No specific gross symptoms have been attributed to either virus. Whereas APBV is a disease of adults, KBV is reported to cause mortality in brood and adult honey bees. APBV and KBV diseases can be diagnosed using immunodiffusion tests. Recently, molecular methods were developed for detecting both diseases. However, immunodiffusion and molecular methods are not routinely used in our laboratory.

Disease Interactions

Paenibacillus larvae produces a potent antibiotic that eliminates competition from other bacteria typically associated with honey bee larvae. For this reason, American foulbrood (AFB) and European foulbrood (EFB) are rarely found in the same colony, except in cases where AFB is just becoming established in colonies that already have EFB.

Ascosphaera apis produces linoleic acid, which inhibits the growth of *Paenibacillus larvae* and *Melissococcus pluton*. Since the introduction of chalkbrood into the United States, the incidence of EFB has fallen dramatically. However, the incidence of AFB appears to have remained constant. It is not unusual to find chalkbrood and sacbrood on the same comb or on a comb with larvae infected with AFB, although no single larva has been found to be infected with more than one disease. This is an important point to remember when selecting a sample for disease diagnosis.

Mixed infections in adult bees are more common. Adult bees could be infected with *N. apis* and also be infected with viruses or spiroplasma. It is also possible for adult bees to be infested with one or more species of mites.

Parasitic Honey Bee Mites

Varroa jacobsoni

The mite *Varroa jacobsoni* can be found on adult bees, on the brood, and in hive debris. The adult female mite is oval and flat, about 1.1 mm long $\times$ 1.5 mm wide, and pale to reddish brown; it can easily be seen with the unaided eye. Because the mites attach to the adult bee between the

abdominal segments or between body regions (head, thorax, abdomen), they are difficult to detect. However, they can be easily seen against the white surface of pupae. Male mites are considerably smaller, are pale to light tan, and are rarely encountered (Delfinado-Baker 1984).

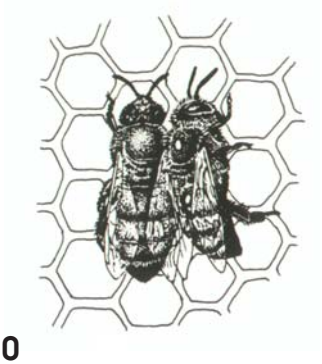
The life cycle of *V. jacobsoni* is summarized in figure 9. It is important to note that the bee-louse, *Braula coeca*, resembles *V. jacobsoni* in size and color. However, being an insect, *Braula* has six legs that extend to the side (fig. 10). *Varroa*, an arachnid, has eight legs that extend forward (fig. 11). The most severe parasitism occurs on older bee larvae and pupae, with the mite preferring drone brood to worker brood (Ritter and Ruttner 1980). In heavy infestations, pupae may not develop into adult bees. Adults that do emerge may have shortened abdomens, misshapen wings, and deformed legs and may weigh less than healthy bees (De Jong et al. 1982b). The misshaped wings are believed to be caused by deformed wing virus. *V. jacobsoni* may act as a vector of the virus, introducing it to the pupae during feeding.

Sampling techniques

When sampling, remember that the number and location of *V. jacobsoni* in a colony vary according to time of year. The number of mites is lowest in spring, increases during summer, and peaks in fall. During spring and summer, most mites are found on the brood (especially drone brood). In late fall and winter, most mites are attached to adult worker bees.

To examine adults, collect 200 to 300 bees. This can be done by brushing the bees off the comb through a large-mouthed funnel (paper or cardboard, for example) or directly into a container. Individual honey bees can be examined with or without the aid of a hand lens or dissecting microscope. When the mites are moving about on a bee, they are fairly easy to detect; but once they attach themselves between abdominal segments, they may be difficult to find. Mites can be collected from adult bees as follows:

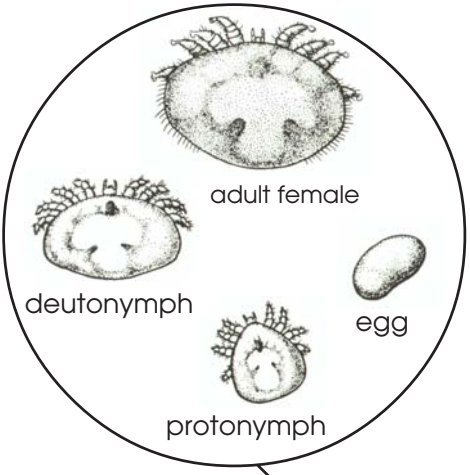
Shaking. *Varroa jacobsoni* can be dislodged by shaking the bees in liquids such as hot water, alcohol, or detergent solution. We recommend 70-percent alcohol (ethyl, methyl, or isopropyl); the alcohol kills the bees and preserves them for use for other purposes, such as examination for *Acarapis woodi*. De Jong et al. (1982a) found that hand-shaking bees in alcohol for 1 minute dislodged about 90 percent of the mites. The mites are collected by passing the bees and alcohol through a wire screen (8- to 12-mesh) to remove the bees and then sieving the alcohol through cotton cloth. The cloth is then examined for mites.



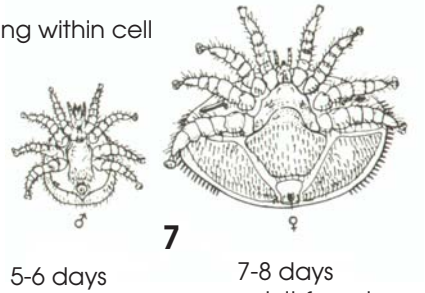
10
Mite transfer through
close contact between bees

1
Adult bee, with
Varroa feeding
on hemolymph

9
Adult females leave cell
with emerging bee.
Male and immature stages
stay in cell.



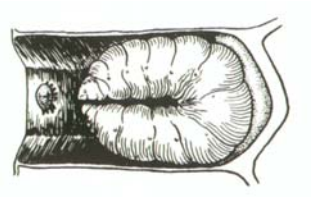
8
Mating within cell



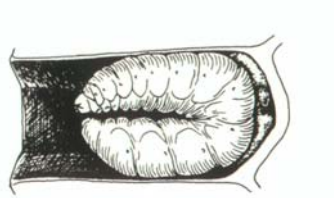
7
5-6 days
adult male
7-8 days
adult female

6
1-6 eggs developing
from egg to larva to
protonymph to
deutonymph

Figure 9. Life cycle of *Varroa jacobsoni*. (Courtesy of Roger A. Morse)



2
Mite enters cell with larva of 5 to 5-1/2 days



3
Mite in bee food

4
Mite feeds on prepupa.

5
Female lays first egg 60 hours after cell capping. Female lays subsequent eggs at 30-hour intervals.

Ether roll. This technique is a rapid and efficient detection method in the field and minimizes the handling, shipping, and time-consuming procedures associated with shaking adult bees in alcohol or other solvents that best require a laboratory. Collect 200 to 300 bees in a jar and anesthetize them with ether delivered from an aerosol can (this aerosol product is sold in auto-parts stores as an aid to start engines). A 1- to 2-second burst of aerosol is adequate. Roll the jars lengthwise for about 10 seconds. The majority of mites dislodge from their hosts and adhere to the inside wall of the jar. To complete the process, deposit the bee sample on a white surface and spread it around. This should cause any remaining mites to fall onto the white substrate. The bees should be immediately emptied from the jar because the mites tend to stick to them if left in the jar for more than a few minutes. The bees can be washed (see shaking method above) if a more precise count is desired.

Powdered sugar method. Reported to be more efficient than the ether roll method, this method does not require bees to be killed. Use a wide-mouth, pint or quart canning jar. Set aside the metal ring from the two-piece lid; discard the center portion. Cut a circle of #8-mesh hardware cloth to fit inside the ring. Collect 200 to 300

Figure 10.
Braula coeca,
dorsal view.

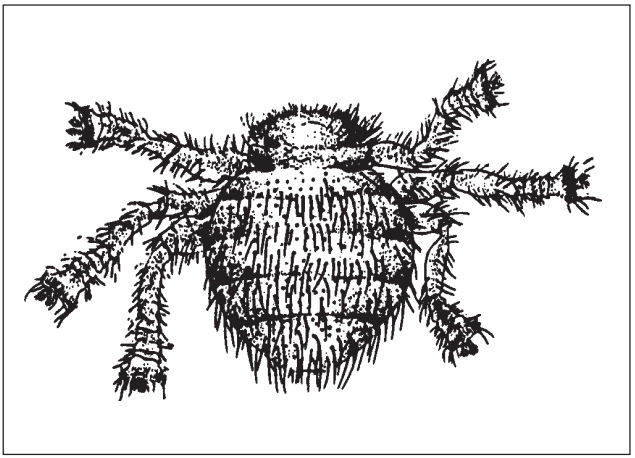
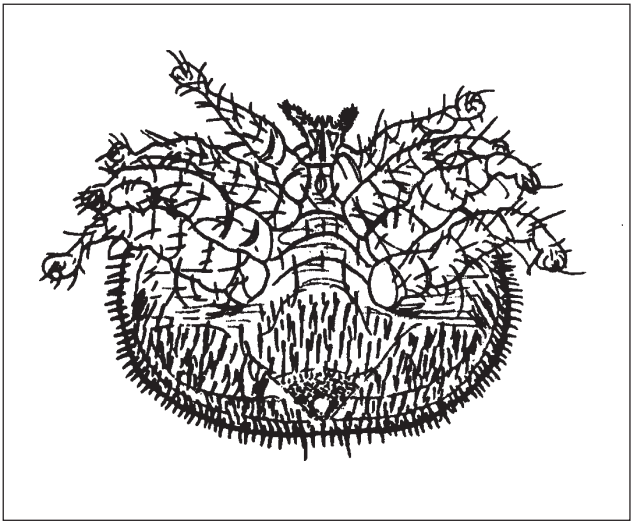


Figure 11.
Varroa
jacobsoni,
ventral view.



bees in the jar. Through the #8-mesh lid, add enough powdered sugar to the jar to coat the bees, about 1 teaspoon to 1 tablespoon. Roll the jar around to distribute the sugar, allow the jar to sit for a few minutes, then invert it, and shake it over a piece of paper to recover the mites. The mites and sugar will pass through the mesh to the paper. Pour the sugar and mites into another jar fitted with a fine-mesh lid and shake, allowing the sugar to escape. Then dump the mites on a clean sheet of paper and count them. A brief shaking will usually recover 70 percent of the mites; if you persist a little longer, 90 percent can be recovered (Ellis 2000).

Heating method. Live adult honey bees can be shaken into a wire-based cage and placed in an oven over white paper. The bees are heated for 10 to 15 minutes at 46° or 47° C. The *Varroa*, if present, fall off the bees and can be observed on the white paper (Crane 1978).

To look for mites on brood, examine the pupae (preferably drone) individually. *V. jacobsoni* can be easily seen against the white surface of worker or drone pupae after they are removed from their cells. Remember to also examine the bottom of the cells. It is suggested that a minimum of 100 drone pupae per colony be examined.

The classic method of pupal collection is to uncap each cell and remove the pupa with forceps. Groups of pupae can be quickly and easily removed by inserting a capping scratcher at an angle through the cappings and lifting the brood and cappings upward (Szabo 1989). Alternatively, the brood comb can be incubated at 37° C, followed by examination of all the emerged bees and remaining brood.

Debris in a hive, such as wax particles, pollen, dead bees and brood, and mites, normally falls to the hive floor and is removed by housecleaning bees during warm weather. The collection and examination of hive debris for mites can be facilitated by placing white construction paper on the hive floor. Staple the paper under a wire frame constructed with a 1/4-inch wood frame and 8- to 12-mesh wire and keeping a 1/4-inch separation between paper and wire. The wire protects the paper and debris from the bees. Examine the paper for mites, which can easily be seen against the white background. A magnifying glass or dissecting microscope may be helpful in locating the mites in the debris. Sticky boards or shelf paper (with the adhesive surface exposed) help hold the mites. Substances such as petroleum jelly, cooking sprays, oils, and tanglefoot have been used for preparation of sticky boards.

The acaricides used to treat mite infestations can also be used in combination with the paper method to detect *V. jacobsoni*. Apistan is currently approved and available for this purpose. After treatment, the mites drop to the paper and are easily detected. It is important that the paper have a sticky surface to hold any recovering mites.

A flotation method can be used to examine debris for *V. jacobsoni*. Place hive debris in a jar or pan and cover with 98-percent alcohol. The mites float to the surface while the heavier debris sinks (Ritter and Ruttner 1980).

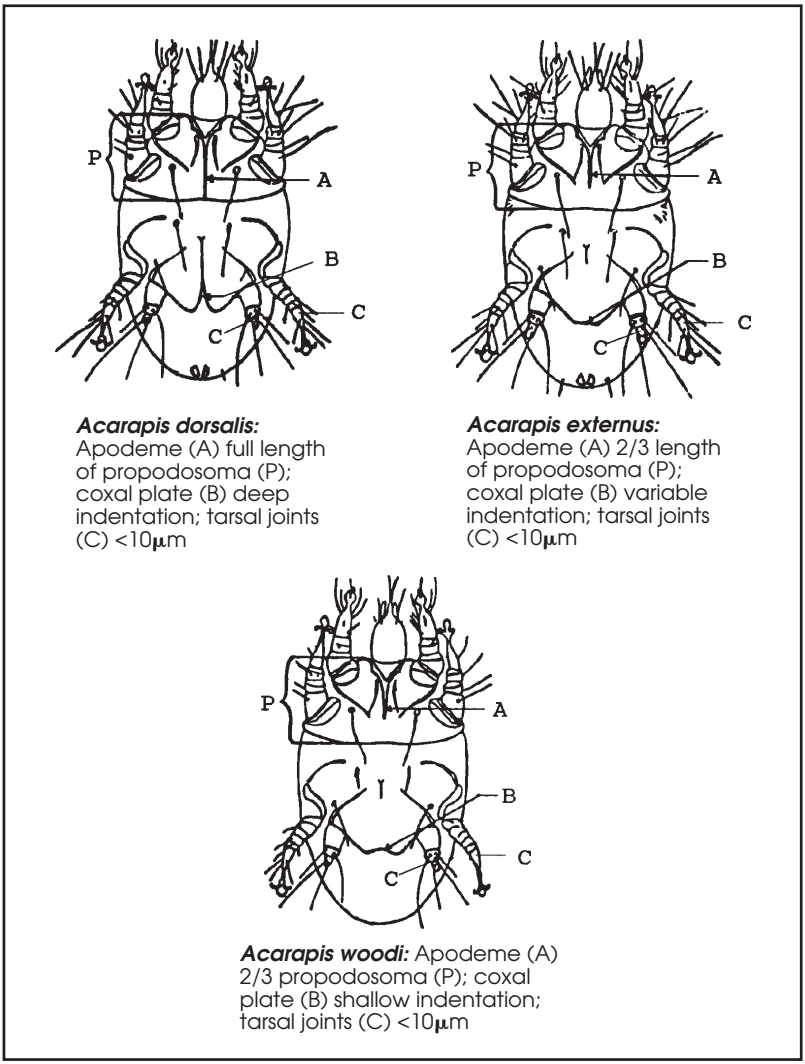
Parasitic mite syndrome

Colonies infested with parasitic mites can display an array of symptoms referred to as the parasitic mite syndrome. The syndrome affects both adult bees and brood. It is quite likely this syndrome is similar to that reported by Ball (1988) who refers to the condition as a "secondary infection" in colonies infested with *V. jacobsoni*. Some of the symptoms are listed below. Not all symptoms may be evident in a colony at a given time:

1. Presence of *V. jacobsoni*
2. Reduction in adult bee population
3. Evacuation of hive by crawling adult bees
4. Queen supersedure
5. Spotty brood pattern
6. Affected brood can vary from C-stage larva to prepupa
7. Individual larva may appear
   - twisted in the cell
   - "molten" in the bottom of the cell
   - light brown, gray to black in color
   - watery to pasty consistency, resembling EFB, AFB, and sacbrood
8. Scales are not brittle and are easy to remove
9. No typical odor
10. No characteristic microflora

Honey Bee Tracheal Mite (*Acarapis woodi*)

Three *Acarapis* species are associated with adult honey bees: *Acarapis woodi*, *A. externus*, and *A. dorsalis*. Only *A. woodi* is known to be harmful. All three species are difficult to detect and identify because of their small size and similarity, so they are frequently identified by location on the bee instead of by morphological characters. *A. woodi* can be diagnosed solely on habitat; the position of other species on the host is a useful but not an infallible characteristic. *A. woodi* lives exclusively in the prothoracic tracheae. *A. externus* inhabits the membranous area between the posterior region of the head and thorax or the ventral neck region and the posterior tentorial pits. *A. dorsalis* is usually found in the dorsal groove between the mesoscutum and mesocutellum and the wing bases. Morphological characters differentiating these species are shown in figure 12. For complete descriptions and illustrations see Delfinado-Baker and Baker (1982).



Acarapis dorsalis: Apodeme (A) full length of propodosoma (P); coxal plate (B) deep indentation; tarsal joints (C) <10μm

Acarapis externus: Apodeme (A) 2/3 length of propodosoma (P); coxal plate (B) variable indentation; tarsal joints (C) <10μm

Acarapis woodi: Apodeme (A) 2/3 propodosoma (P); coxal plate (B) shallow indentation; tarsal joints (C) <10μm

Figure 12. Morphological characters separating *Acarapis* species.

The female *A. woodi*, or honey bee tracheal mite, is 143–174 μm long and the male 125–136 μm. The body is oval, widest between the second and third pair of legs, and whitish or pearly white with shining, smooth cuticle. A few long hairs are present on the body and legs. This mite has an elongate, beaklike gnathosoma with long, bladelike styles (mouthparts) for feeding on the host.

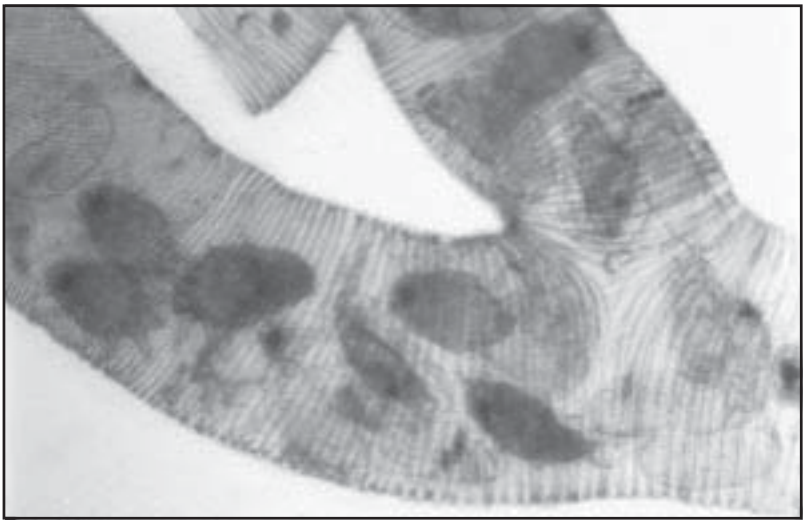


Figure 13. Honey bee trachea containing mites (×100).

No one symptom characterizes this disease. An affected bee could have disjointed wings and be unable to fly, have a distended abdomen, or both. Absence of these symptoms does not necessarily indicate freedom from mites. Positive diagnosis can be made only by microscopic examination of the tracheae. *A. woodi* is the only mite found in the bee tracheae, so this is an important diagnostic feature.

The trachea must be examined carefully for the presence of mites. The trachea of a severely infested bee may have brown or black blotches with crustlike lesions and be obstructed by many mites in different stages of development (fig. 13). However, the trachea may not be discolored when mites are present, and a cloudy or discolored trachea does not always contain mites.

Sample collection and preservation

The population of *A. woodi* may vary seasonally. During the period of highest bee population, the number of bees with mites is reduced. The likelihood of detecting tracheal mites is highest in the fall and winter. In sampling, one should try to collect either moribund bees that may be crawling near the hive entrance or bees at the entrance as they leave or return to the hive (older bees). These bees should be killed and preserved

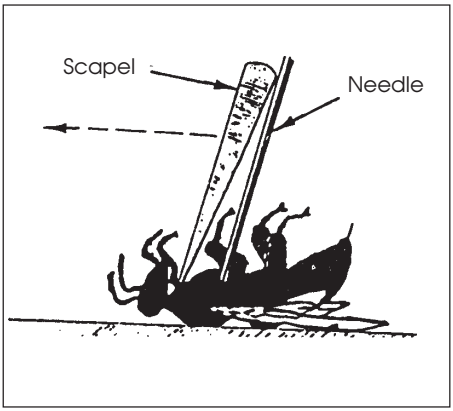


Figure 14. Positioning a bee for dissection.

in 70-percent ethyl, methyl, or isopropyl alcohol as soon as they are collected.

Methods for detecting *A. woodi*

Classic technique. Pin the bee on its back and remove the head and first pair of legs by pushing them off with a scalpel or razor blade in a downward and forward motion (fig. 14). Using a dissecting microscope, remove the first ring of the thorax (tergite of prothorax) with forceps. This exposes the tracheal trunks in the mesothorax (fig. 15). When the infestation is light, it is necessary to remove the trachea. Place the trachea in a drop of 85-percent lactic acid on a glass slide, and cover with a cover glass for examination at 40× to 100× on a compound microscope.

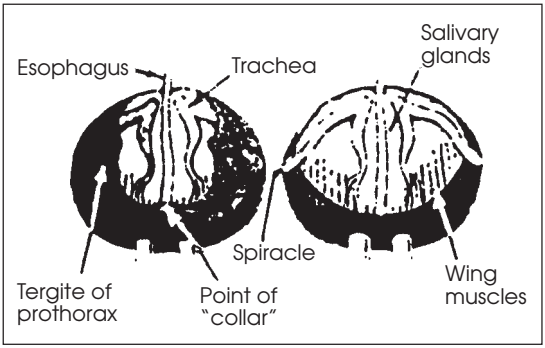


Figure 15. Location of the trachea in the thorax.

Quick examination of a few bees. Put the bee on its back, grasp it with forceps or between your thumb and forefinger, and remove the head and first pair of legs by pushing them off with a scalpel or razor blade in a downward and forward motion. Then with a scalpel, razor blade, or fine pair of scissors, cut a thin transverse section from the anterior face of the thorax in such a way as to obtain a disk. Place the disk on a microscope slide and add a few drops of lactic acid. This makes the material more transparent and helps to separate the muscle. With the aid of a dissecting microscope, carefully separate the muscles and examine the trachea. Suspicious trachea should be removed and examined as in the classic technique above.

Examination of large numbers of bees. Slice transverse-section thoracic disks as described directly above, place them in a small dish containing 5- to 10-percent potassium hydroxide (KOH), and incubate at about 37° C for 16 to 24 hours. The KOH dissolves the muscle and fat tissue, leaving the trachea exposed. Examine the disk-trachea suspension under a dissecting microscope. Suspect trachea should be removed and examined as in the classic technique.

Examination of unpreserved, dry bees. Cut a few thoracic disks as described in the quick method above, place them on a slide, and add a few drops of 10-percent potassium hydroxide. Heat the slide gently for 1 to 2 minutes (do not boil), cover with a cover glass, crush the disks lightly, and examine microscopically.

Methylene blue staining technique (Peng and Nasr 1985). Prepare transverse-section disks from the thoraxes of 50 bees as described in the quick method. Place the disks in a beaker of 8-percent potassium hydroxide solution, and heat to boiling while gently stirring continuously. Remove the solution from the heat and continue stirring until the soft tissues inside the disks are dissolved and cleared (about 10 minutes). Excessive stirring and heating will damage the specimens and subsequently reduce the color intensity of the mites. Recover the disks from the KOH by filtration through a perforated Tissue-Tek processing capsule.

After filtration, cover the processing capsule with a lid, place in a beaker, and wash with tap water to remove the remaining KOH. Transfer the processing capsule to a modified methylene blue staining solution (prepared by first dissolving 1-percent aqueous methylene blue and then adding sodium chloride to make a 0.85-percent sodium chloride solution). Immerse the capsule in that solution for 5 minutes and then in distilled water for 2 to 5 minutes. Finally, rinse the capsule with 70-percent ethyl alcohol. Examine the disks for stained mites within the tracheae under a dissecting microscope.

Differentiation of live mites from dead mites (Eischen et al. 1986). Anesthetize live bees with carbon dioxide and remove the abdomens with a scalpel to prevent being stung during examination. Remove the head and first pair of legs by holding each bee on its back and gently pushing this section off with a downward and forward motion. Holding the bee in this position, place each under a dissecting microscope and remove the first ring of the thorax with fine forceps. This exposes the tracheal attachment to the thoracic wall, which is often the only location of mites in a light

infestation. With tweezers, remove tracheae that appear abnormal and transfer them to a glass slide; as the moist tracheae dry, they will adhere to the slide. Then dissect the tracheae using a pair of fine needle probes. Mites are considered dead if they do not move. Dead mites often appear discolored and desiccated; living mites have a translucent gray or pearl color and move when prodded with a probe. This is the method of choice for evaluating chemicals used to control tracheal mites.

Serodiagnosis. Ragsdale and Furgala (1987) produced an antiserum against extracts of *Acarapis woodi*-infested tracheae to be used as the primary antibody in a direct enzyme-linked immunosorbent assay (ELISA). Ragsdale and Kjer (1989) improved the ELISA technique, making it reliable for the detection of *A. woodi*. Grant et al. (1993) developed a practical ELISA for tracheal mite detection. Their technique consists primarily of the following:

- Salt preservation. Alcohol preservatives destroy the mite components detected by ELISA. Saturated salt preservative is made by dissolving sodium chloride in boiling water until no more will dissolve (>30 percent weight by volume).
- A desalting step in an ELISA buffer solution is required before the bees are homogenized.
- Whole, desalted bee samples (100 bees) are homogenized in ELISA buffer for 60 seconds using a blender.
- A small, filtered sample is used in the ELISA analysis.
- The final steps involve a yellow color reaction and reading this color intensity in an ELISA reader.

This technique is reliable for samples with a greater than 5-percent infestation. For detecting lower infestations, one should analyze larger number of bees in a sample or analyze the thorax or slices of thorax instead of whole bees.

Tropilaelaps clareae

The distribution of *Tropilaelaps clareae* (fig. 16) is still restricted to Asia. These mites parasitize brood. They have also been reported to infest colonies with *Varroa jacobsoni* (Delfinado-Baker and Aggarwal 1987). Female mites are about 1 mm long and 0.6 mm wide; the male is slightly smaller. The mites are difficult to detect because of their small size and their brownish color, but they can be seen under a magnifying glass or by using a dissecting microscope to examine a brood comb suspected of being infested. In the field, when the comb is jarred on a light-colored

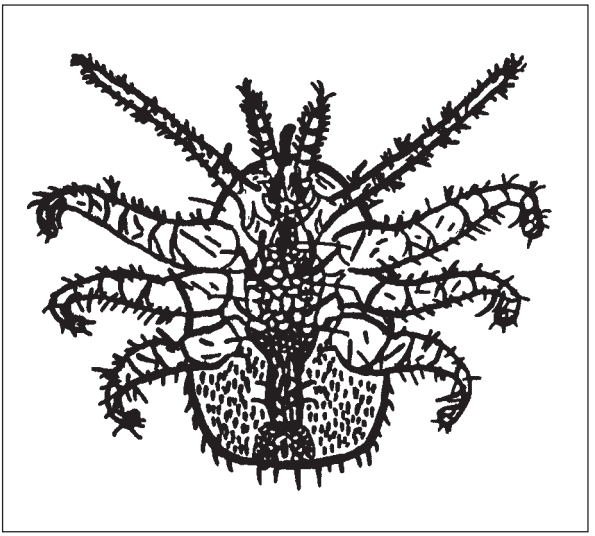


Figure 16. *Tropilaelaps clareae,* ventral view.

surface, dislodged mites may be seen moving on that surface. The mites can be picked up with a fine brush moistened with alcohol.

Pests

Wax Moths

The greater wax moth, *Galleria mellonella* (fig. 17), is the most serious pest of honeycombs. Comb damage can also be caused by the lesser wax moth, *Achroia grisella* (fig. 17), and the Mediterranean flour moth, *Anagasta kuehniella*. These moths are an especially serious problem in tropical and subtropical climates, where warm temperatures favor their rapid development. Female greater wax moths lay their eggs in a cluster, usually in the cracks or between the wooden parts of the hive. The larvae are the destructive stage. They actually obtain nutrients from honey, cast-off pupal skins, pollen, and other impurities found in beeswax, but not from the beeswax itself. Consequently, older combs are more likely to be damaged than new combs or foundation.

Small Hive Beetle

Aethina tumida, the small hive beetle (SHB), was recently discovered in the Southeastern and Mid-Atlantic states. In South Africa, where it was

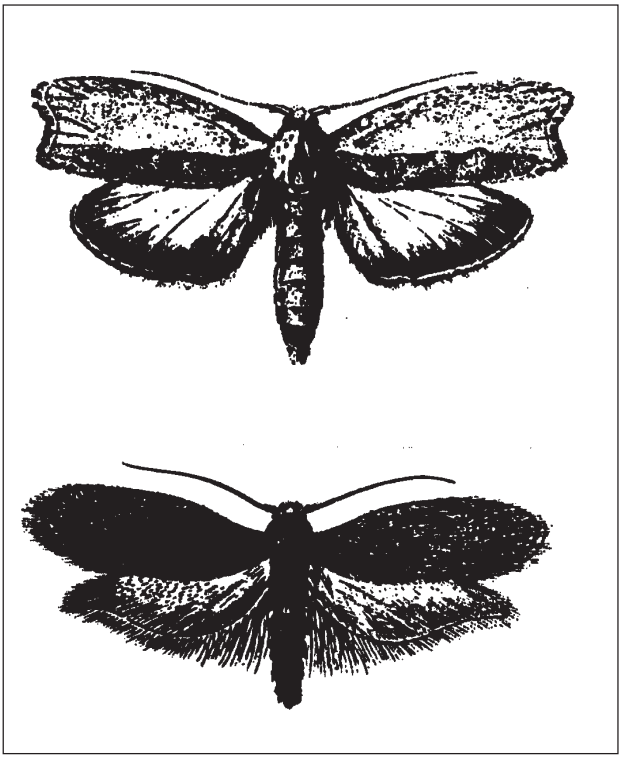
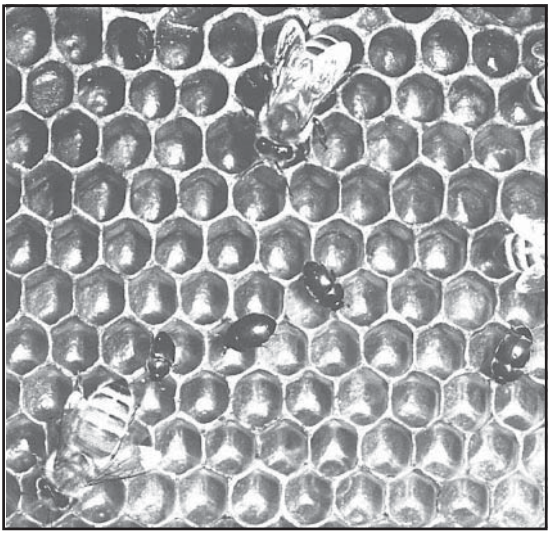


Figure 17. Top, greater wax moth, *Galleria mellonella*. Bottom, lesser wax moth, *Achroia grisella* (not to scale). These species are easily distinguished from each other by the shape of the wings and their comparative sizes. The greater wax moth is about two times larger than the lesser wax moth.

first discovered in association with honey bee hives, the beetle is rarely an economic pest. In the southern United States, it has caused thousands of colonies to abscond, destroyed the marketability of honey, and rendered combs useless.

Adult SHB are reddish brown and about 1/4 inch long, roughly one-third the length of an adult worker honey bee (fig. 18). Adult beetles can be found in brood cells, on the bottom board, or on the inner cover. The eggs are laid in empty cells and are about half the size of honey bee eggs. The larvae can get as large as 1/2 inch (fig. 19). They feed on brood, pollen, and honey, then leave the hive, and pupate in the soil close by. Honey that the larvae have fed upon becomes discolored and frothy and smells like fermented fruit. If you have trouble identifying the insect, contact your state apiary inspector or extension specialist for help. If help is not available, send your sample in 70-percent alcohol to the Bee Research Lab (see appendix C for address).

Figure 18. Small hive beetle, adults.



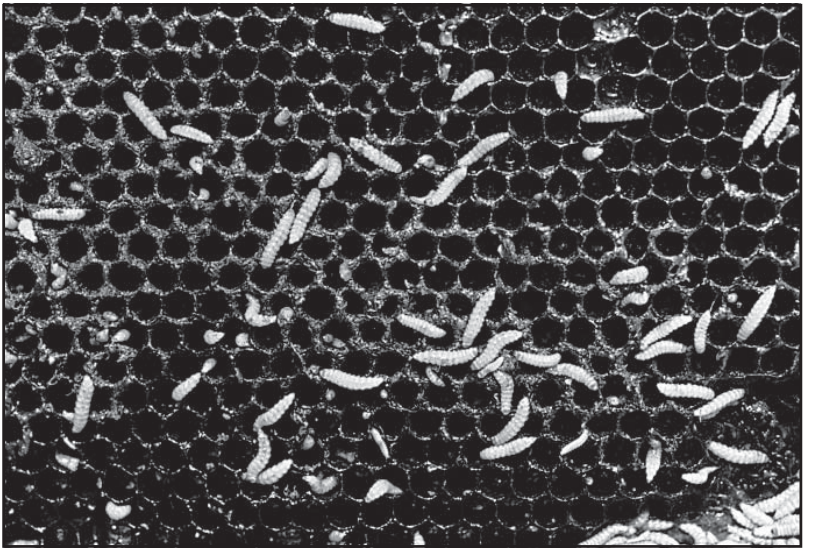


Figure 19. Small hive beetle, larvae.

Bee-louse

Braula coeca, or bee-louse (fig. 10), is actually not a louse but a wingless fly that feeds on honey. No detrimental effect on adult bees has been attributed to the bee-louse, but its larvae can damage the appearance of comb honey. Adult *B. coeca* can be found on adult workers and queens. It is important to note that *B. coeca* resembles *Varroa jacobsoni* in size and color. However, *Braula*, being an insect, has six legs that extend to the side. *Varroa*, an arachnid, has eight legs that extend forward.

Melittiphis alvearius

Melittiphis alvearius is a little-known mite that is associated with adult honey bees but is not considered to be a pest. It is unlikely that *M. alvearius* would be confused with other mites found in honey bee colonies. The adult female mite is ovate, flattened dorsoventrally, 0.79 mm long × 0.68 mm wide, brown, and well sclerotized with numerous stout and spinelike setae. It is included here because it was found in California during a survey for *Varroa* and because of increasing reports of its distribution (Delfinado-Baker 1988).

Noninfectious Disorders

Noninfectious disorders can be the result of neglect, lethal genes, pollen or nectar from poisonous plants, toxic chemicals (pesticides), and the like. Most often, dead or discolored pupae result from a noninfectious condition. For a good review of noninfectious diseases, see Calderone and Tucker (1997).

Neglected Brood

Normally, nurse bees feed and protect the brood. However, if there is a sudden shortage of adult bees, the larvae and pupae suffer and may die of chilling, overheating, or starvation.

Chilled brood

Chilling usually occurs in early spring when brood nests expand rapidly, there is a shortage of adult bees to cover all the brood, and the weather suddenly turns cold. Chilled brood are found most often on the fringes of the brood area, and healthy brood remain at the center. Chilling can also happen during cold weather following any sudden reduction of the worker bee population. Chilled larvae and pupae are often yellowish, tinged with black on segmental margins. They may also be brownish or black,

crumbly, pasty, or watery. In extreme cases, brood cells are punctured and uncapped, and pupae are decapitated by the adult bees. It should be remembered that decapitation can also be caused by the larvae of the lesser wax moth.

Starved brood

Normally when there is a shortage of food in a colony, brood are removed or consumed by the adult bees or both. However, when there is a sudden shortage of adult bees to feed the larvae, the larvae starve. Affected larvae are not restricted to the periphery of brood combs. The most striking feature of starved brood is larvae crawling out of the brood cells in search of food. Starved brood is almost always restricted to the larval stage. However, emerging bees may starve if they were stressed as pupae by chilling or overheating and if there are too few nurse bees to feed them soon after they have chewed through their cappings. In these cases, emerging adult bees usually die with only their heads out of the cells and their tongues extended.

Overheating

The overheating of brood develops when there is a sudden loss of worker bees to cool the colony during hot weather. Larvae that died from overheating become brownish or black and are watery; pupae have a black, greasy appearance. Newly emerged adult bees may be wingless. Cappings of brood cells can appear melted, darkened, sunken, and punctured.

Worker bees can overheat if they are confined in their hives during hot weather without proper ventilation or access to water. Adult bees dying from overheating crawl about rapidly while fanning their wings. They are often wet, and their wings appear hazy. In some cases, an abnormally large accumulation of dead bees may be seen at the hive entrance.

Genetic Lethality

Bees can also die from genetic faults during all stages of development, usually without exhibiting symptoms of known diseases. However, drone brood from laying workers and drone-laying queens often die with symptoms resembling EFB but in the absence of known pathogenic agents. Genetic lethality is the suspected cause of this condition.

Plant Poisoning

Poisonous plants can be a problem under certain conditions in limited areas. If a plant's nectar is poisonous, the symptoms of plant poisoning are limited to the blooming period. If the poison is in the pollen, the symptoms may linger as long as the pollen remains in the combs. There is no clear-cut method for differentiating between plant poisoning and pesticide poisoning. The effects of plant poisoning are usually more gradual and last longer than the effects of pesticide poisoning. Plant poisoning usually occurs in the same geographical area at the same time each year, whereas pesticide poisoning is indiscriminate. Some examples of plant poisoning are listed below and in table 3. For a good review of poisonous plants, see Skinner (1997).

Purple brood

Purple brood occurs when adult bees collect and use the pollen and nectar from *Cyrilla racemiflora* (titi, southern leatherwood). This "disease" is characterized by the blue or purple color of the affected larvae.

Paralysis

Aesculus californica (California buckeye) is probably the best known of the poisonous plants in the United States. Field bees exhibit symptoms similar to those of chronic bee paralysis; specifically, the bees are black and shiny from loss of hair and they tremble. Also, either the eggs do not hatch or the larvae die soon after hatching.

Milkweed pollinia

The pollen of milkweed (*Asclepias* spp.) is produced in pollinia (coherent pollen grains) that are attached in pairs by a slender filament. When removed from a flower, the pollinia resemble a wishbone with pollen masses hanging from the ends. Honey bees become ensnared in the thin pollinia attachment and free themselves by pulling the pollinia from the flower. Honey bees often become seriously encumbered and cannot remove themselves from the flower, or they may have difficulty flying and crawl back to their hives with the pollinia still attached to their body parts.

Pesticide Poisoning

The most apparent indication of serious pesticide poisoning is the sudden loss of adult bees. This loss is characterized by the appearance of many

Table 3. Comparative symptoms in honey bees poisoned by toxic chemicals and selected plants

Source of poison	Stages most affected	Effect on adult	Effect on brood	Effect on colony
Toxic chemicals	Adult	Field bees die in or near hive or in field. Nurse bees may also die. Queens usually not affected.	Usually few larvae killed. Symptoms of starvation may be evident if adult population reduced severely.	Weakened or killed. Many dead bees near hive.
California buckeye (*Aesculus californica*)	Young brood	Emerging young workers often deformed, pale. Some hairless and tremble. Queens lay eggs at reduced rate, cease, or become drone layers.	Eggs normal at outset; later fail to hatch or all are drone eggs. Larvae die soon after hatching and disappear. Little or no capped brood; if present, scattered.	Weakened or killed. May be many dead bees near entrance. Supersedure of queen may fail.
Yellow jessamine (*Gelsemium sempervirens*)	Larva, pupa, and young adult	Young workers affected and soon die. Old adults appear normal.	Pupae die in cells and become mummified.	Slightly to to severely weakened.
Loco plants (*Astragalus* spp.)	Adult and pupa	Field bees die. Some become black and tremble. Queen may die.	Many cells contain dried pupae.	Population dwindles. Colony may die.
False hellebore (*Veratrum californicum*)	Adult	Many field bees die between plants and hive. Adults die in curled state. Queens not affected.	No effect.	Field population lost.
Southern leatherwood (*Cyrilla racemiflora*)	Larva	No effect.	Many blue or purple larvae. Larvae die in cells when nearly mature.	Slight to severe weakening.

Source: Modified from Burnside and Vansell (1936).

dead and dying adult bees and sometimes pupae at the colony entrances. In many instances, however, the bees are lost in the field before returning to the colony. If the pesticide is brought back to the hive by the foragers, the nurse bees die from feeding on contaminated honey or pollen and the brood exhibits symptoms of neglect or poisoning. The symptoms of poisoned honey bees often depend on the class of pesticide involved. For a good review of poisoning from pesticides, see Atkins (1992).

Sample collection and preservation

Sampling must be done in a way that protects the sample from decomposition and further contamination. In the case of suspected pesticide poisoning, a sample of dying bees (at least 100) is placed in a clean, dry, preferably glass container. For comb containing pollen, honey, or brood, an ounce or two can be placed in the same sort of container. Be sure to include the date and time, location of hive, and name of beekeeper. Samples can be stored for a few hours on ice but should be moved as soon as possible to the freezer (the colder the better) until analysis. Leaving samples unrefrigerated will render them useless. Check with your state bee specialist or department of agriculture about laboratories capable of doing pesticide analyses. The ARS Bee Research Laboratory does not do pesticide analyses.

Appendix A. Microscopy

Most bee diseases can be diagnosed by observing the associated pathogens or parasites with a compound light microscope. A stereoscopic (dissecting) microscope is useful in identifying large parasites and pests.

Types of Microscopy

The following types of microscopy are referred to in this handbook:

- Bright-field. In bright-field microscopy, the microscopic field is brightly lighted and the objects being studied appear dark. The microscope should be equipped with two dry objectives (10× and 40×), an achromatic oil-immersion objective (100×), and a good light source. The bright-field microscope is used for most routine microscopic procedures.

- Dark-field. The effect produced by the dark-field technique is a black background against which objects are brilliantly illuminated. This is accomplished by equipping the light microscope with a dark-field condenser. Dark-field microscopy is particularly valuable for examination of unstained microorganisms suspended in fluid.

- Phase-contrast. The phase-contrast technique employs controlled illumination, which is obtained by special phase-contrast objectives and a condenser assembly attached to a conventional light microscope. This system of controlled illumination reveals differences in cells not discernible by other microscopic methods.

- Fluorescence. If a mixture of bacteria is treated with a solution of fluorescent stain, the organisms that combine with or otherwise take up this substance become fluorescent and can be detected in a microscopic field illuminated with ultraviolet light.

- Electron. The electron microscope employs waves of electrons and magnetic fields to produce the image, whereas the light microscope uses waves of light and lenses.

Preparations for Microscopic Examination

Simple stain. Morphological studies of bacteria are generally done on fixed and stained preparations. When a single dye is used, the process is referred to as simple staining. Residue from a suspected cell is first mixed

with water. Then a drop of this suspension (smear) is spread on a glass microscope slide over an area of about 2 c^2.

The smear is dried and fixed under a heat lamp, or the smear can first be air-dried and then heat-fixed by passing it, smear side up, several times over a bunsen burner flame. The fixed smear is stained with a suitable spore stain for 10 seconds. Carbol fuchsin, methylene blue, and safranin are examples of stains that can be used. Enough stain should be placed on the slide to cover the entire smear. The excess stain is then washed off with water. Air-dry or gently blot the stained smear. Place a drop of immersion oil directly on the smear. No cover glass is necessary. Examine the slide, using the oil immersion objective. Results: Organisms are uniformly stained and easily distinguished.

Gram stain. The gram stain is a standard microbiological method that can be substituted for the simple stain. Briefly, the procedure involves a fixed smear that is stained with crystal violet, immersed in iodine solution, decolorized in ethyl alcohol, and counterstained with safranin. Results: gram-positive organisms are blue; gram-negative organisms are red.

Modified hanging drop. The modified hanging drop technique (Michael 1957) can be very useful for differentiating brood diseases. Residue from a suspected cell is first mixed with water. Then a drop of this suspension (smear) is placed on a cover glass. The suspension used should always be dilute and only slightly turbid. The smear is dried and fixed under a heat lamp, or the smear can be air-dried and then heat-fixed by passing it, smear side up, several times over a bunsen burner flame.

The fixed smear is stained with carbol fuchsin[3] or a suitable spore stain for 10 seconds. Enough stain should be placed on the cover glass to cover the entire smear. The excess stain is then washed off with water. While the smear is still wet, the cover glass is placed smear side down onto a standard microscope slide previously coated with a very thin layer of immersion oil. The slide is gently blotted dry and examined with a microscope, using the oil immersion objective. Results: Organisms that are not heat-fixed are caught in areas where pockets of water formed in the oil, and the organisms usually exhibit Brownian movement (see American foulbrood, p. 4).

[3]Solution A: 0.3 g basic fuchsin (90% dye content), 10 mL ethyl alcohol (95%); solution B: 5 g phenol, 95 mL distilled water. Mix solutions A and B.

Negative staining. This is a technique by which, without staining, cells are made readily visible in an otherwise dark film. The advantage of this procedure is that the bacterial cells do not receive vigorous physical and chemical treatment, which could result in distortion of some cells. Residue from a suspected larva is thoroughly mixed with a drop of 5-percent (wt/vol) aqueous nigrosin (India ink and Congo red are alternatives). This suspension is then spread in a thin film across a glass microscope slide and allowed to dry. Results: Bacteria appear transparent and outlined by the dark background (see European foulbrood, p. 9).

Wet mount. The wet mount is especially useful for examining fungi or protozoa. Macerate a portion of the sample in water. Place a drop of the suspension on a microscope slide, and carefully drop the cover glass on it to minimize air pockets. No stain is required. The wet mount is usually examined with the dry objectives of a microscope. Phase microscopy is recommended for examining wet mounts, especially if an oil immersion objective is required. Results: Organisms refract light and are therefore visible on the slide.

Appendix B. Microinjection Techniques

To diagnose some diseases or determine toxic levels of sample materials, it may be necessary to force-feed or inoculate larvae, pupae, or adult honey bees. Michael (1960) developed a technique using a microinjector equipped with a syringe and a 30-gauge needle. The microinjector can be calibrated to repeatedly deliver uniform volumes as small as 1 microliter (μL).

Force-feeding

The microinjector can be used to introduce material orally into the midgut (ventriculus) of larvae or to force-feed individual adult honey bees.

Larvae. Honey bee larvae as young as 3 days and weighing as little as 25 mg can be force-fed by carefully inserting the needle through the mouthparts and into the esophagus (fig. 20). When the actuating lever is depressed, a predetermined volume of material is pushed through the esophagus and into the midgut, without physically damaging the larva. After feeding, the larvae are placed in petri dishes lined with filter paper and incubated at 34° C.

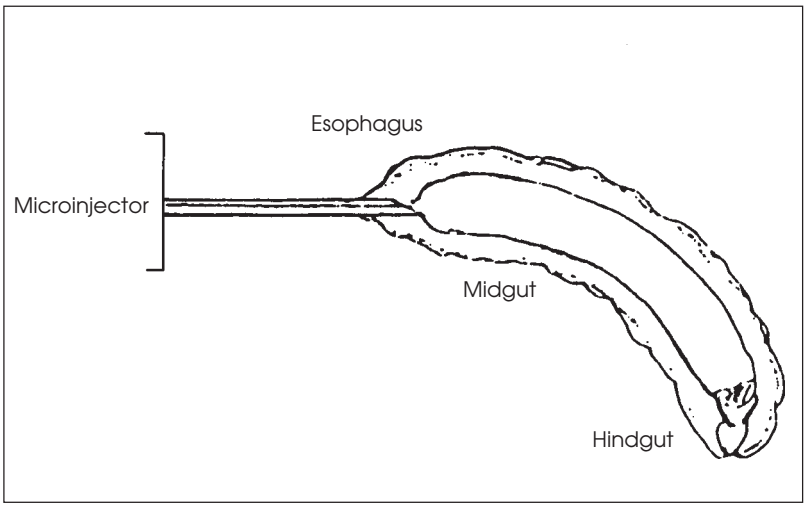


Figure 20. Force-feeding a honey bee larva.

Adults. Individual adult honey bees can also be fed specific volumes with the microinjector. The bees to be fed are held in a cage for about 4 hours without food. The desired concentration of the substance to be fed should be mixed into a sucrose solution to make it attractive to the bees. The microinjector is first actuated to produce a known volume of liquid at the tip of the needle. Then a bee from the cage is grasped by the wings or thorax, held up to the drop of liquid, and allowed to feed. Cold temperatures can be used to slow the bees and make them easier to handle. Avoid the use of carbon dioxide as an anesthetic to aid in handling the bees; the gas makes the bees reluctant to feed and reduces their longevity. After feeding, the bees are placed in small cages with a supply of 50-percent sugar syrup and held in an incubator at 34° C.

Direct Injections

The microinjection technique can also be adapted for direct injections into the body cavity of larvae, pupae, and adults. Care should be taken to insert only the tip of the needle into the hemocoel and not to exceed 2 μL per bee.

Larvae. Injections are usually confined to 4- or 5-day-old larvae. Gently grasp the larva between the first and second fingers and the thumb, and hold it absolutely parallel to the needle. Using gentle pressure, puncture the integument with the needle, expel the inoculum directly into the dorsal blood vessel, and withdraw the needle slowly and steadily. To prevent bleeding, avoid exerting any excessive pressure on the larva with the fingers, particularly when withdrawing the needle.

Pupae. Pupae can be inoculated dorsally between the third and fourth abdominal segments or through the propodeum of the thorax. After injection, place the larvae or pupae in petri dishes lined with filter paper and incubate them at 34° C. Any excessive bleeding will show on the filter paper.

Pupae can also be left in brood combs and inoculated in the head capsule (Wilson 1970). Expose the pupal head by removing the cell cap; then insert the needle between the ocelli or through the clypeal sclerite.

Adults. Adult bees can be injected either through the propodeum of the thorax or dorsally through the intersegmental membrane between the third and fourth abdominal segments. Adult bees should be carefully subjected to carbon dioxide anesthetic before and during the process of injection.

When bee longevity is a factor in the test, cold temperatures can be used as an anesthetic. After injection, place the bees in small cages with a supply of 50-percent sugar syrup and hold in an incubator at 34° C.

Collecting Larvae and Pupae

Honey bee larvae 3 to 5 days old can be readily obtained by removing a brood frame from a colony and placing it horizontally above a towel-lined tray in an incubator at 34° C. Within a few hours, the larvae crawl from their cells and drop to the tray below. Pupae can be easily obtained by collecting 5-day-old larvae as described and incubating them in petri dishes until pupation occurs. This method of collecting larvae and pupae in large numbers saves considerable time and labor. It also eliminates the damage that can occur when attempting removal from cells by mechanical means.

Appendix C. Directions for Sending Diseased Brood and Adult Honey Bees for Diagnosis to the ARS Bee Research Lab

The diagnostic accuracy of any bee disease depends on the condition of the sample. Mail the sample in a wooden or heavy cardboard box. The sample can be loosely wrapped in a paper bag, paper towel, newspaper, and so on, but avoid wrappings such as plastic bags, aluminum foil, waxed paper, tin, or glass because they allow fungi to grow on the samples. The ARS Bee Research Lab accepts samples from anyone and most samples are processed within 1 working day.

Send all samples to

Bee Disease Diagnosis
Bee Research Laboratory
Beltsville Agricultural Research Center-East
Building 476
Beltsville, MD 20705

Include a short description of the problem, along with your name and address. Plainly write your name and address on the outside of the shipping container.

Samples of Brood

- The sample of comb should be about 4 square inches.
- The sample should contain as much of the dead or discolored brood as possible.
- **No honey should be present in the sample.**
- If a comb cannot be sent, the probe used to examine brood should contain enough material for tests. Wrap the probe in paper and send it to the laboratory in an envelope. This method is unsatisfactory for verification of sacbrood disease, which requires special antisera; we rely on gross symptoms for this diagnosis.

Samples of Adult Honey Bees

- Send at least 100 bees in a sample.
- If possible, select bees that do not appear normal or that died recently. Decayed bees are not satisfactory for examination.
- Bees submitted for the identification of mites should be placed in 70-percent ethyl or methyl alcohol as soon as possible after collection. These samples should be in leak-proof containers.

Appendix D. Identification of Africanized Honey Bees

ARS Bee Research Lab Protocol

The ARS Bee Research Lab uses FABIS, the Fast Africanized Bee Identification System Manual (Sylvester and Rinderer 1987) to identify European bees. A full 26-character morphometric analysis is conducted on all samples of honey bees not verified as European by FABIS. When the Bee Research Laboratory declares a sample Africanized, identification is the result of the full 26-character morphometric analysis and is definitive.

Submission of Honey Bees for Identification of Subspecies

The lab accepts samples from the Animal and Plant Health Inspection Service, Plant Protection and Quarantine (APHIS-PPQ) and state regulatory personnel. Preference is given to samples pretested according to FABIS and identified as non-European. A sample of 100 bees should be submitted. Honey bees submitted for identification should be placed in 70-percent ethyl or methyl alcohol as soon as possible after collection. Place samples in leakproof containers and ship to

Honey Bee Identification
Bee Research Laboratory
Beltsville Agricultural Research Center-East
Building 476
Beltsville, MD 20705

Include a short history of the problem along with the sender's name, address, and telephone number. If results are urgently required, please advise the laboratory by telephone (301–504–8205) of the shipment and send the sample via express mail.

Addendum, May 3, 2005: The information on this page is no longer correct. For current information on submitting Africanized honey bees for identification, please visit this website: http://gears.tucson.ars.ag.gov/morphometrics.html.

Appendix E. Reclassification of *Bacillus* spp. Associated With Honey Bees

In 1992, the Subcommittee on the Taxonomy of the Genus *Bacillus* of the International Committee on Systematic Bacteriology decided to undertake a collaborative polyphasic[4] study of the genus *Bacillus*. Among its objectives were the establishment of minimal standards for the description of species and the revision of nomenclature (ICSB 1993). 16S rRNA gene sequence analyses have identified at least 10 phylogenetic groups in the genus *Bacillus*. Five of these groups were reclassified as the new genera *Alicyclobacillus*, *Paenibacillus* (Ash et al. 1993), *Halobacillus*, *Brevibacillus*, and *Aneurinibacillus*.

To further complicate matters Heyndrickx et al. (1996) found *P. larvae* and *P. pulvifaciens* to be similar at the infraspecific level. However, phenotypic and genotypic differentiation is possible and this is consistent with their different pathologies. Therefore, they proposed reclassification of *P. larvae* strains as *P. larvae* subsp. *larvae* and of *P. pulvifaciens* strains as *P. larvae* subsp. *pulvifaciens*.

Bacillus spp. associated with honey bees have been reclassified as follows:

Bacillus alvei = *Paenibacillus alvei*
Bacillus apiarius = *Paenibacillus apiarius*
Bacillus laterosporus = *Brevibacillus laterosporus*
Bacillus larvae = *Paenibacillus larvae* subsp. *larvae*
Bacillus pulvifaciens = *Paenibacillus larvae* subsp. *pulvifaciens*

[4] Polyphasic analysis = morphological and biochemical tests, a variety of chemotaxonomic and genomic fingerprints, and DNA relatedness measurements.

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