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Small hive beetles, *Aethina tumida*, are vectors of *Paenibacillus larvae**

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Abstract – The transmission of honeybee pathogens by free-flying pests, such as small hive beetles (= SHB), would be independent of bees and beekeepers and thereby constitute a new challenge for pathogen control measures. Here we show that larval and adult SHB become contaminated with *Paenibacillus larvae* spores when exposed to honeybee brood combs with clinical American foulbrood (= AFB) symptoms in the laboratory. This contamination persists in pupae and newly emerged adults. After exposure to contaminated adult SHB, honeybee field colonies showed higher numbers of *P. larvae* spores in worker and honey samples after five weeks. Despite these results, the rather low number of *P. larvae* spores on adult SHB suggests that clinical AFB outbreaks are not likely. However, even small spore numbers can be sufficient to spread *P. larvae*. Therefore, our data clearly show that SHB are vectors of *P. larvae*. We suggest considering the role of SHB in AFB control in areas where both pests are established.

Aethina tumida / AFB / American foulbrood / *Paenibacillus larvae* / small hive beetle / vector

1. INTRODUCTION

Among honeybee pathogens, the spore-forming bacterium *Paenibacillus larvae* poses one of the key threats to the health and well-being of colonies (Bailey and Ball, 1991). It is almost globally distributed by now (Ellis and Munn, 2005) and causes American foulbrood (= AFB). *Paenibacillus larvae* produces spores, which can infect honeybee larvae (Ritter, 1996). These spores can remain infectious for decades (Shimanuki and Knox, 1994) and are the prime transmission option. *Paenibacillus larvae* spores can be transmitted via beekeepers as well as via drifting and robbing of honeybees (Hornitzky, 1998;

Lindström et al., 2008). Moreover, it has recently been shown that the mite *Varroa destructor* can act as a vector of *P. larvae* from infected to healthy honeybee colonies (De Rycke, 2002) with robbing being the major contributor for mite exchange between colonies (Greatti et al., 1992). With the exception of beekeeping associated transmission, all other pathways thus require honeybees. Given that a free flying honeybee pest might also assist transmission of *P. larvae*, this would open a novel route for the spread of AFB among honeybee colonies, thereby potentially spoiling some of the pest control approaches (e.g. quarantine areas, please refer to national animal health laws). The small hive beetle, *Aethina tumida*, might be one such pest.

In sharp contrast to the mite *V. destructor*, these beetles are active flyers and have been reported to fly up to 16 km (cf. Neumann and

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Elzen, 2004). As a result, there is occasional dispersal of *A. tumida* among apiaries, although it is more likely to occur within apiaries (Spiewok et al., 2007, 2008). Small hive beetles are parasites and scavengers of honeybee, *Apis mellifera*, colonies native to sub-Saharan Africa (Lundie, 1940; Hepburn and Radloff, 1998; El-Niweiri et al., 2008; Hassan and Neumann, 2008; Neumann and Ellis, 2008). They have become an invasive species and were introduced into a number of countries (Neumann and Elzen, 2004; Neumann and Ellis, 2008). In North America and Australia small hive beetles have managed to establish populations (Neumann and Ellis, 2008) and can cause considerable damage to local apiculture (Elzen et al., 1999; Spiewok et al., 2007).

In the US, both *P. larvae* and *A. tumida* are now well established (Ellis and Munn, 2005; Neumann and Ellis, 2008), suggesting that interactions between the two pests are very likely, which has however not been investigated yet. We here explore for the first time whether small hive beetles are vectors of *P. larvae*. We expect that both larval and adult small hive beetles can become contaminated with *P. larvae* spores when roaming on honeybee brood combs with clinical AFB symptoms and that the contamination is still present in the pupae and newly emerged adults. Further, we hypothesize that such contaminated adults are increasing the number of spores in previously uninfected field colonies.

2. MATERIAL AND METHODS

Experiments were conducted at the USDA-ARS Bee Research Laboratory in Beltsville (Maryland, USA) from 21 July–10 August 2006 (Laboratory) and 7 June–12 July 2007 (Field). At a local quarantine apiary, honeybee colonies ($N = 10$) of mixed European origin, predominantly *A. m. ligustica*, were not treated against AFB to obtain heavily infected colonies. Under each test, the respective working hypotheses are given in *italics*.

2.1. Laboratory test

Hypothesis: Adult and larval small hive beetles become contaminated with P. larvae spores when

exposed to honeybee brood combs with clinical AFB symptoms and the contamination is still present in pupae and newly emerged adults.

Combs with sealed and unsealed brood were taken from 10 naturally infected AFB colonies and arranged into six plastic containers with an equal amount of infected brood cells each. One container with non-infested brood combs served as a control. Adult small hive beetles were reared from field-caught adults following routine protocols (Mürle and Neumann, 2004) and introduced into the containers ($N = 20$ each). The containers were kept in darkness at 30 °C. After seven days, all live adult small hive beetles were collected in Petri dishes (ø 4 cm) and immediately frozen. Three days later, all wandering larvae (= post feeding stage; Lundie, 1940) were transferred into separate containers. Then, 40 larvae each were collected in seven Petri dishes (ø 4 cm, one for each container) and immediately frozen. About 100 wandering larvae of each container were divided into two groups (~50 each) and moved into two sand containers to allow further development in darkness in an incubator at 30 °C. To obtain pupae and adults, individuals were collected from these containers after four and ten days respectively. All samples were immediately frozen and kept separately in Petri dishes (ø 4 cm, one for each container) until the number of *P. larvae* spores was quantified in the laboratory.

2.2. Field test

Hypothesis: Adult small hive beetles contaminated with P. larvae increase the number of spores in uninfected field colonies.

To eliminate the possibility of a previous contamination with *P. larvae* spores, we used only new equipment (18 wooden nucleus colony boxes [Beehive 5 Frame Nuc Box], plastic combs [$N = 72$, Pierco Plastic Frame], feeders [$N = 18$; Division Board Feeder]) and queenright non-infected package bees (European-derived honeybees, presumably of *A. m. ligustica*). Three groups consisting of six nucleus colonies each were established at different isolated apiaries (distance > 3.5 km) to minimize drifting and robbing as alternative transmission pathways of *P. larvae*. Each nucleus box was equipped with four frames and one feeder. On day one, the package bees with their caged, mated queens were introduced into each of the nucleus boxes and the feeders were supplied with sugar water (1:2 by volume). After four days, the queens

were released and all colonies were fed again. Furthermore, bee samples (~ 100 workers) were collected from each colony to double check for potential previous infections with *P. larvae*. One week later, adult small hive beetles (N = 36; 3284 [2119; 3545] spores/beetle, based on 10 SHB), which were previously kept for one week on brood combs with clinical AFB symptoms, were introduced into each colony at one apiary (= treatment apiary). On the same day, the colonies (N = 6) at the second apiary were treated with uncontaminated beetles (N = 36), which were previously kept for one week on brood combs without clinical AFB symptoms (= negative control apiary; 2 [0; 5] spores/beetle, based on 10 SHB). Two brood combs from each of the colonies at the third apiary were sprayed twice on both sides with a *P. larvae* spore suspension (~ 4 mL per comb). The suspension was obtained from the brood combs with clinical AFB symptoms used to contaminate the beetles by mixing the ropy mass and foulbrood scales into aqua dest. (~ 6.5 mill. spores / mL). The spraying was repeated twice, after three and seven days. This apiary served as a positive control for viability and virulence of the *P. larvae* spores used. Five weeks later, all 18 colonies were carefully checked for clinical AFB-symptoms (Ritter, 1996) and adult workers (~ 100) and honey (~ 10 g) were collected. All samples were immediately frozen until they were analysed in the laboratory to quantify the number of *P. larvae* spores per individual.

2.3. Culture of *Paenibacillus larvae*

The culture of *P. larvae* was performed according to routine protocols (De Graaf et al., 2006). Small hive beetle adults, pupae and wandering larvae (N = 12 individuals from each treatment and from the control) were individually squashed in plastic vials (1.5 mL) and diluted in 0.5 mL (dilution factor per SHB: 5) sodium chloride (0.9%). Then, the liquid was transferred into new plastic vials to facilitate pipetting. From each worker sample, 40 individuals together were homogenized in 20 ml (dilution factor per bee: 5) sodium chloride (0.9%) using a stomacher bag. From each honey sample, 5 g were diluted with 5 mL (dilution factor per gram: 20) aqua dest. and vortexed. To select for *P. larvae* spores, all samples were incubated in water for six minutes at 90 °C. Then, all samples were allowed to cool down to room temperature, vortexed again and 100 µL were applied onto each

of three Colombia sheep blood agar (CSA; containing 3 µg/mL nalidixic acid) plates per sample. All plates were incubated at 37 °C and 5% CO₂ for six days. Then, all *P. larvae* colonies were counted on each plate and the average of the plates was calculated. If more than one of the three plates was not countable because of contamination by other bacteria, the individual spore count was not included into the results. Colonies were identified as *P. larvae* visually and confirmed with the Plagemann-Test (Plagemann, 1985) and PCR (De Graaf et al., 2006).

2.4. Statistical analyses

Kruskal-Wallis-Tests with multiple comparisons were applied to compare the number of spores per individual for each of the different SHB adult and pupae containers in the laboratory test. Mann-Whitney U-Tests with Bonferroni adjustments ($\alpha = 0.025$) were used to test if the *P. larvae* spore contamination of honeybee- and honey- samples in the field test differed between treatment and control colonies and between before the treatment and five weeks after. All spore-numbers are given as medians and [1st; 3rd] quartiles.

3. RESULTS

3.1. Laboratory test

The number of *P. larvae* spores on individual adult beetles kept in treatment containers with AFB infected brood combs was for each container significantly different from the controls (Tab. I). Similarly, individual pupae obtained from larvae originating from treatment containers had significantly more spores than the controls (Tab. I). Finally, individual, newly emerged adults from the treatment groups were in four out of five containers significantly more contaminated with spores than the controls (Tab. I). In the case of wandering larvae all CSA plates (N = 216) from treatments became overgrown with *P. larvae* colonies. The number of colonies on those plates was therefore estimated as > 1000, which equates > 5000 *P. larvae* spores per individual. The controls had 0 [0; 0] spores/larvae.

Table I. Results of the laboratory tests. Sample sizes (N) and the number of *P. larvae* spores are shown for each tested life stage, except for the wandering larvae (see text). The *P*-values of the comparisons of the treatments with their respective control groups (Kruskal-Wallis-Test with multiple comparisons as post hoc tests) are also given.

Group	Treatment						Control
	1	2	3	4	5	6	C
Parental adults							
N	12	12	12	12	11	12	12
Minima	473	397	30	283	110	393	0
1st Quartile	674	482	211	410	218	593	1
Median	730	567	351	465	410	847	3
3rd Quartile	1202	730	480	536	432	1105	7
Maxima	1420	1360	625	1267	1313	1733	20
<i>P</i> -values	<0.001	<0.001	0.332	0.007	0.159	<0.001	
Pupae							
N	12	12	12	12	12	12	12
Minima	20	68	17	17	12	40	0
1st Quartile	40	91	19	32	18	69	2
Median	69	128	33	41	28	97	2
3rd Quartile	142	173	82	53	75	225	3
Maxima	262	233	860	1250	335	470	10
<i>P</i> -values	<0.001	<0.001	0.017	0.026	0.049	<0.001	
Newly emerged adults							
N	12	12	12	10	*	11	12
Minima	13	65	12	3		12	0
1st Quartile	35	89	43	13		31	0
Median	55	97	83	18		59	3
3rd Quartile	83	108	149	43		108	4
Maxima	293	215	462	60		149	23
<i>P</i> - values	0.003	<0.001	<0.001	1.000		0.002	

(*evaluation of spore numbers was impossible, due to contamination of the samples)

3.2. Field test

Before the treatments, all tested workers in all groups were free of *P. larvae*. After five weeks, we found significantly more *P. larvae* spores on workers from colonies (N = 6) treated with contaminated beetles (before: 0 [0; 0]; after: 9 [2; 14]; Mann-Whitney U-Test: U = 6, Z [adjusted for ties] = -2.29; *P* = 0.022 [Bonferroni adjusted; α = 0.025]). There was no significant difference in the number of spores at the negative control apiary after five weeks (before: 0 [0; 0]; after: 1 [0; 2]; N = 6 colonies; Mann-Whitney U-Test: U = 9, Z [adjusted for ties] = -1.90; *P* = 0.058 [Bonferroni adjusted; α = 0.025]). No significant differences in spore numbers were found between negative control and treatment group af-

ter five weeks, neither on bees (N = 6 colonies; Mann-Whitney U-Test: U = 9, Z [adjusted for ties] = -1.50; *P* = 0.134 [Bonferroni adjusted; α = 0.025]) nor in the honey (N = 6; Mann-Whitney U-Test: U = 9, Z [adjusted for ties] = -1.62; *P* = 0.102 [Bonferroni adjusted; α = 0.025]). While none of the honey samples of the negative control group was contaminated, two of five honey samples (one colony had no honey) from the treatment apiary were contaminated (0 [0; 6]). While all colonies in the treatment group showed a scattered brood pattern after five weeks, this was not observed in any of the negative control colonies. In the positive control apiary, we found high numbers of *P. larvae* spores on adult workers (15 000 [10 000; 15 000] spores/bee) and in the honey samples (15 000 [2000; 40 000]

spores/g). Moreover, all colonies at this apiary showed clinical AFB symptoms (> five infected cells with ropy mass and several foulbrood scales) and a scattered brood pattern after five weeks.

4. DISCUSSION

Our data clearly show that small hive beetles are vectors of *P. larvae*. Both adult and larval beetles became contaminated with *P. larvae* spores when exposed to honeybee brood combs with clinical American foulbrood symptoms in the laboratory. Indeed, we found spores on all larval and adult small hive beetles kept on infected combs. The contamination persisted in pupae and newly emerged adults, even though those individuals had no further AFB exposure after the wandering phase. As expected, the wandering larvae had the highest spore numbers (> 5000 spores/individual), probably because the larvae fed on infected brood and were mining within the cells with clinical symptoms.

Honeybee field colonies, which were infested with contaminated adult beetles, showed slightly higher numbers of *P. larvae* spores in adult workers and honey samples after five weeks. All colonies in the positive control showed obvious clinical AFB symptoms, thereby proving the viability and virulence of the used spores. However, neither the treatment colonies nor the negative controls showed any clinical symptoms of AFB. Nevertheless, recent data suggest that colonies may develop considerable spore densities on adult bees without exhibiting visible symptoms of disease (Lindström et al., 2008) and this could contribute to further AFB spread.

We found no significant differences in spore numbers on adult workers between the treatment and the negative control apiary. This is most likely due to the contamination of the SHB which were used for the negative control apiary (2 [0; 5] spores/beetle). Since antibiotics were allowed to control AFB in the USA (Morse and Flottum, 1997), *P. larvae* spores, which are able to survive such treatments, have apparently accumulated on the brood combs that were used to feed the SHB for the negative

control. However, in contrast to the continuous brood nests in the negative controls, the brood patterns in the colonies of the treatment apiary were obviously scattered. This might have resulted from hygienic behaviour of the workers removing infected larvae (Spivak and Gilliam, 1998).

Hansen and Brødsgaard (1997) fed honeybee colonies with contaminated honey and found that the minimum dose of *P. larvae* spores necessary to cause an outbreak of American foulbrood is 2.0×10^9 . This suggests that the numbers of spores we found on adult SHB (3284 [2119; 3545] spores/beetle) might be too small to cause a clinical AFB outbreak, even if hundreds of contaminated beetles invade a colony at once (Tribe, 2000). However, Brødsgaard et al. (1998) also fed various doses of *P. larvae* spores to in vitro reared honeybee larvae and reported a lethal dose of only 8.49 spores/larvae which killed 50% of 24–28 h old larvae and a lethal dose of 51.35 spores/larvae which killed 90% of the same group. This suggests that even if only small doses of *P. larvae* spores are transmitted, it is nevertheless possible to infect young honeybee brood (Brødsgaard et al., 1998). Small hive beetle adults are known to consume honeybee eggs and larvae (Swart et al., 2001) and thus could deliver spores directly to the base of brood cells, thus increasing the chances for transmission of AFB.

The rather low number of *P. larvae* spores on adult SHB suggests that clinical AFB outbreaks are less likely. However, even small spore numbers can be sufficient to spread *P. larvae*. This could be especially dangerous for colonies with young brood only, as a result of swarming or requeening after queen loss, because young brood are more susceptible to infection with *P. larvae* (Brødsgaard et al., 1998). We suggest considering the role of SHB in AFB control in areas where both pests are established.

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Le petit coléoptère des ruches, *Aethina tumida*, vecteur de *Paenibacillus larvae*.

***Aethina tumida* / loque américaine / *Paenibacillus larvae* / petit coléoptère des ruches / vecteur**

Zusammenfassung – Kleine Beutenkäfer, *Aethina tumida*, sind Vektoren von *Paenibacillus larvae*. Eine Übertragung von anzeigepflichtigen Krankheiten der Honigbiene durch aktiv fliegende Parasiten wie den Kleinen Beutenkäfer (=KBK) ist unabhängig von Bienen und Imkern und würde somit neue Herausforderungen an Seuchenkontrollmaßnahmen stellen. Wir können hier zeigen, dass sich Adulte und Larven des KBK mit Sporen von *P. larvae* kontaminieren, wenn sie im Labor auf Brutwaben mit klinischen Amerikanischen Faulbrut (=AFB)-Symptomen gehalten werden (Tab. I). Die sich aus diesen Larven entwickelnden Puppen und die neu schlüpfenden adulten KBK waren ebenfalls mit Sporen kontaminiert (Tab. I), obwohl diese Individuen nach dem Wanderlarvenstadium nicht mehr mit infizierter Brut in Kontakt kamen. In einem Feldversuch wurden sechs Völker mit je 36 kontaminierten adulten KBK infiziert. Zuvor entnommene Bienenproben aus allen Völkern zeigten keine Infektion mit *P. larvae* Sporen. Fünf Wochen nachdem die mit *P. larvae* kontaminierten KBK zugefügt wurden, konnten geringe Sporenmengen in Honig- und Bienenproben nachgewiesen werden. Zu diesem Zeitpunkt zeigte keines der infizierten Völker klinische AFB-Symptome, aber das Brutbild war lückenhaft. Die geringe Anzahl der auf den Käfern nachgewiesenen (Tab. I) und im Feld übertragenen Sporen indiziert vermutlich keinen klinischen Ausbruch von AFB. Es reichen jedoch im Prinzip bereits geringe Sporenmengen aus, um *P. larvae* zu verbreiten und insbesondere junge Brut zu infizieren. Der KBK ist somit ein Vektor von *P. larvae*. Unsere Ergebnisse sollten daher insbesondere in Gebieten berücksichtigt werden, in denen sowohl AFB als auch KBK etabliert sind.

***Aethina tumida* / AFB / Amerikanische Faulbrut / Kleiner Beutenkäfer / *Paenibacillus larvae* / Vektor**

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