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MICROORGANISMS IN DEAD BUMBLE BEE LARVAE (*Bombus spp.*) FROM LABORATORY-REARED COLONIES

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Abstract

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It has been found previously that early mortality, the so-called „blackening" of bumble bee larvae, is a phenomenon greatly reducing success in the laboratory bumble bee rearing. Therefore, the aim of this study was to carry out additional isolation of the bacteria and the fungi (*Ascosphaera*) from the dead larvae sampled from the different inter-independent laboratories and from the greater number of colonies. This effort resulted in obtaining 13 types of isolates – nine of them were identified as distinct species, one of them as a species of *Bacillus* sp. and two as gram-negative cocci (bacilli). The yeasts were found only in the one case. Representatives of the genus *Ascosphaera* were not isolated. *Bacillus thuringiensis* was detected in the bumble bee larvae for the first time. *Bacillus pumilus* and *Paenibacillus glucanolyticus* were isolated as a considerable bacteria. These bacteria are well known and have been detected previously. Consequently, they are regarded as potential pathogens in the bumble bee larvae. Potential pathogenity of several microbes is discussed.

Bombus spp., bumble bees, laboratory rearing, sporulating bacteria, *Bacillus thuringiensis*

Early mortality of bumble bee larvae (so-called „blackening of larvae") under laboratory conditions greatly decreases success in the laboratory bumble bee rearing. Larvae blackening syndrome have been described by PŘIDAL, SEDLÁČEK & MARVANOVÁ (1997). In this study, isolations of microbiota were carried out from both the dead (or syndromatic) and the well-growing larvae. Their spectra were different (left part of Tab. II). Some of the microbes isolated from provisions were not found in the dead larvae. In consequence of this fact, the spectrum from the dead larvae was regarded as a potentially pathogenic (harmful). There was discussed the necessity of additional isolation and verifying pathogenity by an experiment in the mentioned study.

The subject of this study was to carry out additional isolation of the sporulating bacteria and fungi of the genus *Ascosphaera* from the dead larvae. The main effort was to carry out the isolations from the greater number of larvae, colonies and above all from inter-independent laboratories. The obtained results compare with the previous results and operating hypothesis.

MATERIAL AND METHODS

a) laboratories and applied nourishment

Larvae were sampled from three laboratories as follows:

1. Bee Research Institute Ltd., Dol - station Prostějov (Dr. Krieg);

2. Masaryk University, Brno - (Assoc. prof. Ptáček);
3. Research Institute for Fodder Plants Ltd., Troubsko (Dr. Hofbauer).

These laboratories were inter-independent – i.e. there was practised neither frequent exchange of the biological material nor provisions. There was one exception – the pollen has been changed between Prostějov and Masaryk University labratories. There were reared mainly *Bombus terrestris* in these laboratories.

The nourishment in the separate laboratories was based on individual variants based on the pollen loads from pollen-traps (on bee hive entrance) and sugar-honey solutions.

b) sampling of the dead larvae

The dead larvae were sampled with clear tweezers into test tubes (with number of laboratories, colonies and dates of sampling). The test tubes were either sterilized or degreased and washed in 75% ethyl alcohol including plugs. Thus sampled larvae were examined immediately or stored at 5 °C until examination. Only sample M1 from Masaryk University has been stored for few weeks at room temperature (below).

c) isolation of sporulating bacteria

The microbiological works were provided at Czech Collection of Microorganisms in Brno (CCM). The isolation of sporulating bacteria was carried out on two basic agar media, nutrient agar and Columbia agar with blood (both OXOID). The samples (part of the larva) were homogenized in 2 ml of saline solution and subsequently inoculated 250 microlitres to agar media. The plates were incubated for 1-4 days at 30 °C under aerobic conditions. Morphologically different colonies were picked up and with the aid of cross spreading the purity of culture was verified on the medium identical with the isolation one. The generic and species identification of pure cultures was based on microscopy (spore formation), agar morphology and biochemical and physiological tests (Parry et al., 1983, Priest et al., 1988) which are essential for a group of gram-positive sporulating rods.

d) isolation of fungi (*Ascosphaera*)

To isolate fibrous fungi two larval samples were used (sample 1 and sample F). Isolation was carried out on the following nutrient media: Sabouraud agar (SaA), Littman agar (LA) (Difco), Czapek-Dox agar (CDA). The fertility and growth of isolated cultures were tested on the following media: water agar with bee larvae (VCL), Brain-Heart Infusion agar (BHI), malt extract agar (SA). The isolation involved dissecting blackened mummified larvae into pieces with a sterile scalpel and placing the pieces on nutrient solutions and their incubation at 30 °C in the dark under aerobic conditions.

The growth and fertility were determined on the basis of the inoculation of a small portion of culture from the actively growing margin to the centre of a Petri dish (9 cm in diameter) with VCL and BHI and the dishes were incubated at 30 °C in the dark.

RESULTS

Seven samples from 153 nests were examined. Their characterisation is below.

Bee Research Institute Ltd., Dol - station Prostějov

sample **P**

- the death entirely blackened larvae from 45 starting colonies with queens (founder) captured in nature during spring–time.

Research Institute for Fodder Plants Ltd., Troubsko

sample **T1a**

- the dead entirely blacked larvae from 9 colonies with bad growth in initial period. Their queens were obtained from the nature or the laboratory rearing.

sample **T1b**

- as well as the sample „T1a" but these larvae were whitish and taken from outside of the nest (comb) from two colonies.

sample **T2**

- as well as sample „T1a" but these larvae arised from two well-growing colonies.

sample **T3**

- the dead entirely blackened larvae from 15 starting colonies with queens captured in nature during spring–time.

Masaryk University in Brno

sample **M1**

- the dead entirely blackened larvae from 23 colonies with queens emerged in laboratory rearing.

sample **M2**

- the dead mainly entirely blackened larvae from 57 badly developing colonies in initial period. Their queens originate either from the laboratory rearing or they were captured in the natural surroundings during spring-time.

RESULTS

Results are given in Tab. II. There are listed all found microbes including microbes which have been isolated by Přidal et al. (1997) - left part of table. Commentary to the potential pathogenity is given. The results according to the individual samples of the larvae are given Tab. I.

Bacillus badius and *B. sphaericus* were found for the first time and in two laboratories (P and M). From the dead larvae, *Bacillus circulans* and *B. licheniformis* were isolated for the first time. These bacteria have been found in provisions of bumble bees so far – in honey and pollen from pollen traps. As well as *Paenibacillus pabuli* has been found only in pollen and well-growing larvae so far.

Species *B. pumilus* and *P. glucanolyticus*, which have been found previously, were found also in this experiment - repeatedly and frequently in pure cul-

I: *The outcomes of identification of microbes according to the place of bumble bee rearing*

type or species of microbe	place of bumble bee rearing						
	Bee Research Institute station in Prostějov	Research Institute for Fodder Plants in Troubsko				Masaryk University in Brno	
	sample P	T1a	T1b	T2	T3	M1	M2
Bacillus sp.					+		+
Bacillus badius							+
Bacillus circulans	+						
Bacillus licheniformis			+	+	+		
Bacillus pumilus						+	
Bacillus sphaericus	+						+
Bacillus thuringiensis	+	+					+
Paenibacillus glucanolyticus						+	
Paenibacillus pabuli						+	
G-cocci					+		
G-coccusbacilli			+	+			
Ascopshaera sp.	neg.	neg.	neg.	neg.		neg.	

ture. Species *B. cereus*, *B. fusiformis*, *B. megaterium* and *B. subtilis* were not isolated in spite of it that in previous study they were found.

Bacillus thuringiensis was found for the first time from bumble bee larvae in the each examined laboratory rearing – in one case also as a pure culture (sample T1a). Any species of entomopathogenic *Ascosphaera* was not found.

Repeatedly, there were also unidentified isolates of different types as follows: *Bacillus* sp. (without specific identification), gram-negative cocci, gram-negative coccusbacilli, rods and one non-typical *Bacillus licheniformis*. Their biochemical characterisation is presented for subsequent opportunities of comparison.

A) result of identification:

Bacillus sp.

source: Masaryk University

1. microscopical features: gram-variable thin rods, in pairs or palisades; ellipsoidal to round spores are produced in swollen sporangia and placed subterminally.
2. morphology on agar: colonies are circular, smooth, shiny, slightly convex, entire margin and about 0.5-1mm in diameter.
3. positive results of biochemical tests: catalase, hydrolyse of gelatine, esculin, ONPG (o-nitrofenyl-beta-D-galaktopyranosid) and DNA.
4. negative results of biochemical tests: growth under anaerobic conditions, in 10% NaCl and at 50 °C; hydrolyse of starch, casein, tyrosin and lecithine; urease, acetoin; reduction of nitrates; acid production from glucose, xylose, mannitol and lactose; gas production from glucose; Simmons citrate, arginin dihydrolase.

B) result of identification: *Bacillus* sp.

source: Research Institute for Fodder Plants

sample: T3

1. microscopical features: gram-variable rods, in single or irregular clusters; ellipsoidal spores are produced in not swollen or in swollen sporangia (group I. - II.) and placed subterminally.
2. morphology on agar: irregular colonies, matt surface, flat, lobate margin, 3-6 mm in diameter.
3. positive results of biochemical tests: catalase; hydrolyse of gelatine, Tween 80 and ONPG; reduction of nitrates; acid production from glucose; growth at 50 °C.
4. negative results of biochemical tests: growth under anaerobic conditions, in 10% NaCl and at 56 °C; hydrolyse of starch, casein, esculine, DNA and lecithine; urease, acetoin; acid production from xylose, mannitol, cellobiose, lactose, fructose and inositole; Simmons citrate, arginin dihydrolase.

C) result of identification: unidentified

source: Research Institute for Fodder Plants

sample: T3

1. microscopical features: gram-negative cocci, in single, in irregular clusters.
2. morphology on agar: colonies are dots, whitish, translucent.
3. positive results of biochemical tests: catalase; acid from glucose by oxidation; acid from fructose and xylose; Simmons citrate; hydrolyse of Tween 80, esculine and ONPG, urease; growth at 37 °C.
4. negative results of biochemical tests: growth in 6,5% NaCl and at 42 °C; acid production from glucose by fermentation; acid production from mannitole and maltose; production sulphuretted hydrogen; reduction of nitrate and nitrite; hydrolyse of gelatine, starch, caseine, tyrosine, lecithine and DNA; decarboxylation of lysine and

ornitine; arginin dihydrolase, acetamid; haemolyse and oxidase.

D) result of identification: *Bacillus licheniformis*
source: Research Institute for Fodder Plants non-typical
sample: T3

1. microscopical features: gram-positive rods, in pairs, in short palisade; ellipsoidal spores are produced in not swollen sporangia and placed centrally.
2. morphology on agar: colonies round, surface smooth, flat with sculptures; margin irregular and about 2–4 mm in diameter.
3. positive results of biochemical tests: catalase; growth at anaerobic conditions and 56 °C; hydrolyse of gelatine, starch, caseine, esculine, ONPG and DNA; acetoin, reduction of nitrate; acid production from glucose, mannitole, celobiose, fructose; Simmons citrate; arginin dihydrolase.
4. negative results of biochemical tests: hydrolyse of Tween 80 and lecithine; urease; acid from xylose, lactose and inositole.

E) result of identification: unidentified
source: Research Institute for Fodder Plants
sample: T2

1. microscopical features: gram-negative coccobacilli or rods, sporadic filaments; in single and irregular clusters.
2. morphology on agar: colonies round, surface smooth, slightly shiny and convex, translucent; margin entire and about 1 - 2 mm in diameter.
3. positive results of biochemical tests: growth in 6,5% NaCl at 37 °C; acid production from glucose by oxidation; Simmons citrate; hydrolyse of esculine and ONPG; urease; acid production from fructose, xylose, maltose and sacharose; beta-glucosooxidase; catalase.
4. negative results of biochemical tests: growth at 42 °C; acid from glucose by fermentation; production fluoresceine, indole, sulphuretted hydrogen; arginin-dihydlolase; acetamid; reduction of nitrite; hydrolyse of gelatine, starch, Tween 80, caseine, tyrosine, lecithine; decarboxylase of lysine and ornitine; acid from mannitole and lactose; oxidase, fosfatase, gama glutamyl-transferase, N-acetyl beta-D glucosaminidase.

DISCUSSION

Ruijter, Eijnde & Steen (1997) studied impact of dried-fruit-moth (*Plodia interpuctella*) on bumble bee colony development within prime eusocial stage. They found that moth larvae are harmful for laboratory-reared colonies of bumble bees. They added young moth larvae into 12 nests – ten of them were disturbed by destruction of nest formation and dead larvae were also found. In the control group the development was normal. The same trials were carried out by Přidal (1999) with the same results. It is possible that the larvae of *Plodia interpuctella* are source of virulent strains of entomopathogenic *Bacillus thuringiensis*. Since *Bacillus thuringiensis* is pathogenic agent in butterflies (*Lepidoptera*) its rate of pathogenity in bumble bee larvae must be verified by experimental infections. Tasei (2000, personal communication) told me that they have mixed „BT" preparation (spores *B. thuringiensis*) into bumble bee provisions to control *P. interpuctella*. He added that they had not found any affect on colony development. *B. thuringiensis* can come from mites; a similar possibility is discussed by Pouvreau (1993). Přidal et al. (1997) isolated *Paenibacillus glucanolyticus* from mesenteron of bumble bee queens captured in natural habitat - it is next potential source of the infection.

It was found that agent, which could cause the death of larvae, was present in every recognized sample. Whether it was well-known: *Bacillus pumilus* and *Paenibacillus glucanolyticus* or newly detected entomopathogenic agent *Bacillus thuringiensis*. No bacteria typical of the dead larvae spectrum were found in sample T2 and T3; in the sample T2 only *Bacillus licheniformis* and gram-negative coccusbacilli; in the sample T3 only *Bacillus licheniformis* typical + non-typical, *Bacillus* sp. and gram-negative cocci. The reason of their death is ambiguous for the present.

Macfarlane et al. (1995) found mainly gram-positive sporulating bacteria in dead larvae of *Pyrobombus melanopygus* that is in conformity with results of this study. Gram - negative sporulating bacteria were detected only exceptionally.

Bailey (1981) states several bacterial groups according to their pathogenity as follows:

a) primary pathogen bacteria - etiological agent which causes disease directly (e.g. *Bacillus thuringiensis* - mainly in *Lepidoptera* or *Paenibacillus larvae* - American foulbrood in honeybees);
b) secondary pathogen bacteria - the death of infected larvae by primary bacteria may be accelerated by secondary bacteria - following bacteria (e.g. *Paenibacillus alvei* - as a etiological agent of European foulbrood which is followed by the secondary bacterium *Brevibacillus laterosporus*);
c) weak pathogens - e.g. *Bacillus cereus* causes Septicaemia in honeybees;
d) commensals;
e) saprophytes.

It is not excluded that *Paenibacillus glucanolyticus* (Alexander & Priest, 1989; in Shida et al., 1989) was confused with *P. alvei* before 1989 because of the similarity of their morphological (motile colonies) and another biochemical features. This similarity can be one of the reason for assumption that *P. glucanolyticus* is also the potential pathogen. Pathogenity of *Bacillus sphaericus* was confirmed in mosquito (Charles et al., 1996, etc.).

Most of the bacteria isolated from the dead bumble bee larvae are positive in degradation of macromolecules that is indicative of potential pathogenity. For example, Priest et al. (1988) stated almost 100 %

degradation of chitine in *B. thuringiensis, B. cereus, B. licheniformis* atd.

It is evident that there is a necessity to carry out experimental infections by spores of mentioned potential pathogens to verify their real pathogenity. Results must be compared with control groups. However, in spite of these exact experiments the pathogenity need not be definitely verified because of above mentioned the weak pathogens.

The dead larvae are often too small for the possibility of dividing of the content of their mesenteron (or whole alimentary canal) from mixocoel. Therefore, the bacteria presented in this study need not come only from mixocoel. *Bacillus licheniformis, B. megaterium* and *B. subtilis* were found in alimentary canal of following bees: honey bees and *Megachile rotundata* (GILLIAM & PREST, 1987; INGLIS, YANKE & GOETTEL, 1998).

SUMMARY

It has been found previously that blackening of bumble bee larvae decreases success in laboratory rearing. Therefore, the aim of this study was to carry out additional isolation of bacteria and fungi (*Ascosphaera*) from dead larvae sampled from different inter-independent laboratories and from greater number of colonies.

This effort resulted in obtaining 13 types of isolates. Following bacteria were isolated from the dead bumble bee larvae for the first time: *Bacillus* sp., *B. sphaericus* and *B. thuringiensis*. Further following bacteria have been found in bumble bee provisions and now they were found also in the dead larvae: *B. circulans, B. licheniformis* and *Paenibacillus pabuli. Bacillus fusiformis, B. pumilus, Paenibacillus glucanolyticus* and yeasts were found again and these bacteria are typical of the syndromatic (black) larvae. Any species of entomopathogenic *Ascosphaera* ware not found.

Bacillus pumilus and *Paenibacillus glucanolyticus* are regarded as potential pathogens in bumble bee larvae in relation to their frequent occurrence in body of the dead larvae. Their pathogenity and pathogenity of *B. thuringiensis* must be verified in individual trial by the experimental infection.

SOUHRN

Mikroorganismy izolované z uhynulých larev čmeláků *(Bombus spp.)* pocházejících z laboratorních chovů

Již dříve bylo zjištěno, že tzv. černání larev výrazně snižuje úspěšnost laboratorního chovu, a proto bylo přistoupeno k dalším analýzám uhynulých larev. Cílem této práce bylo izolovat spektrum sporulujících baktérií a hub rodu *Ascosphaera* z těl uhynulých larev. Přičemž snahou bylo, aby tyto izolace byly provedeny z většího počtu larev, hnízd a především na sobě nezávislých chovů.

Celkem bylo izolováno z těl uhynulých larev 13 typů izolátů. Poprvé byly izolovány z uhynulých larev tyto druhy: *Bacillus* sp., *B. sphaericus* a *B. thuringiensis*. Další druhy byly dosud izolovány pouze z potravy a nyní nově i z uhynulých larev: *B. circulans, B. licheniformis* a *Paenibacillus pabuli*. Opakovaně byly v uhynulých larvách zjištěny tyto druhy: *Bacillus fusiformis, B. pumilus* a *Paenibacillus glucanolyticus* a kvasinky. Zástupce rodu *Ascosphaera* nebyl zjištěn v žádném vzorku.

S ohledem na opakovaná zjištění *Bacillus pumilus* a *Paenibacillus glucanolyticus* v tělech uhynulých larev považujeme oba druhy za potenciální patogenní agens v chovech čmeláků. Jejich skutečná míra patogenity musí být však spolu s druhem *Bacillus thuringiensis* ověřena experimentálními infekcemi.

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II: *General survey of still isolated microorganisms from bumble bee rearing*

	the results of isolation (Přidal et al., 1997)							the results of this study			COMMENTARY
	dead larvae	larvae from badly developing colonies or colonies in switch point	well growing larvae	pollen loads from pollen traps	honey	sugar	mesenteron of bumble bee queen	Bee Research Institute - station Prostějov	Research Institute for Fodder Plants	Masaryk University	
Bacillus sp.	+				+				+	+	see for the characterisation in results
Bacillus badius										+	isolated for the first time
Bacillus cereus		+									potential pathogen; also ehiological agent of Septicaemia in honey bees; pathogen in Bombyx mori (Steinhaus, 1949)
Bacillus circulans				+	+			+			typical bacterium of pollen loads (as well Gilliam, 1979)
Bacillus fusiformis		+							+		only one isolation
Bacillus licheniformis				+	+	+			+		for the first time isolated from the dead larvae; typical bacterium of pollen loads (as well Gilliam, 1979)
Bacillus megaterium	+										also in pollen (Gilliam, 1979); pathogen in Bombyx mori (Steinhaus, 1949)
Bacillus pumilus	+	+			+				+	+	POTENTIAL PATHOGEN, Gilliam (1979) also in pollen
Bacillus sphaericus								+		+	for the first time isolated from the dead larvae
Bacillus subtilis	+										also in pollen (Gilliam, 1979)
Bacillus thuringiensis								+	+	+	typical entomopathogenic agent
Brevibacillus laterosporus			+								also as a secondary infection in European foulbrood (Bailey, 1981); athogen in Bombyx mori (Steinhaus, 1949)
Enterococcus faecalis	+										also as a secondary infection in European foulbrood (Bailey, 1981)
Flavimonas oryzihabitans				+							
Paenibacillus glucanolyticus	+						+			+	POTENTIAL PATHOGEN
Paenibacillus pabuli			+	+						+	for the first time isolated from the dead larvae
Pantoea agglomerans				+							
Gram-negative cocci									+		possible patogenity must be verified
Gram-negative coccobacilli									+		possible patogenity must be verified
Ascoshaera sp.	+			+				neg.	neg.	neg.	pathogenity is not excepted
yeasts	+	+			+		+			+	pathogenity is not excepted

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