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Original article

Horizontal transmission of *Paenibacillus larvae* spores between honey bee (*Apis mellifera*) colonies through robbing*

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Abstract – Surprisingly little is known about transmission rates between honey bee colonies of *Paenibacillus larvae,* the causative agent American foulbrood. We studied the rate of horizontal transmission of *P. larvae* spores between colonies as a function of physical distance between colonies by culturing for the spores from sequential samples of adult bees. The results demonstrate a direct effect of distance to clinically diseased colonies on the probability of contracting high spore levels, as well as on the probability of developing clinically visible disease symptoms. The results also demonstrate that colonies may develop considerable spore densities on adult bees without exhibiting visible symptoms of disease. Furthermore, the data suggest that transmission of AFB between apiaries occur within 1 km distance from clinically diseased colonies, but is significantly lower at 2 km distance or longer when colonies dead from AFB are allowed to be robbed out.

horizontal transmission / *Apis mellifera* / *Paenibacillus larvae* / robbing / American foulbrood

1. INTRODUCTION

American foulbrood (AFB) is a common bacterial disease of honey bees (*Apis mellifera* L.) caused by *Paenibacillus larvae* (Genersch et al., 2005). It occurs on all continents where beekeeping is practiced (Ellis and Munn, 2005). The bacterium produces extremely resilient spores whereby transmission within and between hosts is accomplished. When clinical disease symptoms appear in infected colonies they are likely to succumb to the disease if left untreated (Hansen and Brødsgaard, 1999).

The mode of transmission (horizontal vs. vertical) of disease agents between hosts is thought to be crucial for determination of virulence in pathogens (Lipsitch et al., 1996). Compared to vertical transmission, horizontal parasite transmission is expected to select for increased virulence (Lipsitch et al., 1996). This is expected because when parasites depend mainly on horizontal transmission, the fitness interests of the host and the parasites are not necessarily aligned, as is the case in vertically transmitted pathogens.

Transmission rates and main mode of pathogen transmission are key factors for the evolution of virulence in any host-pathogen system (Dieckmann et al., 2002). It was recently demonstrated that vertical transmission of AFB spores between colonies occur as infected colonies divide by colony fission (swarming) (Fries et al., 2006). However, such transmission of spores to new colonies rarely results in clinically diseased daughter colonies (Fries et al., 2006). Horizontal spore transmission between colonies has been demonstrated (Goodwin et al., 1993, 1994; Hornitzky, 1998) but the importance of distance to the infection source for disease transmission was never

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investigated. Nevertheless, proximity to documented cases of clinical AFB cases appears to increase the probability of detection of spores in honey from neighbouring apiaries (de Graaf et al., 2001).

Robbing is generally considered to be of major importance for the transmission of AFB (Hansen et al., 1988; Ratnieks, 1992; Goodwin et al., 1993; Shimanuki, 1997; Hansen and Brødsgard, 1999; Fries and Camazine, 2001; de Graaf et al., 2001). However, there are no studies of transmission rates of *P. larvae* spores between colonies during robbing. Nor has the importance of the distance between the robbing colonies and robbed out, clinically diseased colonies been investigated.

Robbing behaviour is a phenomenon that occurs regularly when nectar is scarce, primarily at the end of the flowering season, but also in the spring (Winston, 1987). During robbing events, honey bees attack weaker colonies and try to steal their honey stores. If colonies are weakened, for example, by disease, pathogens may spread to the robbing colonies by means of contaminated honey. *Paenibacillus larvae* appears to be well adapted to this horizontal transmission route (Fries and Camazine, 2001), where the resilient spores contaminate the honey in infected colonies (Hansen and Rasmussen, 1986). When robbing bees bring back and store spore contaminated honey, spores will be released into the colony as the honey is utilized. If a sufficient number of spores are released, chances are that some larvae will become infected. Furthermore, honey bees robbing out AFB infected hives are likely to become externally contaminated by *P. larvae* spores, although such documentation is lacking.

In the field, detection of AFB is based on the appearance of diseased brood where the infected dead larvae exhibit a typical consistency (ropiness). As the dead larvae dries up, hardened scales appear that adhere firmly to the cell wall. A single dead larva may contain as much as 2.5×10^9 spores (Sturtevant, 1932; Lindström et al., 2008). In colonies without clinical symptoms of disease, spores of the pathogen can be detected in samples of adult bees (Hornitzky, 1988; Nordström et al., 2002). Thus, spore loads on adult bees allow quantification of pathogen transmission between colonies (Fries et al., 2006).

Here, we use samples of adult bees to describe the rate of horizontal transmission of *P. larvae* spores and clinical symptoms between honey bee colonies, as a function of their physical distance to colonies clinically diseased by AFB.

2. MATERIALS AND METHODS

2.1. Experimental setup

The experiment was set up in west-central Finland in the end of July the first experimental year. Three heavily AFB infected colonies (> 500 colony forming units/100 adult bees, and > 500 diseased larvae/colony) were placed in an apiary together with three colonies. Four apiaries in total containing 11 colonies were set up at 0.5, 1, 2 and 3 km distance from the AFB diseased apiary, containing 4, 2, 2 and 3 colonies respectively. A total of 14 experimental colonies were used, including three AFB diseased colonies. Half (7) of the experimental colonies were not entirely free from *P. larvae* spores at the beginning of the experiment, but showed very low levels of spores (0.3–3.7 colony forming units /100 bees). The contaminated colonies did not show any clinical signs of American foulbrood prior to the experiment, nor did the colonies at 2 or 3 km show any disease symptoms during the experiment. The contaminated colonies were randomly distributed over the experimental apiaries. There were no other known colonies within a 5 km radius of the experimental apiaries.

2.2. Fate of the colonies

Some of the honey stores in the original infected AFB source colonies at the 0 km apiary were robbed in the fall of the first experimental year and those colonies died during the winter. In the spring the second experimental year, the remaining stores in the dead colonies were robbed, after which the colonies were removed from the apiray. At the end of the same summer three new heavily infected colonies where brought to the same apiary to be target colonies for robbing. The honey stores in the new colonies where robbed in the fall, although not completely robbed out, and the colonies died during the winter. The stores of the dead colonies were

Table I. Colony distribution over the five apiaries, colony survival and disease incidence. Colonies denoted with a letter (A–F) are target colonies and colonies denoted with a number (1–14) are experimental colonies. A superscript "a" denotes a colony where clinical symptoms were discovered and a "b" denotes a colony that either died or was removed with severe AFB symptoms.

	Year 1	Year 2	Year 3	Year 4
Targets 0 km	A[a]	A[b]	D[a]	D[b]
	B[a]	B[b]	E[a]	E[b]
	C[a]	C[b]	F[a]	F[b]
Exp col at 0 km	1	1[ab]		
	2	2[ab]		
	3	3[ab]		
0.5 km	4	4	4[ab]	
	5	5	5[a]	5[b]
	6	6	6	6
	7	7	7[a]	7[b]
1 km	8	8	8	8[ab]
	9	9	9[ab]	
2 km	10	10	10	10
	11	11	11	11
3 km	12	12	12	12
	13	13	13	13
	14	14	14	14

robbed in the spring of the third experimental year. A schematic view of the development of clinical symptoms and the survival of colonies is given in Table I.

2.3. Samples

Samples consisted of >100 live adult bees, shaken from the brood chamber and placed in a plastic bag and frozen as soon as possible after collection. All colonies were sampled once a week the first season, from July to November, and then once a month the following seasons from April to October. All colonies were examined for clinical symptoms at the same time as the samples were collected.

Samples were cultured according to the protocol in Lindström and Fries (2005). One hundred bees were counted, thawed and put in a Neogen™ plastic DNA extraction bag. To this bag 20 mL of sterile water was added and the bees were crushed. The sludge was poured out into a centrifugal tube and centrifuged (15 050 rpm/27 000 G, 10 min). The supernatant was discarded and the remaining pellet was resuspended in 2 mL of sterile water. The suspension was heat treated in a water bath (Grant™ GLS 400) for 10 minutes at 91 °C to reduce contamination. The suspension was plated onto MYPGP agar with 3 μg nalidixic acid/mL using a 10 μL plating loop. Plates were incubated for 7 days in 36 °C with 5% CO_2. The bacterial colonies were counted manually.

2.4. Statistical treatment

For the statistical analysis a generalized linear mixed model ANOVA by using the GLIMMIX procedure of the SAS® statistical software (version 9.13) was used. Apiary was used as the class variable and the log 10 of the cfu was used as the response variable.

3. RESULTS

The factors that influence the development of spore load in the apiaries are shown in Table II. The original spore load of the apiaries

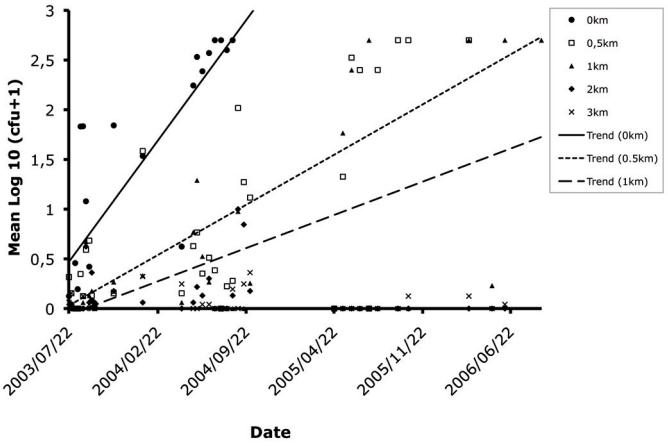

Figure 1. The spore loads in the different apiaries. For apiaries at 0, 0.5 and 1 km a trend line has been included. The functions of the trend lines are in the text.

Table II. ANOVA table of significant factors and interactions for the five apiaries for the duration of the study. The significance of time x apiary term show that the shape of the curves in Figure 1 differ significantly.

Effect	Num DF	Den DF	F Value	Pr > F
apiary	4	9	1.22	0.3667
time	1	385	6.59	0.0107
time*apiary	4	385	6.08	<.0001

was also tested but showed no significant effect on the development of spore loads in subsequent samples.

Data show that colonies in apiaries up to one kilometer away actively participate in robbing (Fig. 1, Tab. I). The increase in mean log 10 spore load over time for colonies in the 0 km apiary can be described by the function $y = 0.006x - 206$ ($r^2 = 0.67$). At 0.5 km the increase can be described by $y = 0.0024x - 86$ ($r^2 = 0.51$) and at 1 km by $y = 0.0016x - 57$($r^2 = 0.33$). For apiaries at 2 and 3 km there was no significant trend over time and, thus, no functions were calculated.

A least squares mean test was performed to reveal differences in spore load between apiaries at different distances to the target apiary. The result is shown in Table III.

The mean log10(cfu) of all samples from an apiary for the whole experimental period was calculated and plotted in Figure 2, to visualise the average spore loads in apiaries at different distances from the target apiary.

4. DISCUSSION

Our data clearly demonstrate that transmission of *P. larvae* spores through robbing is an important transmission route between honey bee colonies over short distances and that the transmission of sufficient spore levels to produce clinical disease is directly related to the distance from AFB diseased colonies or colonies dead from AFB. Three of the four colonies at 0.5 km, and both colonies at 1 km developed clinical symptoms and died or were moved away with clinical symptoms of AFB (Tab. I). This data corroborates earlier reports (Hansen et al., 1988; Ratnieks, 1992; Goodwin et al., 1993; Shimanuki, 1997; Hansen and Brødsgaard, 1999; Fries and Camazine, 2001; de Graaf et al., 2001) where robbing is described as one of the most common and serious transmission routes of *P. larvae* spores within apiculture. However, the presented data also quantifies the importance of distance to diseased colonies, for effective transmission, with the most effective disease transmission occurring within 1 km from the spore source.

Table III. Differences of apiary Least Squares Means to identify apiaries which differed significantly in adult honey bee spore load. The symbol "*" denotes apiaries that differ significantly with regard to spore load.

Apiary vs.	apiary	Estimate	Std Error	DF	*t* Value	Pr > |*t*|
0 km	0.5 km*	1.6892	0.2180	9	7.75	<.0001
0 km	1 km*	2.1296	0.3430	9	6.21	0.0002
0 km	2 km*	2.7195	0.4187	9	6.49	0.0001
0 km	3 km*	3.5184	0.4934	9	7.13	<.0001
0.5 km	1 km	0.4405	0.3605	9	1.22	0.2528
0.5 km	2 km*	1.0303	0.4331	9	2.38	0.0413
0.5 km	3 km*	1.8292	0.5057	9	3.62	0.0056
1 km	2 km	0.5898	0.5077	9	1.16	0.2752
1 km	3 km*	1.3887	0.5709	9	2.43	0.0378
2 km	3 km	0.7989	0.6193	9	1.29	0.2292

Seven of the experimental colonies showed slightly raised spore levels prior to the experiment. These colonies were distributed randomly over the 4 apiaries and the colonies in the apiaries at 2 and 3 km never developed any clinical symptoms. Further, our analysis showed no significant relationship between the original spore load and the development of subsequent spore loads. Therefore it seems highly likely that the results (Fig. 1) of this study were due to spores disseminated through robbing and, correspondingly, highly unlikely that they were a result of the slight intial spore loads.

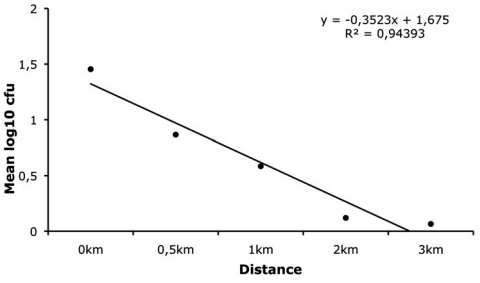

Figure 2. The mean spore load of all samples from all years for each apiary.

With the impact of robbing within the target apiary and at 0.5 and 1 km distance, it is surprising that the colonies at 2 and 3 km only showed slightly elevated spore levels in some samples with irregular intervals, but never developed any clinical symptoms. Whether or not these slightly raised spore levels originated from irregular participation in robbing activities or if the colonies had continuous low infection levels without manifesting clinical symptoms at inspection, remains unknown. It has been shown (Hansen and Rasmussen, 1986; Fries et al., 2006) that colonies can maintain low levels of spores for several years without developing clinical disease symptoms.

Previously it has been demonstrated that the risk for elevated spore levels in honey was three times higher in apiaries close to apiaries with clinical symptoms than in other geographic locations (de Graaf et al., 2001). The definition of close in the study of de Graaf et al. (2001) was based on postal code areas of undefined size within a 5 km radius of a different postal code area containing colonies manifesting clinical symptoms of AFB. The raised spore levels in geographic areas close to colonies with clinical symptoms was assumed to be due to robbing (de Graaf et al., 2001). Our data clearly support the findings in that study (de Graaf et al., 2001), but also define the effect from distance on the risk of spore transmission more precise (Tabs. I, III).

Time (sampling occasion) has a strong effect on the over all development of spore loads in the apiaries (Tab. II). There is also an

interaction effect between time and apiary, implying that the spore loads develop differently over time in different apiaries (Tab. II, Fig. 1), The logical interpretation of this result is that the spore loads of adult honey bees are not only linked to the robbing event, but the contaminated honey stores work as a reservoir for spores, releasing spores into the colony when it is utilised. The importance of honey as an intra-colony reservoir for *P. larvae* spores has been documented by Lindström et al. (2008). By adding infectious material to experimental colonies through contaminated honey and dead larvae respectively, it was demonstrated that the spore load of the honey has a delayed effect on spore prevalence in adult bee samples, and that spore loads of adult honey bees increase during periods when nectar is not available. In a temperate climate this can lead to high spore loads in the spring when rearing of brood commences, thus, probably increasing the risk for infection (Lindström et al., 2008), since there is a general high spore load and only few larvae.

Besides robbing, horizontal spore transmission may be caused by adult bees drifting into foreign colonies or mechanical transmission of spores by the beekeeper, exchanging combs or hive parts between colonies. In our experiment, beekeeper transmission was kept at a minimum with no material exchanged between colonies within apiaries or between apiaries. Drifting is probably of minor importance for transmission of sufficient number of *P. larvae* spores between colonies to produce clinical disease symptoms, even within apiaries. Out of 25 pairs of one healthy (controls) and one clinically diseased colony, with the hives in each pair touching and the entrances facing the same direction Goodwin et al. (1994) failed to create clinical disease due to drifting in more than 90% of the control colonies with an average exposure period of 103 days. Even removing clinically diseased colonies during day time, leaving all foraging bees to drift into foreign colonies, does not significantly increase the risk for further disease outbreaks in the remaining colonies, compared to removing diseased colonies at night, when all foragers are within the hives (Goodwin and Haine, 1995). Thus, for this study it is highly unlikely that drifting was a major source for spore transmission between apiaries, but that the increased spore loads and subsequent development of clinical disease symptoms recorded were dependent on the documented robbing behaviour of the bees.

There is a fundamental difference in the spatial distribution of colonies between managed and natural systems, if a local scale is considered. Under managed conditions local colony densities may be extreme, compared to natural systems, even if the km^2 density may be similar. An apiary containing 10 colonies, covering say 20 m^2, corresponds to a colony density of 50 000 colonies per km^2. In a natural system individual honey bee colonies are scattered, and the distance between colonies is larger than in apiculture where extreme local colony densities are the rule. Local high colony densities give fundamentally different possibilities for disease transmission through robbing in apiculture compared to natural systems. In natural systems, the impact on spore transmission from robbing is probably lower because of the average larger distances between colonies. In apiculture the impact is likely to be considerably higher since colonies are aggregated and the risk for spore transmission through robbing increases with decreased distance between colonies as demonstrated here. Because of this increase in horizontal disease transmission in apiculture, compared to natural systems, it is likely that increased colony level pathogen virulence will evolve (Dieckmann et al., 2002), although this has not been documented and may be difficult to measure. Recently it has been demonstrated that different strains of *P. larvae* differ in virulence at least at the individual larval level (Genersch et al., 2005), suggesting that *P. larvae* may use different strategies for increased fitness depending on the selection pressure.

Here we document the importance of colony density for the transmission of AFB between honey bee colonies through robbing. It needs to be further investigated if the extreme local colony densities created by apiculture also selects for more virulent pathogens, due to increased rates of horizontal transmission, compared to natural systems.

Transmission horizontale des spores de *Paenibacillus larvae* entre colonies d'abeilles (*Apis mellifera*) par le pillage.

***Apis mellifera* / *Paenibacillus larvae* / loque américaine / transmission horizontale / pillage**

Zusammenfassung – Horizontale Übertragung durch Räuberei von *Paenibacillus larvae* Sporen zwischen Honigbienenvölkern (*Apis mellifera*). Ziel dieser Untersuchung war die Ermittlung der Entfernung, über welche Räuberei als Übertragungsweg von *Paenibacillus larvae* Sporen effektiv sein kann. Vierzehn Völker wurden in Entfernungen von 0, 0,5, 1, 2 und 3 km von dem Zielbienenstand aufgestellt, der drei Völker mit schweren AFB Infektionen enthielt. Die kranken Völker wurden im Herbst des ersten Experimentaljahres beräubert, starben im Winter und wurden im Frühjahr des zweiten Experimentaljahres erneut beräubert. Drei neue, hoch infizierte Völker wurden auf den gleichen Bienenstand gebracht. Sie wurden im Herbst beräubert, starben über den Winter und ihre verbliebenen Honigvorräte wurden im Frühjahr des dritten Experimentaljahres ausgeräubert. Als Proben wurden Adultbienen genommen und entsprechend den Standardprotokollen auf *P. larvae* Sporen hin kultiviert.

Die Ergebnisse zeigen, dass Völker sich bis zu einem km Entfernung von dem Zielbienenstand an der Räuberei beteiligten (Abb. 1, Tab. I). Alle Völker auf diesen Bienenständen bis auf eins entwickelten klinische Krankheitssymptome. Hierdurch ist klar gezeigt, dass die Übertragung von Sporen durch die Räuberei auf kurze Entfernungen einen sehr wichtigen Übertragungsweg für *P. larvae* Sporen zwischen Bienenvölkern darstellt, und dass weiterhin die Übertragung von für die Entwicklung eines klinischen Krankheitsbildes ausreichenden Sporenanzahlen direkt mit der Entfernung von an AFB erkrankten Bienenvölkern zusammenhängt.

Die Bienenstände in 2 und 3 km Entfernung zeigten keinen zeitlichen Trend, wiesen allerdings bei einigen Proben in unregelmäßigen Zeitabständen leicht erhöhte Sporenlevel auf. Sie entwickelten allerdings keinerlei klinische Symptome. Es ist bekannt, dass Völker über mehrere Jahre niedrige Sporenbelastungen aufweisen können, ohne jemals klinische Symptome der Krankheit zu entwickeln. Weitere Ergebnisse zeigten, dass die Zeit der Probennahme einen starken Einfluss auf die Gesamtentwicklung von Sporenbelastungen auf den Bienenständen hat (Tab. II). Weiter zeigte sich ein Interaktionseffekt zwischen dem Probenzeitpunkt und dem Bienenstand, was darauf hinweist, dass sich die Sporenbelastungen in den Bienenständen unterschiedlich über die Zeit entwickeln (Abb. 1, Tab. II). Die logische Schlussfolgerung dieses Ergebnisses ist, dass die Sporenladungen der adulten Bienen nicht nur auf das Räubereiereignis zurückzuführen sind, sondern weiterhin die Honigvorräte als Reservoir für die Sporen dienen und diese in die Kolonie freisetzen, wenn der Honig genutzt wird.

Horizontale Übertragung / *Apis mellifera* / *Paenibacillus larvae* / Räuberei / Amerikanische Faulbrut

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