Distribution and Transmission of American Foulbrood in Honey Bees

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Abstract

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The distribution of *Paenibacillus larvae* spores, the causative agent of American foulbrood was studied on three different levels in the honey bee system; the apiary level, the colony level and the individual honey bee level. The increased understanding of spore distribution has been used to give recommendations regarding sampling of adult honey bees. The vertical transmission of *P. larvae* spores through natural swarms has been described for the first time and artificial swarming as a method for control of American foulbrood have been evaluated.

The results demonstrated that there is no practical difference in spore load between supers and brood chambers, and that the spore load in samples of adult honey bees on the different levels correspond to the clinical disease status of the colony. The study on individual bees showed that spores are unequally distributed among the bees and that as more bees get contaminated each positive bee also contains more spores. This may present a problem when sampling from colonies with low levels of clinical disease, although the study on colony and apiary level showed no false negatives. A model for calculating the number of bees that needs to be sampled to detect *P. larvae* in a composite sample of adult bees, given certain detection levels and proportions of positive honey bees in the sample, was developed The swarm study demonstrated vertical transmission of *P. larvae* spores. Furthermore, the artificial swarm study showed that single and double shaking are equally effective treatment methods, and that the original disease status is of little importance for the spore load decrease.

Keywords: Apis mellifera, honey bee pathology, epidemiology, shaking, evolutionary epidemiology, foulbrood, AFB, adult bee sampling, transmission.

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Till min familj!

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Lindström, A. & Fries, I. 2005. Sampling of adult bees for detection of American foulbrood (*Paenibacillus larvae* subsp. *larvae*) spores in honey bee (*Apis mellifera*) colonies. *Journal of Apicultural Research* 44, 82-86.

II. Lindström, A. Distribution of American foulbrood (*Paenibacillus larvae*) spores among adult honey bees (*Apis mellifera*). (Submitted manuscript).

III. Fries, I., Lindström, A. & Korpela, S. 2006. Vertical transmission of American foulbrood (*Paenibacillus larvae*) in honey bees (*Apis mellifera*). *Veterinary Microbiology*. In press.

IV. Lindström, A. Control of American foulbrood (*Paenibacillus larvae*) in honey bees (*Apis mellifera*) using artificial swarming. (Submitted manuscript).

Introduction

Most diseases that affect honey bees are little more than a nuisance, but some are serious and a few are lethal not only to the individual bees but to the whole colony (Fries & Camazine, 2001). To diminish the impact of disease in honey bees is of interest not just because of the well-being of the insects, and the value of the honey they produce for the beekeepers, but the value of pollination is estimated to exceed the value of the products from beehives manyfold (Delaplane & Mayer, 2000). This is reflected in legal restrictions around several diseases and parasites of honey bees (*i.e* American foulbrood (AFB)) and various forms of government support within the European Union, to combat disease in honey bee colonies.

Throughout the world, one of the most severe honey bee diseases is AFB (Shimanuki, 1997) caused by the spore-forming bacterium *Paenibacillus larvae* (Genersch *et al* 2006). This disease is considered to be especially severe because it can kill entire colonies, and because it is hard to eradicate once it has been established in a beekeeping operation. The spores are extremely infective and resilient, and one dead larva may contain billions of spores (Hansen & Brødsgaard, 1999). Contaminated hive material or products can cause outbreaks many years after the original disease was treated. Because AFB is very contagious, hard to cure, and lethal at colony level, it is of paramount importance to have reliable methods to detect outbreaks before they spread and become more difficult to control. Reliable detection methods are also of great importance for studies of pathogen transmission within and between colonies. Of the methods available today, adult bee sampling has been shown to reflect the current disease status of the colony most correctly (Nordström *et al*., 2002). However, the method needs further evaluation at different organisational levels to determine its usefulness and limitations both for practical screening purposes as well as for epidemiology and transmission studies.

It has been hypothesised that one of the important factors that mold the virulence of a pathogen is the main route of transmission between hosts (Lipsitch, Siller & Nowak, 1996). Horizontal transmission refers to pathogen transmission between individuals within generation equivalent to transmission between colonies in the honey bee system. Vertical transmission refers to pathogen transmission between individuals of different generations. AFB has been thought to be mainly horizontally transmitted, which hypothetically could explain its exceptional colony level virulence (Fries & Camazine, 2001). However, little is known about AFB modes of transmission, or colony level transmission rates, in apiculture and under natural conditions. A deeper understanding of the mechanisms that cause some pathogens to be more virulent than others may offer improved possibilities for disease control and management schemes to reduce pathogen virulence and impact.

Aims of the thesis

The aim of this thesis was to investigate some practical aspects of AFB diagnostics and control. The aim was also to study a neglected process for understanding the epidemiology of the disease: colony level pathogen transmission. Article I investigates the possibility to use composite sampling for diagnosis of AFB in apiaries through composite sampling of adult bees. The scope of article II was to study the distribution of AFB spores among individual adult bees to further elucidate the efficiency of adult bee sampling. In article III the aim was set on quantification of vertical transmission rates of AFB spores in natural honey bee swarms for a better understanding of how the pathogen is adapted to the honey bee system. Finally, in article IV, the aim was to evaluate artificial swarming as an apicultural treatment method for clinically diseased colonies and to compare spore transmission in artificial and natural swarms.

The studies in this thesis have been interpreted from an apicultural perspective as well as from an evolutionary epidemiology perspective. This aspect of honey bee pathology is in its infancy. The distribution and transmission of pathogens on different levels of the honey bee system is poorly known and this thesis aims to shed some light on distribution and transmission of one important honey bee pathogen. In this respect article I and II aim to describe the distribution of AFB spores on different organisational levels, namely the apiary level, the colony level, and the individual honey bee level. Article III and IV aim to describe vertical transmission of AFB spores; possible epidemiological implications are discussed.

Study organism: the Honey Bee

The honey bee, *Apis mellifera* Linneaus, 1758, together with the *Drosophila* fly and the *Anopheles* mosquitoes, is one of the most well-studied insects. For a thorough account of honey bee biology, see for example Winston (1987). Here only a brief description of some features of the honey bee that are important for understanding the host-pathogen system that this thesis explores, will be given.

Colony organisation and transmission of AFB

Honey bees are eusocial insects. This mean that they form perennial colonies with overlapping generations, have cooperative brood care, and a reproductive division of labour. A colony has one reproductive female, the queen. The workers, that constitute the bulk of individuals in a colony, are non-reproductive females and can number somewhere between 40.000 and 60.000 when the colony peaks during the summer. Drones are haploid reproductive males that do not serve any other purpose than mating with virgin queens. Thus, the queen and the drones are responsible for the reproduction within the colony. But there is also a form of vegetative reproduction at colony level when swarms bud off from the mother colony and start new daughter colonies. For the survival of the honey bee species, this colony level reproduction is imperative since colonies may die from many causes such as starvation or disease and the colony have no other means of reproduction.

The workers carry out different tasks during their lifetime. Although these age-related activities is very flexible, and workers can go back and forth between different tasks, they tend to follow a sequence of tasks through their lifetime. Some of the tasks might have an impact on the transmission of AFB spores within the colony and some on the transmission of spores between colonies. When the bee has hatched from its cell the first task is to clean the surrounding cells, the next task is tending and feeding of larvae. Here, the risk of transmitting AFB spores is particularly great if larvae that succumbed to AFB are cleaned out prior to feeding of susceptible larvae. After cleaning and nursing, the young worker bees take on multiple tasks like comb building, wax production, ventilation, and guarding before they become foragers. The foragers bring nectar, pollen, and water to the colony. At the end of summer when flowering plants become scarce, foragers actively scout other honey bee colonies for food. If a colony is sufficiently weakened, for example by AFB, so that it cannot defend itself, it will be robbed out. Robbing means that all the honey is stolen and taken to another colony. Because the honey in an AFB-weakened colony will be heavily contaminated by AFB spores this means that the robbing bees also bring a large amount of spores with them. Robbing may be one of the main routes of horizontal transmission of AFB under natural conditions (Fries & Camazine, 2001).

Drifting occurs when worker bees enter another hive than the one they were born in by mistake. It has been shown that drifting may cause spore transmission of *P. larvae* between colonies but that drifting is of minor importance in generating clinical cases of AFB (Goodwin, Perry & Ten Houten, 1994). Their (Goodwin, Perry & Ten Houten, 1994) estimate was based on the approximation that 6% of the marked bees drifted to other colonies. However, it has been estimated that close to 50 % of the workers in a colony can be of alien origin, depending on the layout of the apiary (Pfeiffer & Crailsheim, 1998). This may of course lead to more efficient inter-colony spore transmission.

Superorganism

The colony itself can be regarded as analogous to an organism, a superorganism. It seems clear that there is a selective pressure on honey bees at the colony level and not only on individual bees. Single bees are dispensable to the colony in the same way that single cells are dispensable to human bodies, and a single bee is just as doomed as a single human cell should it be withdrawn from the body. In this context swarming can be regarded as reproduction at the colony level (Moritz & Southwick, 1992). From a disease point of view a honey bee pathogen has to be efficient at several different levels (Fries & Camazine, 2001). It has to establish itself in a colony and successfully infect individual bees or larvae and be transmitted between individual bees within the colony, but it also has to spread between colonies for increased fitness. In this thesis, the concept of regarding the honey bee colony as a superorganism has been applied when considerations concerning the evolution of pathogen virulence are discussed.

AFB resistance

Many honey bees exhibit hygienic behaviour by cleaning out dead or diseased larvae from their cells (see review in Spivak & Gilliam, 1998a, b). If the bees are very hygienic the only trace of disease might be irregular patterns of empty cells on the brood combs. Hygienic behaviour is an inheritable trait and can easily be tested by killing off brood and then measuring the time it takes the bees to clean out the larval cadavers (Spivak & Reuter, 1998). Consequent breeding of hygienic lines have created bees that show increased resistance to AFB infections. So far, no totally AFB-resistant lines of bees have been bred (Hansen & Brødsgaard, 1999).

The larval susceptibility to infection shows considerable variation. After establishing that there are differences in survival of bee larvae between different inbred lines of honey bees (Rothenbuhler & Thompson, 1956), it was shown that there were variations in the age at which the larvae become resistant (Bamrick & Rothenbuhler, 1961). Recently, it has been demonstrated that a substance in honey bee larvae inhibits the growth of P. larvae, and that this inhibitory effect increases as the larvae grow older (Wedenig, Riessberger-Gallé & Crailsheim, 2003). In addition, the larval food has inhibitory properties that differ between susceptible and resistant lines (Rose & Briggs, 1969). Lastly, the proventricular valve shows variation between resistant and susceptible lines in its efficiency in removing solid particles from the proventriculus, the honey sac (Sturtevant & Revell, 1953).

To conclude, there are several possible mechanisms for AFB resistance in the honey bee colony. This give potential for breeding more resistant lines of bees. Some of the traits are hard to test whilst others, like the hygienic behaviour of adult bees, are easier to test and to include in a breeding programme.

Study organism: *Paenibacillus larvae*

American foulbrood is caused by the spore-forming bacterium *Paenibacillus larvae* (Genersch *et al*, 2006). It infects honey bee brood, and for individual larvae the infection is fatal. The trivial name of the disease, American foulbrood, was coined not because it originated on the American continent, but because the causative agent of the disease was described for the first time by an American scientist (White, 1906). For more detailed descriptions of American foulbrood disease see Bailey & Ball (1991), Shimanuki (1997) and Hansen & Brødsgaard (1999).

Taxonomy and nomenclature

The pathogen presently known as *Paenibacillus larvae* (Genersch *et al*, 2006) was originally described as *Bacillus larvae* (White, 1906). Another species called *Bacillus pulvifaciens* was described in 1950 (Katznelson, 1950). The species rank of "*pulvifaciens*" was rejected in 1980, only to be restored in 1984 (Nakamura, 1984). In 1993, it was proposed that *B. larvae* and *B. pulvifaciens*, among others should be moved to the new genus *Paenibacillus* (Ash, Priest & Collins, 1993).

Drobníková *et al.* (1994) proposed that *P. pulvifaciens* and *P. larvae* should be treated as one species. This position was supported by Heyndrickx *et al.* (1996) who also proposed that *P. larvae* and *P. pulvifaciens* should be treated as subspecies of one species, e.g. *P. larvae larvae* and *P. larvae pulvifaciens*. Recently, however, it has been demonstrated that this subspecies classification was ill founded and that '*larvae*' and '*pulvifaciens*' should not be treated as distinct taxa, because there are no consistent differences between them (Genersch, Ashiralieva & Fries, 2005).

Biology

Paenibacillus larvae is a spore forming, gram-positive bacterium. It measures 2.5-5 μm by 0.5-0.8 μm (Bailey & Ball, 1991). The spores measure 1.3 μm x 0.6 μm. *Paenibacillus larvae* only infects bee larva from the genus *Apis*. The younger the larvae the more susceptible they are. For larvae up to about 24 hours old it will suffice with as few as around 10 spores to start an infection (Brødsgaard, Ritter & Hansen, 1998). If the larvae are older the infective dose needs to be many times higher in order to cause infection. There is great variation in infectivity among different strains of *P. larvae*, and some strains need many more spores to infect a host larva (Genersch, Ashiralieva & Fries, 2005).

The spores germinate in the gut lumen of the larvae and the bacteria penetrate the gut wall and enter the hemocoel where they multiply. Clinical disease is often manifested by larvae that have died after the cell has been capped. Recent research has shown that there is great variation in virulence, expressed as LT_{50} (the time it takes 50% of the larvae to die), among different strains of the bacterium where some isolates kill most larvae before they are sealed (Genersch, Ashiralieva & Fries, 2005. This could give rise to the paradox that the most virulent strains at the individual larval level in fact might be less virulent at the colony level, since dead or diseased unsealed larvae are easier to detect and remove and the bacteria produce fewer spores because of the lower body mass of the larvae.

The remains from dead brood in sealed cells are typical: a brownish, sticky substance with a unpleasant odour that sometimes can be noticeable. If a match or straw is inserted into the cell and then pulled out, the remains of the larvae will typically form a thread. This is regarded as a diagnostic field test of AFB (Shimanuki, 1997). If the dead larvae are not cleaned out, the remains will dry out and a blackish scale is formed. This scale will adhere to the bottom of the cell and can be almost impossible to remove without destroying the wax cell wall. These scales can be hard to detect and often require that the comb be held in an angle so that light enters the cells. Each dead larva or scale contains approximately 2.5 billion spores (Sturtevant, 1932). The spores are extremely long lived and have been cultured from scales 69 years after collection, although the viability is reported to be much reduced (Shimanuki & Knox, 1994).

Sampling

The most common method for detection of AFB is visual inspection of the brood combs for clinical symptoms (Shimanuki, 1997). If many colonies have to be inspected this is laborious even for experienced beekeepers. To overcome this unwieldy procedure, culturing of honey samples in the laboratory has been widely used as a screening method (Hansen, 1984; Hansen & Rasmussen, 1986; Hornitzky & Clark, 1991; Steinkraus & Morse, 1992; Alippi, 1995; von der Ohe, 1997). This method has been criticized because it does not necessarily reflect the current disease status of the colony and occasionally produces false negative results (Kabay, 1995; Nordström, Forsgren & Fries, 2002). Hornitzky & Karlovskis (1989) introduced the method of culturing adult honey bees for AFB and demonstrated that spores can be detected also from colonies without clinical symptoms. Recently, culturing of *P. larvae* from adult honey bee samples has been shown to be a more sensitive tool for AFB screening compared to culturing of honey samples (Nordström, Forsgren & Fries, 2002). When samples of adult bees are used, the detection level of *P. larvae* is closely linked to the distribution of spores among the bees. The samples are plated on agar plates and the number of bacterial colonies that grow is referred to as "colony forming units" or "cfu".

Some studies on the horizontal transmission and distribution of AFB spores at the colony and apiary level have been published (Goodwin, Perry & Haine, 1996; Hornitzky, 1998), but so far no studies explicitly discuss the spore load and spore distribution among individual honey bees.

Treatment and control

In Sweden, no treatment is allowed for clinically diseased colonies. Instead a stamping out policy is employed, which means that the bees have to be destroyed and the contaminated equipment destroyed or thoroughly cleaned (Anon., 2002). Several treatment strategies are allowed in other countries, such as treatment with antibiotics and apicultural techniques like artificial swarming. Treatment with antibiotics is not allowed in the EU, but is common in, for example, USA and Canada where preventive treatments with antibiotics is considered a routine procedure to avoid outbreaks of AFB. Not surprisingly, antibiotic resistant strains of *P. larvae* have evolved (Miyagi *et al.*, 2000). Another problem with this practice is residues of antibiotics in honey and other hive products (Bogdanov, 2006).

The main apicultural technique used worldwide to treat AFB-infected colonies is artificial swarming or shaking (Shimanuki & Knox, 1997; Hansen & Brødsgaard, 2003; Article IV). This method was described for the first time already in 1769 by Schirach (Howard, 1907) and was rediscovered by McEvoy in the beginning of the 20th century (Howard, 1907). By depleting the bees of their contaminated honey stores and brood combs, and supplying them with clean hive material, the transmission cycle is thought to be broken and the bees will become free from disease symptoms. Although it is reported to be successful in many cases (Del Hoyo *et al.*, 2001; Hansen & Rasmussen 1986; Knox, Shimanuki & Caron, 1976), several authors report recurring disease (Pankiw & Corner, 1966; Cantwell, 1980;

Hornitzky & White 2001). There are two shaking methods that are practiced, single and double. It is mainly the double shaking (shaking bees onto clean equipment in two subsequent steps) that has been promoted, but there are no data available to support that this method should be preferred compared to a single shaking event.

Epidemiology

The field of evolutionary epidemiology is vast and rapidly expanding. Here, only some key concepts will be discussed to give a background for some of the reasoning in later sections and in the articles. For a brief and instructive introduction to epidemiology of social insects in general see Schmid-Hempel (1998). Fries & Camazine (2001) give a thorough introduction to the main epidemiological concepts discussed in this thesis and apply them to the honey bee system, as do Brown & Fries (2006).

A critical trait for the evolution of virulence of a pathogen according to the theories of evolutionary epidemiology is the mode of pathogen transmission between host individuals. Horizontally transmitted pathogens have the potential to evolve higher virulence than vertically transmitted pathogens. Horizontal transmission refers to pathogen transmission between individuals within a generation (Ewald, 1994) equivalent to transmission between colonies in the honey bee system (Fries & Camazine, 2001). Vertical transmission refers to pathogen transmission between individuals of different generations, typically from parent to offspring (Ewald, 1994). In the honey bee system this corresponds to transmission from a mother colony to a daughter swarm (Fries & Camazine, 2001). Horizontal transmission is thought to select for more virulent pathogens compared to vertical transmission, because vertically transmitted pathogens are dependent on host reproduction (Ewald, 1994). Consequently, the pathogen cannot afford to substantially reduce the reproductive fitness of the host. Horizontally transmitted pathogens do not have to consider host fitness as long as transmission is secured. Estimates of horizontal AFB spore transmission between colonies have been published (Goodwin *et al.*, 1993; Hornitzky, 1998), but prior to the work in this thesis no quantification of vertical transmission of honey bee pathogens has been published. This is surprising given the fundamental importance of pathogen transmission rates for understanding disease epidemiology.

Some horizontally transmitted pathogens rely on vectors for transmission. A vector typically carries and transmits a pathogen without being harmed by it. It has been hypothesized that vector-borne pathogens can evolve even higher virulence than other horizontally transmitted pathogens because the welfare of the host is of even less importance to the pathogen if a vector transmits it anyway (Ewald, 1994). Some recent, more theoretical work supports this hypothesis under certain conditions (Boots & Sasaki, 1999). The possible vector role of apiculturists for AFB is discussed in the conclusions in this thesis.

Another trait that has been forwarded as an important factor for the evolution of AFB virulence is the existence of free-living propagules (Fries & Camazine,

2001). Partly the same arguments that predict increased virulence in vector-borne diseases can be used for spore-forming pathogens. Similarly, host fitness is of reduced value to the pathogen if long-lived spores ascertain its transmission even if the host dies. Some pathogens, such as AFB for the individual larva, even rely on the death of the host for spore transmission.

Results and discussion

Composite sampling (Article I)

Early detection of AFB infection in honey bees is critical to avoid that infectious spores are distributed throughout a beekeeping operation. Thus, it is important to have a method that allows quick and reliable sampling of large beekeeping operations, or geographical areas, for clinically diseased colonies. Because honey bee colonies in rational beekeeping always are organized in apiaries, it is desirable to compare the distribution of AFB spores both at the colony and at the apiary levels.

To understand how composite samples of adult bees at the apiary level reflect the clinical disease status of the colonies, 489 colonies from 59 apiaries were visually inspected and composite bee samples were taken from the supers and from the brood chambers. The composite samples were taken as apiary samples, where >100 adult honey bees from each colony in the apiary were put in a single sample representing the whole apiary.

The spore load of individual colonies within apiaries were studied by samples of >100 adult honey bees from 94 individual colonies from 10 apiaries with simultaneous inspection for clinical disease symptoms of AFB. All samples were cultured in the laboratory for *Paenibacillus larvae*. A 10-fold dilution series was used to be able to count the spore load of every sample.

In the samples from the individual colonies there were no significant difference in spore load between supers and brood chambers, a result reflected in the samples from individual bees in Article II. Twenty-two percent of the individual colonies were clinically diseased. All samples from clinically diseased colonies were positive. Of the remaining colonies, 77% were positive although they had no visible symptoms of AFB. We found a significant relationship between the number of clinically diseased cells in the colony and the number of colony-forming units (cfu) in the laboratory cultures. Colony-forming units are the number of bacterial colonies that grow on the agar plates.

Fifty-four percent of the apiaries contained clinically diseased colonies. In the lab cultures, however, 70 % of the clinically healthy apiaries were positive for AFB. At the apiary level, there was a significant difference in spore load between supers and brood chambers that was not reflected in the colony samples.

In this study it was established that from a practical point of view, composite sampling from the supers in an apiary is a reliable and efficient method for screening of larger beekeeping operations for AFB. However, see Article II for a discussion on detection levels and sample sizes.

The results show that there is no difference of practical importance in spore load between supers and brood chambers. The slightly higher spore load in the brood chambers from the apiary samples corresponds to previous findings (Goodwin, Perry & Haine, 1996), but does not contradict the statement that adult bee sampling from the supers is useful. The sensitivity of the sampling method was 100% (i.e. samples from all colonies with clinical symptoms were positive for *P. larvae*), but the specificity was only around 30 % (i.e. samples from colonies with no clinical symptoms were often positive for *P. larvae*) (I). The specificity can be raised but only at the expense of the sensitivity. If the purpose of the sampling is to find all colonies with clinical symptoms decreasing sensitivity is not an option. Low specificity is likely to be a problem mainly when the prevalence is high, as in the beekeeping operation in this study. In most situations prevalence is much lower, for example in Sweden the annual prevalence is below 1% (Anon., 2005). Under such circumstances composite sampling of adult bees is likely to be an efficient tool when screening for clinically diseased colonies.

False negative results (clinical symptoms present but negative culturing results) can probably not be avoided in the long run for apiary composite samples, although none were found in this study (I). In an apiary with several healthy colonies, and single infected colonies, the dilution effect of the AFB-negative bees on the AFB-positive ones could potentially cause false negative results (II). Because composite sampling from the supers is fast and simple however, and it is known that adult honey bee samples are more sensitive than honey samples (Nordström, Forsgren & Fries, 1995), this method can still be recommended for screening purposes.

It should be noted that the “false positive” culture results (no clinical symptoms but positive culturing results) do not represent false diagnostic results in the visual inspection. In this context false positives are likely to represent colonies that are infected by the pathogen, but where clinical symptoms are not manifested at the time of inspection. It is tempting to hypothesize that this is the natural type of infection, where a low-grade infection exists by producing occasional diseased larvae that keep the disease cycle running. From a transmission perspective these sub-clinical infections should not be neglected because they may still be responsible for considerable horizontal spore transmission within and between apiaries as beekeepers move material between colonies and apiaries. The importance of these infections in disease transmission within apiculture needs further study.

Another interesting question, that needs further research, is why some honey bee colonies maintain a sub-clinical infection and some develop symptoms, even if they have similar spore loads. Somehow a certain level of infection is maintained within the colony and if the right conditions are present an outbreak of the disease may occur. This could be attributed either to variation in bee tolerance, bacterial virulence, or to abiotic or even random factors. We know that there is great

variation in susceptibility to AFB in honey bees (Spivak & Gilliam, 1998a). The variation in honey bee resistance can be attributed to different factors; 1) adult honey bee hygienic behaviour (Woodrow & Holst 1942; Spivak & Gilliam, 1998a; Spivak & Reuter 1998), 2) physiological traits of the honey bee larvae (Rothenbuhler & Thompson, 1956; Crailsheim & Riessberger-Gallé, 2001, Wedenig, Riessberger-Gallé & Crailsheim, 2003), 3) composition of the larval food (Thompson & Rothenbuhler 1957). Whether or not these different factors can interact in a synergistic or even antagonistic way is unknown.

Variation in virulence of different *P. larvae* strains has recently been demonstrated using laboratory infection studies (Genersch, Ashiralieva & Fries, 2005). How this variation is manifested under field conditions needs to be investigated, as does the colony level impact of variations in individual level virulence expressed by different strains.

This study clearly demonstrates that sampling at the apiary level is a fast and reliable method to apply when the number of colonies that needs to be screened is too large to allow visual inspection. It also shows that the distribution of spores at the colony and at the apiary level is similar. However, the distribution of spores at the individual honey bee level still needs to be studied in order to dimension samples correctly to minimize the risk of false negative culturing results.

Spore distribution (Article II)

Knowledge of the distribution of *Paenibacillus larvae* spores among individual adult bees is crucial for the dimensioning of composite samples of adult bees. To study the spore distribution at the individual honey bee level, 532 honey bees were collected from different parts of 9 clinically diseased colonies and individually analysed for *P. larvae*. The colonies were concurrently visually inspected. Clinical disease ranged from one cell to about 400, in which case the number of cells demonstrating symptoms of AFB were approximated rather than counted.

As the rate of *P. larvae* contaminated bees increased, each bee also contained more spores. It was also demonstrated that the spores were unequally distributed among the bees and that the spore load ranged over several orders of magnitude. In congruence with Articles I and IV there was a strong correlation between the disease status of the colonies and the number of colony-forming units in the bee samples from the same colonies. A significant relationship was also found between the disease status of the colonies and the proportion of bees positive for *P. larvae* from the same colonies. Based on the culturing results, a model for calculating the number of bees (N) that needs to be sampled to detect *P. larvae* in a composite sample of adult bees, given certain detection levels and proportions of positive honey bees in the sample, was developed.

$$N = \frac{\mathrm{Ln}(1 - DC)}{\mathrm{Ln}\left(1 - \frac{\sum_{i=1}^{n}\left(1 - e^{-0.1178 x_i}\right)}{n_i + n_h}\right)}$$

In this model *DC* denotes the degree of certainty (to detect positive bees in a subsample taken from a composite sample), x_i denotes the number of clinically diseased cells in a colony and n_i and n_h denote the number of clinically diseased colonies and healthy colonies, respectively. It should be pointed out that this formula is based on data from a limited number of colonies and do not represent the great variation in virulence of the bacterium, nor the variation in resistance on behalf of the bees. It is, therefore, possible that if this experiment is repeated in populations that differ in the aforementioned parameters, results can vary considerably. Nonetheless, it is the first attempt to validate adult bee sampling and give recommendations on the calculation of sample sizes.

The presented data demonstrate that the proportion of positive bees increases as the number of clinically diseased cells increases. Already when 40 cells are clinically diseased, 99% of the bees are positive for *P. larvae*. Using the regression equation above for the relationship between proportion of positive bees and the number of clinically diseased cells, it was found that one diseased cell corresponds to about 11% of the bees positive for *P. larvae* in a composite sample of adult bees.

Because the spore distribution on individual bees is skewed we concluded that composite sampling from large apiaries with few infected colonies might potentially give false negative test results. We also described the distribution of spores among individual adult honey bees. Some bees carry a large spore load whereas others have few or no detectable spores. It was also clearly shown that as the proportion of positive bees increases, each positive bee tends to carry a heavier spore load. Previously, the proportion of positive bees in AFB-infected colonies has been reported (Goodwin, Perry & Haine, 1996), but the proportions of *P. larvae* positive bees were never correlated to the clinical disease status of the colony, or the spore load of the individual bee.

The results suggest that false negative culturing results from individual colonies with clinical symptoms of AFB are highly improbable. At the apiary level the outcome is strongly dependant on the number of healthy and infected colonies in the apiary, the proportion of positive bees and the detection level one is ready to accept. In large apiaries with single infected colonies, detection may present a problem, although the presented study gave no false negatives, neither at colony nor at apiary level (I).

The strong correlation between the number of clinically diseased cells and the number of colony forming units in the adult bee samples (II) is congruent with data from composite samples of adult bees (I). As the spore load of the adult bees rise, the numbers of clinically diseased cells increase. If the infection level is low

and kept under control by the colony through hygienic behaviour or by other means, then clinically diseased cells may only be manifested at irregular intervals. Then the proportion of adult bees that carry spores will indeed be small and samples from such colonies may suggest that the disease disappears, only to reappear, although it is a continuously ongoing low-grade infection. Spore distribution and transmission are closely linked and therefore it is necessary to study transmission of the spores to understand how host and pathogen have coevolved.

Natural swarming (Article III)

The distribution of spores among adult bees is important for dimensioning samples for AFB surveys (II). However, the spore distribution is also likely to be important for transmission of *Paenibacillus larvae* spores between colonies. Furthermore, the rate of vertical versus horizontal transmission is of importance in order to understand how *P. larvae* is adapted to the honey bee. Prior to this work there is no information available of vertical colony level transmission of this pathogen.

To investigate vertical transmission of *P. larvae*, the spore load of 25 pairs of mother colonies-daughter swarms were followed for up to two seasons. Most queens in the mother colonies were individually marked prior to the start of the experiment. The colonies were devoid of supers and monitored for swarm preparations. When queen cells started to appear the colonies were monitored daily for swarms. A total of 25 swarms with the swarm issuing mother colony identified were captured. Samples were taken from the mother colony and from the swarm when the swarm was issued and successfully collected. Samples were cultured for *P. larvae* as in Article I. The swarms were transported to a separate apiary outside the flight distance of any other apiary. The mother colonies and the swarms were visually inspected for symptoms of AFB and sampled on a weekly basis for the first four weeks after swarming. Subsequently, sampling and inspection was done on a monthly basis.

Twenty-two of the 25 swarms and 21 of the mother colonies were positive for *P. larvae* at the time of swarming. There was a significant correlation between the spore loads of the daughter swarms and mother colonies at the time of swarming. All swarms reduced their spore load significantly, as did the mother colonies without clinical symptoms. There was no difference in spore load decrease between swarms and mother colonies in the colonies without clinical symptoms of AFB. The clinically diseased colonies, however, showed significant differences in spore load decrease between mothers and daughters.

In this study vertical transmission of AFB spores through natural swarms was described and quantified for the first time. Previously, there have been studies that describe horizontal transmission of AFB spores between colonies (Goodwin, Perry & Brown, 1993; Goodwin, Perry & Ten-Houten, 1994; Hornitzky, 1998). All swarms in this study, both from colonies with and without clinical symptoms, decreased their spore loads to very low levels. None of the swarms showed any clinical disease symptoms at any time. This indicates that the amount of spores

needed to produce clinical disease are not transmitted by swarms, or at least that they are not readily available to the larvae. If clinical symptoms appear, it is on a non-detectable level. It seems reasonable that a "no brood, no food" argument is valid here, as well as in the artificial swarm case. Because the bees do not have any stored food they will consume whatever contaminated honey they have in their honey sac. Also, there are no larvae available to the swarm to which they can transmit spores before most contaminated food carried from the mother colony is consumed. Nevertheless, the samples pick up irregular low levels of AFB spores in some swarms, as well as from the mother colonies, more than one year post-swarming. This may again suggest that the disease actually is present, and that the hive environment provides a continuous inoculum of infectious spores infecting larvae and producing new spores, but that the bees are able to remove infective material below a level where it is detected by the beekeeper as clinical disease symptoms.

This paper provides data on vertical transmission rates of one of the most serious diseases of honey bees in apiculture, data that are imperative to understand the epidemiology of this disease. We demonstrate vertical transmission of the pathogen, and demonstrate that sub-clinical disease levels may be maintained over extended periods allowing the pathogen to rely also on vertical transmission, just like most diseases of honey bees (Fries & Camazine, 2001). Surprisingly, there was no difference in spore load reduction in the swarms with respect to the original spore load in mother colony. Furthermore, the results suggest that the problem with AFB experienced in apiculture may primarily be dependent on apicultural practices, increasing the infection pressure and changing the pathogen transmission routes in the system.

Artificial swarming (Article IV)

The results from Article III clearly demonstrate that swarms decrease their spore load significantly and none of the swarms showed any clinical symptoms, even if issued from clinically diseased colonies. An apicultural method that mimics natural swarming (artificial swarming) has been used to cure AFB for many years, but data on spore loads of adult bees in treated colonies over extended periods are lacking. Therefore it is desirable to evaluate this control method and monitor the spore loads of adult bees in treated colonies over several breeding seasons. The artificial swarming study involved 45 colonies shaken once (29 colonies) or twice (16 colonies) that were monitored for up to three seasons. All hive material that was used was bought new to ensure that it was not contaminated with AFB spores.

At the end of the experiment 19 colonies (42.2%) out of 45 were still alive. Eight out of 16 colonies treated twice remained, as did 11 out of 29 treated once. There was no significant difference in mortality between the two treatments. None of the treated colonies showed any clinical disease symptoms of AFB subsequent to the treatments. The second season there was a slight increase in spore load from some colonies that again disappeared over time. There were no differences in the decrease of spore load over time between the treatments even though the functions had slightly different shapes. The disease status pre-shaking influenced the spore

load significantly, but all colonies that survived eventually decreased their spore load to undetectable levels.

This study shows that artificial swarming is an efficient treatment method for AFB. The results from artificial swarming of bees are congruent with the study of vertical transmission of *P. larvae* spores in natural swarms (III). The fact that no colony (or swarm) showed any clinical disease post-shaking, and that the decrease rate was similar for all colonies, shows that there is some mechanism that aid the bees to reduce the spore load they carry before they have any brood. It is probably the same mechanism that reduces spore loads in natural swarms, but the nature of this mechanism needs further study.

Somewhat surprising is the result that single shaking is equally efficient to double shaking. Most authors promote double shaking (Howard, 1907; Shimanuki & Knox, 1997; Hansen & Brødsgaard, 2003), but there are no studies that compare single and double shaking to confirm this recommendation. In the light of the present study there seem to be no reason to promote the more work intensive and more expensive double shaking method.

Although shaking AFB infected hives is an effective control method, there are also good arguments to continue stamping out of clinically diseased colonies where this method is used. In Sweden, this system has dramatically diminished the rate of clinically diseased colonies since applied in 1974 (Anon., 2005). Data from New Zealand also show that stamping out of clinically diseased colonies has decreased the number of colonies that become infected each year (Goodwin & Van Eaton, 1999). In Denmark, where shaking of AFB-diseased colonies is allowed, the prevalence of AFB is higher than in Sweden (Hansen, 1992).

It is an economic loss to the individual beekeeper to burn AFB infected colonies, but there is also a substantial cost to shaking in manual labour and investments in clean equipment. Unless queen excluders are used, colonies may also abscond (Hornitzky & White, 2001) and queen losses do occur in the process of shaking (Hansen & Brødsgaard, 2003). If the most virulent strains of AFB are constantly removed by burning, selection for more benign forms of the disease will result (Ewald, 1994; Ebert & Bull, 2003). It was recently demonstrated that large variations in virulence do occur between different isolates of *P. larvae* (Genersh, Ashiralieva & Fries, 2005) giving further emphasis to this argument. By removing clinically diseased bees we also select for more disease tolerant bees (Spivak & Reuter, 2001). If stamping out is practiced and the horizontal transmission of spores induced by apiculture is diminished, we may have powerful tools in the battle for healthier beekeeping, with a reduced need for chemical treatment.

This study clearly demonstrates that artificial swarming is a useful tool for curing clinical symptoms of AFB. Furthermore, it shows that there is no difference between single and double shaking and that the original disease status of the colony is of no importance for the outcome of the treatment.

Conclusions

In this thesis adult bee samples have consistently been used to estimate the spore load on different organisational levels in the honey bee system because it has proved to be a sensitive tool for measuring of spore loads (Nordström, Forsgren & Fries, 2002; Article I). While the method has its limitations when applied at the apiary level, these limitations are not greater than those of any other method for monitoring transmission or prevalence of *Paenibacillus larvae* spores. On the contrary, because the spore load of the adult bees reflects the actual status of the colonies at the time of sampling, rather than the status at the time of nectar collection, it gives a more accurate picture of the conditions in the apiary and greater possibilities to correlate clinical disease to the spore load. Understanding the transmission of AFB, as well as the distribution of spores in the colonies and among the bees, opens possibilities for theoretical modeling of this system. Theoretical modeling of honey bee – pathogen relationships should be pursued to further the understanding of AFB epidemiology.

The model in Article II implies that one clinically diseased cell corresponds to about 11% positive bees and that already when 40 cells are diseased then 99% of the bees are positive for *P. larvae*. As the proportion of positive bees increases the spore load of each individual bee increases. This could have epidemiological implications because the transmission of spores could be more effective if heavily contaminated bees are involved in feeding of susceptible larvae. But it also has implications for the sampling procedure. The steep rise in both proportion of positive bees and spore load per bee as colonies contain more clinical disease means that, provided the size of the apiary is within the range of 8-12 colonies often used by commercial beekeepers, composite sampling in apiaries are likely to reveal most of the apiaries where clinically diseased colonies appear. In Article II we also give a formula based on the collected data for calculation of sample sizes needed to detect a proportion of positive bees with a defined probability. For individual colonies adult bee sampling is highly unlikely to ever produce any false negative results. Adult bee sampling is the most effective method for monitoring of larger beekeeping operations or geographical areas for AFB. It could be a fast and reliable method for professional beekeepers to screen their apiaries to identify emerging disease problems and to follow up on treatment success.

American foulbrood has long been regarded as a primarily horizontally transmitted disease. The extremely long-lived propagules has been thought to be an adaptation to that strategy by allowing transmission from dead colonies through occupation of contaminated nest sites, or from dying colonies through robbing. Indeed, this is what we see in beekeeping, where beekeepers transmit the disease by using contaminated hive material or by neglecting the signs of diseased colonies, thereby allowing such colonies to be robbed out. However, AFB evolved without beekeepers in a totally different setting. In a natural system, nest sites are probably a limiting factor (Ratnieks, Pirey & Cuadriello, 1991), and the bees will have to compete for this resource with other nest-building insects like wasps and hornets. Birds, such as jackdaws, starlings, stock pigeons and several species of

owls, will also use many of the available nest sites. Therefore it is likely that nests occupied by honey bees were scattered.

There are no published data available regarding transmission of AFB in natural systems, but since colony density can be expected to be low, the opportunity for horizontal transmission through robbing or contaminated nest sites should also be low (Fries & Camazine, 2001). It has been shown that adult bees from wild colonies in areas without beekeeping rarely contain detectable spore levels, but swarms in areas with beekeeping are often contaminated with AFB spores (Hornitzky, Oldroyd & Sommerville, 1996). Furthermore, clinical cases of AFB have never been found in honey bees south of the Sahara where beekeeping is scarce (Fries & Raina, 2003), but *P. larvae* spores have been reported from honey (Hansen *et al.*, 2003). This can be interpreted as if *P. larvae* may primarily rely on vertical transmission under natural conditions using swarms, as demonstrated in Article III. Thus, it can be argued that under natural conditions, *P. larvae* may be more dependent on colony fitness for its survival.

In apiculture on the other hand, crowding of colonies and exchange of hive material and bees between colonies by the beekeeper optimize horizontal transmission opportunities. To make matters worse, it can be argued that beekeepers actually are selecting for even more virulent strains of *P. larvae* by increasing the rate of horizontal transmission and virtually ceasing the vertical transmission. If we add the vector-role of the beekeeper to this equation it may explain why AFB is regarded as a dangerous disease for apiculture, but may be less severe under natural conditions, as suggested in Article III.

It seems clear that on the individual bee level, *P. larvae* is what Ewald (1994) calls a "sit-and-wait" pathogen because of the extremely long-lived spores (Haseman, 1961). However, a case can also be made to characterize *P. larvae* as an attendant-borne pathogen since nurse bees transmit the spores from dead or diseased larvae to susceptible larvae (Bailey & Ball, 1991). An epidemiological interpretation is that AFB may have a sit-and-wait strategy coupled with attendant-borne transmission during disease outbreaks. Both modes of transmission are predicted to increase virulence (Ewald, 1994). The example of a cadaver-to-patient attendant-borne disease (Ewald, 1994) could be analogous to the cleaning-bee/nurse-bee situation in the honey bee system. It is predicted that this type of transmission should promote virulence since the fitness of the host is of no importance for the transmission cycle. This could be an adaptation to maintain a low level infection where larvae occasionally become infected causing small outbreaks in the colony. The long-lived propagules will then be in dormancy for long periods until they are picked up again and fed to receptive larvae and the cycle starts over. With spores retaining their viability for decades, random events are likely to produce occasional infection in individual larvae from time to time. If a larva is of the right age it will suffice with only a few spores to become infected, and once it is infected it will not recover (Brødsgaard, Ritter & Hansen, 1998). We hypothesize that this could be a strategy for the pathogen to survive and maintain itself in the honey bee system (III). It seems likely that colonies where spores are detected on adult bees more than one year post shaking are sub clinically diseased

colonies, colonies with low levels of infection cleaned out by the bees producing no visible symptoms to the inspecting beekeeper.

The synergy between the sit-and-wait strategy and the attendant-borne transmission may have produced a pathogen that can be extremely virulent at the colony level if opportunities for extensive horizontal transmission are available, what occurs when *P. larvae* enters into managed apiary conditions. The beekeeper then functions as a cultural vector (Ewald, 1994) and may further enhance the colony level virulence of the pathogen. Based on our swarm data and data from feral colonies, we propose that AFB is not different from other honey bee pathogens, mainly relying on vertical transmission for its maintenance in a natural system (III).

References

Alippi, A.M. 1995. Detection of *Bacillus larvae* spores in Argentinian honeys by using a semi-selective medium. *Microbiología SEM* 11: 343-350.

Anon., 2002. Föreskrifter om ändring i Statens jordbruksverks föreskrifter (SJVFS 1992:38) om bekämpning av amerikansk yngelröta och varroasjuka hos bin. *SJVFS* 2002: 46.

Anon., 2005. Amerikansk yngelröta i Sverige. Available 2005-11-14. (http://www.jordbruksverket.se/amnesomraden/djurveterinar/biodling/sjukdomsbekampning/amerikanskyngelrota/statistik.4.7502f61001ea08a0c7fff36043.html)

Ash, C., Priest, F.G. & Collins, M.D. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie van Leeuwenhoek* 64: 253-260.

Bailey, L. & Ball, B.V. 1991. *Honey Bee Pathology*. 2nd edition. Academic Press Limited, London, UK. 193 pp.

Bamrick, J.F. & Rothenbuhler, W.C. 1961. Resistance to American foulbrood in honey bees. VI. Spore germination in larvae of different ages. *Journal of Insect Pathology* 3:381-390.

Bogdanov, S. 2006. Contaminants of bee products. *Apidologie* 37: 1-18.

Boots, M. & Sasaki, A. 1999. 'Small worlds' and the evolution of virulence: infection occurs locally and at a distance. *Proceedings of the Royal Society. Biological Sciences* 266: 1933-1938.

Brown, M.J.F. & Fries, I. 2006. Evolutionary epidemiology of virus infections in honey bees. In: (eds. Aubert, M., Ball, B., Fries, I. & Moritz, RFA) *Virology and honey bees*. European Commission, in press.

Brødsgaard, C., Ritter, W. & Hansen, H. 1998. Response of in vitro reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores. *Apidologie* 29: 1-10.

Cantwell, G.E. 1980. The use of ethylene oxide to fumigate honey bee equipment in the United states and Canada during the 1970s. *American Bee Journal* 120: 840-843.

Crailsheim, K. & Riessberger-Gallé, U. 2001. Honey bee age-dependent resistance against American foulbrood. *Apidologie* 31: 91-103.

Delaplane, K.S & D.F. Mayer 2000. *Crop pollination by bees*. CABI Publishing, New York, U.S.A. 352pp.

Del Hoyo, M.L., Basualdo, M., Lorenzo, A., Palacio, M.A., Rodriguez, E.M. & Bedascarrasbure, E. 2001. Effect of shaking honey bee colonies affected by American foulbrood on *Paenibacillus larvae larvae* spore loads. *Journal of Apicultural Research* 40: 65-69.

Drobníková, V., Richter, V., Häusler, J. & Pytelová, I. 1994. Characterization of *Bacillus larvae* and related bacilli by chromatography of cell fatty acids. *Journal of Apicultural Research* 33: 69-74.

Ebert, D. & Bull, J.J. 2005. Challenging the trade-off model for the evolution of virulence: Is virulence management feasible? *Trends in Microbiology*, 11: 15-20.
Ewald, P.W. 1994. *Evolution of Infectious Disease*. Oxford University Press, Oxford, New York, USA. 298 pp.
Fries, I. & Camazine, S. 2001. Implications of horizontal and vertical pathogen transmission or honey bee epidemiology. *Apidologie 32*: 199-214.
Fries, I. & Raina, S. 2003. American foulbrood (*Paenibacillus larvae larvae*) and African honey bees (*Apis mellifera scutellata*). *Journal of Economic Entomology* 96: 1641-1646.
Genersch, E., Ashiralieva, A. & Fries, I. 2005. Strain- and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees. *Applied and Environmental Microbiology* 71: 7551-7555.
Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J. & Fries, I. 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. *International Journal of Systematic and Evolutionary Microbiology* 56: 501-511.
Goodwin, R.M., Perry, J.H. & Brown, P. 1993. American foulbrood disease Part III: Spread. *The New Zealand Beekeeper* 219: 7-10.
Goodwin, R.M., Perry, J.H. & Haine, H.M. 1996. A study on the presence of *Bacillus larvae* spores carried by adult honey bees to identify colonies with clinical symptoms of American foulbrood disease. *Journal of Apicultural Research* 35:118-120.
Goodwin, R.M., Perry, J.H. & Ten-Houten, A. 1994. The effect of drifting honey bees on the spread of American foulbrood infections. *Journal of Apicultural Research* 33: 209-212.
Goodwin, R.M. & Van Eaton, C. 1999. *Elimination of American Foulbrood Without the Use of Drugs. A Practical Manual for Beekeepers*. National Beekeepers Association of New Zealand, Napier, New Zealand. 78 pp.
Hansen, H. 1984. Methods for determining the presence of the foulbrood bacterium *Bacillus larvae* in honey. *Tidsskrift for Planteavl* 88: 325-328.
Hansen, H. 1992. Forekomst af ondartet bipest og bipest-bakterien I Danmark. *Tidsskrift for Biavl* 126: 125-128.
Hansen, H. & Brødsgaard, C. 1999. American foulbrood: a review of its biology, diagnosis and control. *Bee World*, 80: 5-23.
Hansen, H. & Brødsgaard, C.J. 2003. Control of American foulbrood by the shaking method. *Apiacta* 38: 140-145.
Hansen, H., Brodsgaard, C.J., Kryger, P. & Nicolaisen, M. 2003. A scientific note on the presence of *Paenibacillus larvae larvae* spores in sub-Saharan African honey. *Apidologie* 34: 471-472.
Hansen, H. & Rasmussen, B. 1986. The investigation of honey from bee colonies for *Bacillus larvae*. *Tidsskrift for Planteavl* 90: 81-86.
Haseman, L. 1961. How long can spores of American foulbrood live? *American Bee Journal* 101: 298-299.
Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P.,
Logan, N.A., Ali, N., & Berkeley, R.C.W. 1996. Reclassification of *Paenibacillus* (formerly *Bacillus*) *pulvifaciens* (Nakamura 1984) Ash *et al.* 1994, a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) *larvae* (White, 1906) Ash *et al.* 1994, as a subspecies of *P. larvae* with emended descriptions of *P. larvae* as *P. larvae* subsp. *larvae* and *P. larvae* as *P. larvae* subsp. *pulvifaciens*. *International Journal of Systematic Bacteriology* 46: 270-279.
Hornitzky, M.A.Z. 1988. The detection of *Bacillus larvae* (American foulbrood) in adult honey bees. *Australasian Beekeeper* 90: 11-12.
Hornitzky, M.A.Z. 1998. The spread of *Paenibacillus larvae* subsp. *larvae* infections in an apiary. *Journal of Apicultural Research* 37: 261-265.
Hornitzky, M.A.Z. & Clark, S. 1991. Culture of *Bacillus larvae* from bulk honey samples for the detection of American foulbrood. *Journal of Apicultural Research* 30: 13-16.
Hornitzky, M.A.Z. & Karlovskis, S. 1989. A culture technique for the detection of *Bacillus larvae* in honeybees. *Journal of Apicultural Research* 28: 118-120.

Hornitzky, M.A.Z., Oldroyd, B.P. & Sommerville, D. 1996. *Bacillus larvae* carrier status of swarms and feral colonies of honeybees (*Apis mellifera*) in Australia. *Australian Veterinary Journal* 73: 116-117.
Hornitzky, M.A.Z. & White, B. 2001. Controlling American Foulbrood – Assessing Effectiveness of Shaking Bees and Antibiotic Therapy Strategies. *RIRDC publication No. 01/048.* Available 2006-01-25 at: http://www.rirdc.gov.au/reports/HBE/01-048.pdf
Howard, L. O. 1907. Report of the meeting of inspectors of apiaries, San Antonio, Texas, November 1906. *USDA, Bureau of Entomology, Bulletin 70*.
Kabay, M.J. 1995. Evaluation of the culture of honey to detect American foulbrood. *Australian Veterinary Journal* 72: 33-34.
Katznelson, H. 1950. *Bacillus pulvifaciens* (n. sp.), an organism associated with powdery scale of honeybee larvae. *Journal of Bacteriology* 59: 153-155.
Knox, D.A., Shimanuki, H. & Caron, D.M. 1976. Ethylene oxide plus oxytetracycline for the control of American foulbrood in honey bees. *Journal of Economic Entomology* 69: 606-608.
Lipsitch M., Siller S. & Nowak M.A. 1996. The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution* 50: 1729-1741
Miyagi, T., Peng, C.Y.S., Chuang, R.Y., Mussen, E.C., Spivak, M.S. & Doi, R.H. 2000. Verification of oxytetracycline-resistant American Foulbrood pathogen *Paenibacillus larvae* in the United States. *Journal of Invertebrate Pathology* 75: 95-96.
Moritz, R.F.A.; Southwick, E.E. 1992. *Bees as superorganisms.* Springer Verlag, Berlin, Germany. 395 pp.
Nakamura, L.K. 1984. *Bacillus pulvifaciens* sp. nov., nom. rev. *International Journal of Systematic Bacteriology* 34: 410-413.
Nordström, S., Forsgren, E. & Fries, I. 2002. Comparative diagnosis of American foulbrood using samples of adult honey bees and honey. *Journal of Apicultural Science* 46: 5-12.
Ohe, W. von der. 1997. Efficient prophylactic measures against American foulbrood by bacteriological analysis of honey for spore contamination. *American Bee Journal* 137: 603-606.
Pfeiffer, K.I.J. & Crailsheim, K. 1998. Drifting of honeybees. *Insectes Sociaux* 45: 151-167.
Pankiw, P. & Corner, J. 1966. Transmission of American foulbrood by package bees. *Journal of Apicultural Research* 5: 99-101.
Ratnieks, F.L.W., Piery, M.A. & Cuadriello, I. 1991. The natural nest and nest density of the Africanized honey bee (Hymenoptera, Apidae) near Tapachula, Chiapas, Mexico. *The Canadian Entomologist* 123: 353-359.
Rose, R.J. & Briggs, J.D. 1969. Resistance to American foulbrood in honey bees. IX. Effects of honey bee larval food on the growth and viability of *Bacillus larvae*. *Journal of Invertebrate Pathology* 13: 74-80
Rothenbuhler, W.C. & Thompson, V.C. 1956. Resistance to American foulbrood in honey bees. II. Differential survival of larvae of different genetic lines. *Journal of Economic Entomology* 50: 731-737.
Schmid-Hempel, P. 1998. *Parasites in Social Insects*. Princeton University Press, Princeton, New Jersey, U.S.A. 392 pp.
Shimanuki, H. 1997. Bacteria. In: Morse, R.A. & Flottum, K. (eds.) *Honey Bee Pests, Predators, and Disease*s. 3:rd ed. A.I. Root Company, Medina, Ohio, U.S.A. 718 pp.
Shimanuki, H. & Knox, D. 1994. Susceptibility of *Bacillus larvae* to Terramycin. *American Bee Journal* 134: 125-126.
Shimanuki, H. & Knox, D. 1997. Summary of control methods. In: Morse, R.A. & Flottum, K. (eds.) *Honey Bee Pests, Predators, and Diseases*. 3:rd ed. A.I. Root Company, Medina, Ohio, U.S.A. 718 pp.
Spivak, M. & Gilliam, M. 1998a. Hygienic behaviour of honey bees and its application for control of brood diseases and varroa. Part I. Hygienic behaviour and resistance to American foulbrood. *Bee World* 79: 124-134.
Spivak, M. & Gilliam, M. 1998b. Hygienic behaviour of honey bees and its application for control of brood diseases and varroa. Part II. Studies on hygienic behaviour since the Rothenbuhler era. *Bee World* 79: 169-186.

Spivak, M. & Reuter, G.S. 1998. Honey bee hygienic behaviour. *American Bee Journal* 138: 283-286.
Spivak, M. & Reuter, G.S. 2001. Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behaviour. *Apidologie* 32: 555-565.
Steinkraus, K.H. & Morse, R.A. 1992. American foulbrood incidence in some US and Canadian honeys. *Apidologie* 23: 497-501.
Sturtevant, A.P. 1932. Relation of commercial honey to the spread of American foulbrood. *Journal of Agricultural Research* 45: 257-285.
Sturtevant, A.P. & Revell, I.L. 1953. Reduction of *Bacillus larvae* spores in liquid food of honey bees by action of the honey stopper, and its relation to the development of American foulbrood. *Journal of Economic Entomology* 46: 855-860.
Thompson, V.C. & Rothenbuhler, W.C. 1957. Resistance to American foulbrood in honey bees. I. Differential protection of larvae by adults of different genetic lines. *Journal of Economic Entomology* 50: 731-737.
Wedenig, M., Riessberger-Gallé, U. & Crailsheim, K. 2003. A substance in honey bee larvae inhibits the growth of *Paenibacillus larvae larvae*. *Apidologie* 34: 43-51
White, G.F. 1906. The bacteria of the apiary, with special reference to bee diseases. *USDA, Bureau of Entomology, Technical Series 14*.
Winston, M.L. 1987. *The Biology of the Honey Bee*. Harvard University Press, London, UK. 281 pp.
Woodrow, A.W. & Holst, E.C. 1942. The mechanism of colony resistance to American foulbrood**.** *Journal of Economic Entomology* 35: 327-330.

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Jag tanker inte trötta eventuella läsare med att räkna upp namnen på alla vänner, utan nöjer mig med att säga: Kära vänner, ni vet vilka ni är, hos er känner jag mig alltid välkommen, och vad mer kan man begära!?

Birgitta och Göran, Cecilia och Tore, Anders och Karin; ni har fått mig att känna mig som en del av familjen. Tack!
Christina och Benny, mina föräldrar, har alltid trott på mig och stöttat mig. Jag kan tänka mig att det kan ha varit svårt att behålla tron hela tiden! Ord är överflödiga! Tack för allt! Pelle, min kära bror! Vad ska man säga? Jag älskar dig!

Sist, men inte minst! Johanna och Klara (och nr. 2!), ni är stjärnorna i mitt liv! Jag älskar er!