

MICROBIOLOGY OF *BOMBUS TERRESTRIS* L. LARVAE (HYMENOPTERA: APOIDEA) FROM LABORATORY REARING

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Abstract

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The paper gives results from the studies of early mortality of larvae of the primary brood under the laboratory rearing of *Bombus terrestris*. A dynamics of changes — symptoms before death was described. The larvae gradually changed their colour until they turned completely black and the queen (or the worker) removed them from the nest. In some cases removed from the nest were also living larvae which did not change their colour but on the contrary they continuously consumed the provisions. The dead larvae were used for isolation of bacteria and fibrous fungi. The following species were detected: *Bacillus* sp., *B. cereus*, *B. fusiformis*, *B. pumilus*, *Paenibacillus glucanolyticus*, *B. megaterium*, *B. subtilis*, *Ascosphaera* sp. In larvae without symptoms the following species were determined: *Brevibacillus laterosporus*, *Paenibacillus pabuli* but also no species were reported. *B. circulans*, *B. licheniformis*, *Paenibacillus pabuli* and *Ascosphaera apis* were isolated from pollen and *Bacillus* sp., *B. circulans*, *B. licheniformis* and *B. pumilus* were isolated from honey. On the basis of these observations none of the contagious bacterial diseases of *Apis mellifera* was proved. With respect to the differentiation in the spectrum of microbiota from larvae without symptoms and dead larvae we assume that the species isolated from the dead larvae can be harmful to bumble bee larvae. This hypothesis is also supported by the fact that the food contained many more species than was later obtained from the dead larvae. The source of contamination is bee honey and the gut of bumble bee queens captured in the open air. In the case of *Ascosphaera* sp. the source is pollen collected by honey bees. In the case of *B. cereus* and *B. fusiformis* the source was not detected. It is necessary to experimentally test the effects of isolated species of microbiota on bumble bee larvae.

Bombus terrestris, bumble bees, laboratory rearing, microbiology

At the very beginning of our efforts to develop bumble bee rearing it was clear that the health will be a great problem. In closed laboratory rearing the problems with parasites were eliminated (MACFARLANE et al., 1995). These authors also reported that unlike honey bees (*Apis mellifera* L.) no contagious diseases were found in bumble bees (*Bombus* sp.) but it was found that gram-positive sporulating bacteria were involved in 25 % larval mortality in large colonies of *Bombus melanopygus* in Washington, USA. However, GILLIAM et al. (1994) first

isolated the pathogenic fungus *Ascosphaera apis* from the dead larvae of *Xylocopa californica arizonensi*. They reported that the larvae were not mummified, but their development went on right under the integument.

The source of larval contamination is predominantly their provisions. The microbiology of the pollen collected by honey bees and bee bread was investigated by GILLIAM (1979^{ab}). The spectrum of yeasts and bacteria of the genus *Bacillus* was described in pollen collected manually, pollen collected in pollen traps and bee

bread obtained at different fermentation stages from almond (*Prunus dulcis*). The following species of the genus *Bacillus* were isolated: *B. subtilis* (abundant), *B. megaterium*, *B. licheniformis*, *B. pumilus*, *B. circulans*. She also studied the spectrum of microorganisms in the contents of mesenteron in bees (GILLIAM *et* VALENTINE, 1976) and performed the isolation of microorganisms connected with the provisions and the life of stingless and wild bees (GILLIAM *et*, 1990^{ab}). In stingless bees even *Paenibacillus alvei* was isolated, among others were *B. megaterium* and *B. circulans*. In wild bees *B. licheniformis*, *B. cereus*, *P. subtilis*, *B. circulans* and *B. pumilus* were isolated.

The microorganisms present in the provisions of *Megachile rotundata* did not occur at the same species spectrum in the mesenteron any longer (INGLIS *et al.*, 1993) and only the species which are considered to be part of the mesenteron microflora remained there. Also the populations of bacteria and fungi (not yeasts) were higher in the larvae infected with *Ascosphaera aggregata*.

A reduction in the number of microorganisms in the food (using gamma radiation or surface treatment with dry chlorine) resulted in significantly lower mortality of *Megachile rotundata* Panz. larvae (INGLIS *et al.*, 1992).

Soon after the activation of the queen in the laboratory rearing of the bumble bee at the Forage Crop Research Institute in Troubsko u Brna early mortality of larvae from the first brood occurred during their development and growth. A similar problem existed in commercial rearing in Israel. It was believed that the causal agent was *Paenibacillus larvae* (WEISER, 1996 — personal communication). The objective of the present study was to identify external symptoms of larval black discoloration, to isolate and detect microorganisms from dead and living larvae of the bumble bee and to determine their source.

MATERIAL AND METHODS

Bumble bee rearing was performed in a room heated to the temperature of 28°C and relative humidity of 65 ± 5 %. Sucrose solution was prepared from water and sucrose (1:1) with 300 mg of vitamin E, 2 g of Fumagilin, 0.3 g of sorbic acid per 1 kg of solution. This solution was only given for two weeks. For another two weeks the same solution free from Fumagilin was provided (TASEI *et* AUPINEL, 1994). Another solution (another treatment) was composed of water, sucrose, honey contains 18 % water in proportion 1:2:1 with 300 mg vitamin E, 2 g

of Fumagilin per 1 kg of solution. Pollen was offered compact at the rim of a Petri dish to reduce the rate of its drying. In the course of experiments pollen from rapeseed (*Brassica napus* L. var. *olearacea*) and cultivated poppy (*Papaver somniferum* L.) was used. When the queen established the first brood cell the date was recorded and the brood developed under supervision. It means that observations were made if the brood pocket with larvae expands adequately and if larvae are not removed from the pocket by the queen.

The samples of black larvae for microbiological analysis were transferred directly from the nest into sterile test tubes and examined immediately or stored at 5°C until examination. The larvae, which were not quite black and wilt were washed with physiological solution to reduce the risk of surface contamination. The larvae were taken both from the nests which exhibited insufficient growth and from the nests in which larvae developed normally. Larvae without symptoms were subjected to dissection by which the contents of mixocoel and the contents of the mesenteron were separated under aseptic conditions and each of them was examined separately or only the contents of the mesenteron were analyzed.

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 : agar from bumble bee larvae (CLA), 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 CLA, VCL and BHI and the dishes were incubated at 30°C in the dark.

The isolation of sporulating bacteria was carried out on three agar media, viz. meat-pepton agar (OXOID), Brain-Heart Infusion agar (OXOID) and Columbia agar (OXOID) with blood and on two nutrient-rich media recommended for culture of bee pathogens - Melisococcus pluton medium (Bailey and Collins, 1982) and MYPGT-medium (Digman and Stahly, 1983). The samples (part of the

larva, hemolymph, provisions) were direct sown on the media or propagated in the liquid medium and subsequently inoculated to agar media. The culture of samples lasted for 2–6 days at 30°C under aerobic conditions. Macroscopically different colonies were removed 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 biochemical and physiological tests (PARRY *et al.*, 1983, PRIEST *et al.*, 1988) which are essential for a group of gram-positive sporulating rods.

RESULTS

The mortality of larvae was observed especially at the time primary brood development. Another outbreak was recorded in other stages of brooding in laboratory colonies, mostly in middle-sized larvae.

Larval mortality was also reported in colonies in the field. In one rare case the whole outdoor colony collapsed and the syndromes were identical with those observed in laboratories (not yet sufficiently verified by microbiological analysis).

Our observations were that the larvae before dying turned rather dark (creamy yellow to ochre brown in colour). They were still alive at this stage but they did not take any food (confirmed "in vitro"). This stage, however, did not last long (approximately 24 hours at a maximum) before the larvae died. At this stage the microbial processes were very rapid, the larvae turned wilt and changed their colour from brown to black. The intensity of the process was varied.

If the queen did not remove the diseased larvae from the nest before, they did not adhere to the bottom of the cell and it was easy to remove them from the cell. During the wilting process the larva did not have a pastelike consistency like a glue which state is typical for brood pest, but the symptoms of the process reminds us of the disease caused by the bacterium *Paenibacillus alvei* in *Apis mellifera* (European foulbrood). The odour in small larvae is not bad and need not be reported. In older larvae the odour is very unpleasant.

It was also observed that the queen threw larvae out of the nest when they were relatively white and did not show a tendency toward colour change, were viable and busy consumed the food.

Table 1 gives results of the isolation of microorganisms from *Bombus terrestris* larvae closely reared in Troubsko. Dominant isolates were gram-positive sporulating rods from the

group of bacilli. Apart from the genus *Bacillus* the group comprises also several other related genera of which the genera *Paenibacillus* (SHIDA *et al.*, 1997) and *Brevibacillus* (SHIDA *et al.*, 1996) were reported in the study. In the first rearing cycle only the genera *Bacillus* and *Paenibacillus* were isolated from the dead syndromic larvae. The contaminating microbiota on bacterial isolation media included representatives of yeasts (*Endomycetes*), which were beyond identification and isolation. Of the genus *Bacillus* the most widespread were *B. cereus*, *B. fusiformis*, *B. pumilus* and *P. glucanolyticus*.

The source of contamination was partly discovered (Tables II and III). The source of *B. pumilus* is honey. *Paenibacillus glucanolyticus* comes from the gut (in section of abdomen) of the queens captured in the open air (Table III). Table II presents isolates from provisions given to bumble bees. In pollen and honey the same species such as *B. licheniformis*, *B. circulans*) were detected. This is logical because both were obtained from the same bee colonies. The isolation performed on pollen collected by honey bees from *Papaver somniferum* (given in the second rearing cycle) revealed other new species of bacteria. Only *Paenibacillus pabuli* was isolated from the mesenteron of larvae without symptoms. Beet sugar which was used for the preparation of feeding syrup in both rearing cycles contained only *B. licheniformis*, but it was not isolated from dead larvae.

Interesting is that in the second rearing cycle no sporulating bacteria were found in larvae (ca 30) on 20 February 1997. In some larvae the process of blackening was rather varied, it was similar to the process of mummification — *Ascospaera* sp. was isolated on the locality of Troubsko u Brna.

From Sample 1 a species of the genus *Ascospaera* was isolated. It resembles to some extent the species *Ascospaera apis* but the range of the size of ascospores is wider. Compared with the species *Ascospaera maior* which has ascospores of greater dimensions it has smaller mycelia. According to the latest monographic description of the genus (BISSETT, 1988) and on the basis of the recently published key to all the known species (BISSETT *et al.*, 1966) the isolate cannot be unambiguously identified. From Sample F a culture was isolated which morphologically corresponds to the species *Ascospaera apis*. To make a comparison of the isolates from bumble bees with a typical representative of the species *Ascospaera apis* several cultures were isolated from bee larvae with typical

Date of isolation	Rearing cycle	Description of source of microorganisms	Identified genera and species of microorganisms
6.VIII.1996	I.	syndromic larvae sample 1	Paenibacillus glucanolyticus Bacillus pumilus Ascosphaera sp.
10.X.1996	I.	badly - growing larvae a - isolation from mixocoel b - isolation from mesenteron	negativ Bacillus pumilus yeasts
		well growing larvae a - isolation from haemolymph b - isolation from mesenteron	negativ negativ
		badly - growing larvae isolation from whole larva body	yeasts
		larvae from the colony at switch point a - isolation from haemolymph b - isolation from mesenteron	Bacillus pumilus - without washing of larva surface Bacillus cereus Bacillus fusiformis Bacillus pumilus
		badly developing colony a - isolation from sample of dead syndromic larvae b - isolation from sample of living larvae	yeasts Bacillus cereus Bacillus fusiformis Bacillus pumilus yeasts
16.X.1996	I.	larvae fed "in vitro"	Bacillus cereus Bacillus fusiformis Bacillus pumilus Bacillus pumilus - non typical Paenibacillus glucanolyticus
20.II.1997	II.	syndromic larvae	yeasts
		larvae with no symptoms of black colour	yeasts
		syndromic larvae sample F	Ascosphaera sp.
7.III.1997	II.	syndromic larvae	Bacillus sp. yeasts
		larvae with no symptoms of black colour	Bacillus sp.
15.IV.1997	II.	well growing larvae	Brevibacillus laterosporus
		isolation from mesenteron	Paenibacillus pabuli

1: Survey of microorganisms isolated from larvae of *Bombus terrestris* L.

symptoms of "chalkbrood". Although the mycelia on bee larvae morphologically corresponded to the species *A. apis*, the isolates on SA and other agars (VCL, CLA) remained after the first re-inoculation from isolation layers infertile, so the comparison of fructification structures in cultures was impossible.

The source of contamination of bumble bee larvae by the fungus from the species *Ascosphaera* was not detected. From the samples of pollen a species morphologically corresponding to *Ascosphaera apis* and an infertile mycelium similar to isolates from bee larvae were isolated.

The above stated facts suggest that in the samples of dead bumble bee and honey bee larvae and in the pollen studied 3 types of representatives of the genus *Ascosphaera* were detected: 1) an isolate morphologically different from *Ascosphaera apis*, fertile on common agar media at 25 and 30°C, 2) an isolate morphologically corresponding to *A. apis*, fertile under the same

conditions as in the previous case, 3) a mycelium characteristic of *Ascosphaera apis* but infertile under the conditions mentioned above.

The question whether the fungus on bumble bee larvae is identical with the fungus on bee larvae, has not been answered yet. It has not been proved yet that the infestation with the fungus is the primary cause of mortality of bumble bee larvae. An alimentary source of contamination is probable but it has not been proved yet.

In the subsequent isolation from larvae (25 larvae) the representatives of the genus *Bacillus* were obtained in high numbers, but species identification was not successful because the cultures were rather non-typical. In syndromic larvae yeasts were also found. In the larvae which were removed from the nest by adults and did not show symptoms of infection, the representatives of the genus *Bacillus* (quite non-typical and non-classifiable) and yeasts were isolated. This indicates that bumble bees can recognize "infected

Date of isolation	Rearing cycle	Description of source of microorganisms	Identified genera and species of microorganisms
26.XI.1996	I.	polen from pollen traps mainly from rape (unknown habitat)	<i>Bacillus circulans</i> <i>Bacillus licheniformis</i> <i>Ascosphaera apis</i>
20.II.1997	I. + II.	sugar	<i>Bacillus licheniformis</i>
7.III.1997	II.	polen from pollen traps mainly from <i>Papaver somniferum</i> L. and honey bee colonies in Troubsko	<i>Flavimonas oryzihabitans</i> <i>Paenibacillus pabuli</i> <i>Pantoea agglomerans</i>
15.IV.1997	I. + II.	honey from honey bee colonies in Troubsko	<i>Bacillus</i> sp. <i>Bacillus circulans</i> <i>Bacillus licheniformis</i> <i>Bacillus pumilus</i>

2: Survey of microorganisms isolated from provisions of bumble bees

Description of source of microorganisms	Identified genera and species of microorganisms
syndromic larvae	<i>Bacillus megaterium</i> <i>Bacillus subtilis</i>
syndromic larvae from field colonies	<i>Bacillus pumilus</i> <i>Enterococcus faecalis</i>
isolation from bumble bee queen	<i>Paenibacillus glucanolyticus</i>

3: Survey of microorganisms from *Bombus terrestris* L. coming from rearing at Masaryk University

larvae" well before their death.

We were also provided with the larvae from the experimental rearing performed at Masaryk University. Of the detected species only *B. pumilus* from larvae and *P. glucanolyticus* from the adult queen (Table III) were isolate. As far as *B. megaterium* and *B. subtilis* are concerned, they were not detected in our rearing.

In the mesenteron and hemolymph obtained under sterile conditions from the larvae which did not develop any symptoms of infection the results were negative. When similar isolation was made in larvae suspicious of infection, *B. pumilus* was isolated in the hemolymph. Together with *B. fusiformis* and *B. cereus* it was also isolated from the mesenteron. But there is a presumption that the organs are infected during dissection. In the course of the isolation of the mesenteron which was carried out later in 25 larvae without symptoms and normal development from 5 colonies, no microorganism, which occurred in dead larvae, was isolated although the representatives were detected in the provisions (made up of honey).

In the larvae fed on a mixture of pollen and honey "in vitro", which died later, the species spectrum of the genus *Bacillus* was very rich (Table 1).

DISCUSSION

We believe that the species of the genus *Bacillus* which occurred repeatedly in individual

analyses can be pathogenic to bumble bees and have a toxic effect on them. This hypothesis is supported by the fact that the same species of microorganisms were repeatedly isolated from the mesenteron of larvae in the first rearing cycle. They did not occur sporadically but in great numbers. And moreover, pollen and sugar contained also other species of microorganisms which were never before isolated from the larvae. For this reason we believe that this is not accidental infection. However, we do not state that this is the common microflora of the mesenteron as reported by INGLIS *et al* (1993). The infection can be specific to the type of rearing (different species isolated from the rearing at Masaryk University). Most probably essential for its development is the concentration of contaminating microorganisms in the provisions or in the nest, important may also be some abiotic factors. High hydrolytic activity of sporulating bacteria (gelatinase, lipase, amylase, DNase, lecithinase, etc.) which can promote the penetration of microorganisms into the host. The development of infection can also be affected by the ratio of natural to contaminating microflora in bumble bee larvae and the production of their metabolites. The present results do not suggest clearly the presence of specific species of microorganisms as etiological agents of larval disease. The contamination of larvae is apparently not accidental because the microbial spectrum of isolates from larvae without symptoms and dead larvae differed in all cases. The only exception

when high numbers were detected was the species *B. pumilus*. The species is primarily isolated from the soil, but there are no literary data available on its entomopathogenic effects.

It is known that a higher number of yeasts in the mesenteron of bees is the result of stress (MÁCHOVÁ 1997, personal communication). Therefore, it is very surprising that yeasts were always isolated with the bacteria *Bacillus*. The presence of yeasts may signal a high level of stress which reduces most probably the resistance of larvae to bacteria. According to the results of experiments with the honey bee (MÁCHOVÁ, 1997 — personal communication) *B. subtilis* markedly reduced the longevity of workers. The mortality of larvae from the rearing at Masaryk University might have been caused by this microbe.

It is necessary to test the hypothesis on the pathogenic effect of isolates in the experiment in which bumble bee larvae will be given inocula of isolated microorganisms at different concentrations (including *Ascosphaera apis* or *A. maior*). The toxicity of *B. cereus* was proved in weakened insects (WEISER, 1966). The mummification of bumble bee larvae was not so high as in "chalkbrood" in *Apis mellifera*.

Similarly like GILLIAM *et al.* (1994) we found that the development of the fungus goes on first under the integument. Only after a longer period of mortality mycelia can be observed on the surface of the larvae.

We presumed that the potential causal agent of larval contamination was the honey bee (GILLIAM, 1979b), which brings microorganisms into a hive. However, they were not isolated in pollen collected by honey bees. It is also very interesting that in the study of GILLIAM (1979b) the species *Bacillus* isolated from pollen were completely identical with our isolation from dead larvae. *Bacillus pumilus*, which occurred repeatedly in the isolates from dead larvae was later also isolated from the honey which was in close contact with the bees. As for larvae without symptoms, it seems interesting that there are differences in the bacterial spectrum of larvae without symptoms and dead larvae although they are given the same provisions.

We suppose that the difficulties arising in the course of species identification of bacteria are caused by the disinfection which contributes to the genetic drift.

GILLIAM (1979a) confirmed that the spectrum of yeasts decreased in fermented pollen. Controlled fermentation of pollen in greenhouses might possibly replace pollen sterilization

performed by INGLIS *et al.* (1992).

SUMMARY

In the study Laboratory rearing of *Bombus terrestris* L. we described the external symptoms of the syndrome "larval blackening" and the isolated representative of sporulating bacteria of the genus *Bacillus* and *Paenibacillus* from the larvae. We isolated large numbers of representatives of the yeast *Endomycetes* which were not precisely identified. From some dead larvae which never highly nummified the fungi *Ascosphaera* sp. and *Ascosphaera apis* were isolated. The development of the fungus occurs predominantly under the integument of the larvae, only long after death of larvae, the mycelia appear also on the surface of the larvae. However, it was not proved that fungus contamination is the primary cause of mortality.

The pollen and sugar used were not the source of bacterial contamination. *B. pumilus* was isolated from honey and *Paenibacillus glucanolyticus* was isolated from the gut (in section of abdomen) of the adult queen captured under natural conditions. Blackened larvae contained *B. pumilus*, *B. fusiformis*, *B. cereus*, *Paenibacillus glucanolyticus*. In the separate rearing *B. megaterium* and *B. subtilis* were isolated.

On the basis of the present preliminary study and the differences in the microbial spectrum in blackened and larvae without symptoms we presume that *Paenibacillus glucanolyticus*, *Bacillus pumilus*, *B. fusiformis*, *B. cereus* impose stress on the organisms of bumble bee larvae. The contamination of the rearing can be specific depending on the level of contamination of the food which is usually provided by honey bees. The level of contamination of the queens captured under natural conditions determines the nature of the microbial spectrum of the rearing.

None of contagious bacterial diseases of the brood of *Apis mellifera* was proved.

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SOUHRN

Mikrobiologie larev čmeláka zemního (*Bombus terrestris* L.) z laboratorního chovu

V laboratorním chovu *Bombus terrestris* L. jsme popsali vnější příznaky syndromu "černání larev" a izolovali z těchto larev zejména zástupce sporulujících bakterií rodů *Bacillus* a *Paenibacillus*. Ve větší míře jsme izolovali i zástupce třídy kvasinek *Endomycetes*, které nebyly blíže určovány. Z některých uhynulých larev, které však nikdy nebyly silně mumifikovány, jsme izolovali houbu *Ascosphaera* sp. a *Ascosphaera apis*. Vývoj houby probíhá především pod integumentem larev, až delší dobu po úhynu se objevují plodnice i na povrchu larev. V těchto případech nebylo prokázáno, že je napadení touto houbou prvotní příčinou úhynu.

Používaný cukr a pyl nebyli zdrojem kontaminace. Izolací mikroorganismů ze včelího medu jsme získali *B. pumilus* a z mesenteronu dospělé matky odchycené původně v přírodě se podařilo izolovat *Paenibacillus glucanolyticus*. Ve zčernalých larvách se nacházeli *B. pumilus*, *B. fusiformis*, *B. cereus*, *Paenibacillus glucanolyticus* dále v odděleném chovu *B. megaterium* a *B. subtilis*.

Na základě této předběžné studie a odlišnosti mikrobiálního spektra larev bez symptomů černání a zčernalých larev předpokládáme, že *Paenibacillus glucanolyticus*, *Bacillus pumilus*, *B. fusiformis*, *B. cereus* larvám čmeláků zatěžují organismus. Kontaminace chovů může být specifická podle úrovně kontaminace krmiva, které je ponejvíce získáváno z chovu včely medonosné. Rovněž úroveň kontaminace matek odchycených v přírodě zřejmě určuje povahu mikrobiálního spektra v chovu.

Nebyla dokázána žádná z nebezpečných bakterií plodu *Apis mellifera*. Skutečné působení zmíněných sporulujících bakterií a houby *Ascosphaera* sp. na larvy je nezbytné prověřit záměrnou infekcí.

Bombus terrestris, čmeláci, laboratorní chov, mikrobiologie

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