EFFECTS OF THREE BACTERIA SPECIES ON Bombus terrestris MALE LARVAE UNDER LABORATORY CONDITIONS

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Abstract


It has been found previously that the several bacteria are repeatedly isolated from the dead bumble bee larvae with syndrome of blackening of larvae, which greatly reduces success in the laboratory bumble bee rearing. Therefore, the aim of this study was to find out what influence these bacteria on the bumble bee larvae and thus to verify their potential pathogenicity. The experimental infestations were carried out in so-called pseudoccolonies – i.e. pseudosocial units, which consist of three workers from mother colony and put in the plastic box with one – three male cocoons. As soon as one of the workers had laid the first eggs the artificial infestation was carried out in experimental groups (on eggs and in pollen cup). On the base of comparison success in development of experimental groups and control groups was found that Bacillus pumilus and Paenibacillus glucanolyticus show only a harmful effect on bumble bee larvae, therefore, they are agents with weak pathogenicity at most. Bacillus thuringiensis caused also less development but the differences in comparison with the control group were not statistically significant. The method and the results are discussed.

Bombus terrestris, bumble bee, laboratory rearing, pseudoc colony, bacteria, Bacillus thuringiensis

It has been mentioned previously (PŘIDAL et al., 1997) that early mortality, the so-called „blackening“ of bumble bee larvae, is a phenomenon greatly reducing success in the laboratory bumble bee rearing during the solitary stage of developing bumble bee colony. The dead larvae were microbiologically analysed and there were found especially gram-negative aerobic bacteria in these larvae (PŘIDAL et al., 1997; PŘIDAL, 2001). The following bacteria were found repeatedly as follows: Bacillus pumilus and Paenibacillus glucanolyticus. Therefore, these species were indicated as a potentially pathogenic (PŘIDAL, 2001). Bacillus thuringiensis was detected in the dead bumble bee larvae for the first time in the year 2000 (PŘIDAL, 2001) and, considering its pathogenicity for Lepidoptera, it was also indicated as a potentially pathogenic agent.

The aim of this study was to find out how mentioned bacteria affect the growth and total condition of bumble bee larvae and thus to verify their actual rate of the potential pathogenicity.

MATERIAL AND METHODS

Following three experiments were carried out during years 2000 and 2001:

a) Experiment No. 1; the experimental infestation by the bacteria Bacillus pumilus and Paenibacillus glucanolyticus [control group C, two experimen-
b) Experiment No. 2; the experimental infestation by the bacterium B. thuringiensis [control group C, experimental group BT]; 4.10. - 15.12.2000.
c) Experiment No. 3; experimental infestation by the bacterium B. thuringiensis [control group C, experimental group BT]; 11.7. - 29.8.2001.

Remark: Control group is a group which is not exposed to the experimental stimulus under study.

**Pseudocolonies.** The experimental infestations were carried out in pseudosocial units, so-called pseudocolonies. This method has been used with little differences by REGALI & RASMONT (1995). Three young workers taken from laboratory queen-right Romus terrestris colony establish the pseudocolony. These workers are put in the plastic box (8,5 × 12,5 × 12,5 cm) with one or up to three male cocoons from the mother colony. Thus the workers are out of the queen feromon; therefore, in haemolymph one of the workers, which will become dominant worker, titer of juvenile hormone will increase (LARRERE & COULAUD, 1993; BLOCH et al., 1996). This physiological change in one of the workers is followed by oviposition of unfertilised haploid eggs (ROBINSON & VARGO, 1997). The cocoon insertion advances oviposition. In every experiment, a control and one or two experimental groups always with 15 plastic boxes in every groups were established. The workers and their brood were fed by pollen from pollen traps put on the hives entrance of honey bees (Apis mellifera) and by honey (both provisions have origin in south Moravia, CZ). The pollen was fed in little cups with circle platform and capacity 1,5 cm²; the honey was fed in automatic drinking tubes with capacity 25 ml.

The plastic boxes were kept in water thermostat at temperatures 28 - 29 °C and humidity 70±5 %. The boxes were controlled every 2nd day. During these controls boxes were purified of feces and the fresh pollen were fed. At every control, the condition of pseudocolony was recorded as follows: presence and growth of the brood, behaviour of the workers and number of the dead larvae. At the end of every experiment, the final condition of pseudocolony was evaluated by following parameters: number of eggs, larvae, cocoons and total condition classified by subjective scale 1 – 4 (from “very good” to “bad” condition). The dead larvae were stored until their microbiological analysis at 4 °C.

**Experimental infestation.** The experimental infestation was performed with micropipette and inoculum as soon as the first eggs have been laid. It was made as follows: wax pocket with the eggs was carefully (i.e. without damage of eggs) invaded by the micropipette apex and inoculum was carefully injected. The pollen cup was also infested by 0,3 – 0,4 ml of inoculum.

**Preparation of the inoculum.** The cultures were incubated on the sporulating medium MPA plus mangan for 24 – 48 hours. Control of the sporulation was made in the microscopic preparation – minimum 50 % spores (i.e. maximum 10 % of vegetative cells). The culture was transferred in physiological solution. The concentration was measured by comparing of its cloud with McFarland’s scale (LAUDERDALE et al., 1999). The inoculum cloud was agreed with 4th degree, it is 4 × 10⁶ spores/ml. The inoculum was stored at 4 °C.

**Microbiology analysis of the dead larvae.** The microbiological tasks were provided at Czech Collection of Microorganisms 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 homogenised in 2 ml of saline solution and subsequently inoculated 250 µl 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 were verified on the medium identical with the isolation one. The species identification of pure cultures of suspected B. pumilus, P. glucanolyticus or B. thuringiensis was based on some key characteristics and confirmed by biochemical and physiological tests (PARRY et al., 1983, PRIEST et al., 1988) which are essential for three mentioned species.

Presumptive B. pumilus: ellipsoidal spores are produced in not swollen sporangia, growth at 50 °C, reduction of nitrates and hydrolyse of starch; presumptive B. glucanolyticus: ellipsoidal spores are produced in swollen sporangia, reduction of nitrates and nitrates, do not grow at 50 °C and do not hydrolyse urease and casein; presumptive B. thuringiensis: presence of parasporal corpuscle.

Other species were identified in the pure cultures on the base of microscopy (spore formation), agar morphology and biochemical and the physiological tests, which are essential for a group of gram-positive sporulating rods.

The dead larvae were sampled and analysed according to pseudocolonies only in case of the 1st experiment. From the 3rd experiment only mixed samples (sample C – the control group, sample BT – experimental group, i.e. without sorting according to individual pseudocolonies) were analysed. No samples were analysed from the 2nd experiment.

**Statistics.** Results were verified by one-way analysis of variance (ANOVA), methods on minimal differences and confidence interval. In some cases the same difference was tested by all methods concurrently. The analysed data were usually out of the normal distribution, therefore, ones have been logarithmised.
RESULTS
The workers began to lay eggs usually soon after the insertion into the plastic box. There was an exception in case of older workers (threadbare wings and hairs), which a short time after insertion into box have died. The ability of pseudocolonies to establish their own brood on the inserted male cocoons is summarised in Fig. 1 and 6. The brood was the most frequently established on 8th day from the start of experiment No. 1, 2 and 3. In average the brood was established on 11.2 days. After excluding of distant values the average amounts 9.9 days.

![Graph](image)

1: Temporal distribution of the 1st brood foundation by workers (1st experiment)

The perception of the experimental infestation was different among pseudocolonies. Usually the brood was cancelled and a new brood has not been established. If the new brood was established once again it has been artificially infested by inoculum repeatedly. The absolute collapse of pseudocolonies (acute death of all larvae) happens not always. The crumbly porous wax was concomitant circumstance. Pseudocolony reactions can be divided into three following groups:

1) without reaction – it occurs only exceptionally in several cases of experiment No. 2 and 3;
2) high larval mortality but without the brood re-establish and the absolute collapse, pseudocolony developed more or less poorly;
3) the absolute collapse – the total larval mortality, disintegration of the nest and the wax.

However, also in the control groups the phenomenon No. 2 occurred with approximately 20% frequency; i.e. great dead black larvae were discovered comparatively often. The phenomenon of crumbly and porous wax has never been observed in the any boxes of the control groups. Additionally, the infestation of experimental bacteria has never been found in the body of the dead larvae from the control groups.

Experiment No. 1. In Fig. 2 is figured the average time until to the first brood and cocoons foundation among individual groups. The confidence intervals (P<0.05) overlaps each other, therefore, differences are not statistically significant. However, time until the first cocoons was significantly longer in the experimental groups beside the control group (P<0.05, tested by confidence intervals). Tested by ANOVA, the differences were even high statistically significant (P<0.01). The difference between the experimental groups was not statistically significant. The cocoons did not develop in 4 pseudocolonies of the group G and in 3 cases of the group P because of their absolute collapse (always from 15 possible pseudocolonies). In the control group this phenomenon has never been observed.
2: Average time until to the 1st brood and cocoon (1st experiment)

The temporal distribution of the 1st cocoon appearance of individual groups is figured in Fig. 3. This chart demonstrates how many pseudocolonies (in percentages) looked after their own cocoons on certain experiment days. It is visible that the experimental groups were delayed in comparison with the control group. In addition, in the experimental groups, there were several pseudocolonies without presence of cocoons during the whole experiment (the columns of experimental groups are not reaching to the 100% at their end).

3: The temporal distribution of the 1st cocoons appearance (the 1st experiment)
The average mortality in individual experimental groups according to size of dead larvae is figured in Fig. 4. The larvae of approximately 1st–3rd instar were considered as little larvae. In spite of the most larvae have died in the experimental groups G and P beside the control group C the differences were not statistically significant. The mortality in the control group was entirely a normal phenomenon and the sample of larvae for microbiological analysis was obtained from the each pseudocolony of control group. On the contrary, some larvae in some experimental pseudocolonies survived till imaginal stage in spite of the experimental infestation. The absolute collapse was observed only in 5 pseudocolonies of the group G and 8 pseudocolonies of the group P, in each group from 15 pseudocolonies.

![Graph showing average number of dead larvae (1st experiment)](image)

4: Average number of the dead larvae (1st experiment)

The results of microbiological analysis of the dead larvae are summarised in Tab. 1. Each sample from experimental pseudocolonies of the group P contained always and only B. pumilus. There was one exception in case of the sample P6 (i.e. 6th pseudocolonies of group P) where B. subtilis was isolated beside B. pumilus. In the group G the situation was not so definite. Two G group samples (G4 a G14) were negative for *P. glucanolyticus*. Instead of the tested bacterium, only gram-negative rods without their specification were detected. In the G6 *Brevibacillus* sp. was detected; it was very similar to *Brevibacillus cf. parabrevis*. Beside of *P. glucanolyticus* as well the non-specificated microflora (gram negative or labile rods or coccii) gram-negative rods were isolated from samples G7 and G8. Analysis of samples from pseudocolonies of the group C was negative for *B. pumilus* and *P. glucanolyticus*. These samples were positive in gram-positive cocci, non-sporulating rods and coccosbacilli. There were also very abundant yeasts in each of C samples.

In order to evaluate success in development of pseudocolonies among individual groups there was compared the time from the 1st brood to the emerging of the 1st male among individual groups. This parameter was in average for group C 36.2 days, G group 40.8 days and P group 38.9 days. The averages were tested by ANOVA and differences C-G and C-P have been highly statistically significant at level P=0.1032. The most males emerged in the control group besides of P and G groups and differences were very highly significant (P<0.001). In C group 7,2 males emerged, G group 3.06 males and P group 2.0 males emerged in average per one pseudocolony.

The average final condition at the end of experiment is figured in Fig. 5 according to the groups. The best condition with the most number of larvae and cocoons were found in control group. However, the differences tested by confidence intervals (P<0.05) and ANOVA (P<0.012) are statistically significant only for the subjective classification and the larvae number. In case of the cocoon number the differences are not significant.
I: Results of microbiological analysis of the dead larvae (1<sup>st</sup> experiment)

<table>
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<tr>
<th>experimental group</th>
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<th>8</th>
<th>9-13</th>
<th>14</th>
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<td>P. glucanolyticus</td>
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<td>or B. pumilus</td>
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<tr>
<td>dominant G + cocci, non-sporulating rods and coccusbacilli, yeasts</td>
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</table>
5: Condition of pseudocolonies at the end of the 1st experiment

6: Temporal distribution of the 1st brood foundation by workers (3rd experiment)
Experiment No. 2. Results from this experiment have not been summarised because of inappropriate time of its establishment – October 2000. The used workers were consequently out of good condition (too old) and in several cases they have not been able to establish a viable pseudocolony. If they started to lay it has been usually longer than two weeks.

Experiment No. 3. The average time until the 1st brood and 1st cocoon is figured in Fig. 7. The confidence intervals (P<0.05) overlap each other, therefore, the difference among the groups C and BT are not statistically significant in contrast to the 1st experiment. Rejection of H0 hypothesis were tested also by ANOVA and the differences are significant nor at level P = 0.05.

The temporal distribution of the 1st cocoon appearance of individual groups is figured in Fig. 8. This parameter shows some delay in development of the experimental group BT but there were more colonies with occurrence of the cocoons in the BT group than in the control group.

In average the most larvae per pseudocolony dead in the BT group (3.00) in comparison with the C group (2.55). The difference between mean values of the both groups (sampling populations) is not statistically significant. The mortality in the control group was a normal phenomenon and from each pseudocolony, the mixed sample of larvae for microbiological analysis was obtained. On the contrary, some larvae in some experimental pseudocolonies survived till imaginal stage in spite of the experimental infestation. The absolute collapse was observed only in 2 pseudocolonies of group BT from 15 pseudocolonies.

The results of microbiological analysis of the mixed samples C and BT of the dead larvae are summarised in Tab. II. Bacillus thuringiensis was as a dominant and single microflora in sample BT. In the sample C the B. thuringiensis has not been found. Bacteria Bacillus fusiformis and B. lentus were isolated from bumble bee larvae for the first time, as a predominant microflora.

The period from the 1st brood to the emergence of the 1st male was longer in the experimental group BT (27,33 days) than in the group C (26,13 days). Tested by ANOVA, the difference is not significant (P>0.05). More males emerged in the group C (4,7) in comparison with the BT group (3,46) but the difference is also not significant!

In Fig. 9 the average final condition at the end of experiment is figured according to the groups. The result is not as definite as in the 1st experiment. In spite of the control group was better in the subjective classification and in the higher number of the cocoons, the experimental group BT had more larvae at the end of the experiment. Therefore, the results are not clear. In addition, none of the differences among groups are statistically significant (confidence interval, ANOVA, P>0.05).

![Graph showing average time from the start of experiment to foundation of the 1st brood and to the 1st cocoon for groups C and BT.](image-url)
8: The temporal distribution of the 1st cocoons appearance (3rd experiment)

II: Results of microbiological analysis of dead larvae (3rd experiment)

<table>
<thead>
<tr>
<th>experimental group</th>
<th>mixture samples from the all pseudocolonies of given group</th>
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<tbody>
<tr>
<td>BT</td>
<td>B. thuringiensis positive</td>
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<tr>
<td>other bacteria</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>B. thuringiensis negative</td>
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<td>C</td>
<td></td>
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<tr>
<td>other bacteria</td>
<td>B. fusiformis, B. lentus</td>
</tr>
</tbody>
</table>
DISCUSSION

Experiment No. 1. As can be seen in fig. 2, the pseudocolonies of all groups manifested the same fitness – the capability of founding of the first brood, because the differences of the average time from the start of the experiment to the foundation of the 1st brood are not significant among the individual groups. In view of the fact that the ability to found the first brood was approximately equal, the construction of the first cocoons lasted significantly longer just in the experimental group. Initially, the first brood had not developed or its larvae died. As far as the larvae that died shortly after emerging, it is highly probable that these tiny larvae of the 1st instar were overlooked regardless of all endeavour devoted to them. Hereupon, the final result was clear – the following oviposition and the prolongation of the period between the first brood foundation and the presence of the first cocoons. It is presumed that the tiny larvae died due to the experimental infestation before the next larvae started to emerge from the eggs. This presumption is supported by the fact that the characteristic collapse followed only in pseudocolonies of the experimental groups, but not in the control group.

In view of the fact that all larvae died in the experimental groups only sporadically, B. pumilus and B. glucanolyticus are not primary pathogenetic bacteria belonging only into the class of „weak” pathogens (BAILEY, 1981) and playing some role only in stressing situations. In our case this stressing factor was the high concentration of the served inoculum. The larvae usually do not meet with such amounts of pathogens and, moreover, the male larvae being haploid are less resistant to infection. It seems consequently that the „blackening” syndrome is not primarily affected by the bacteria, but rather by stressing factors. The actual experiences from the laboratory rearing of bumble bees indicate that the nutrition is probably the stress factor. Moreover, the blackening of larvae is typical of the solitary stage of colony development and during the social stage the blackening is not so frequent. There is an assumption that the nutrition of larvae by queen is not so valuable as by the workers.

Experiment No. 2. Results of this experiment indicate that the experiments with the pseudocolonies should be started so that their end should coincide with the end of the vegetation period. Such experiments are otherwise distorted by the indisposed workers and possibly also by the season of the year reflected by the workers even inside the lab. In view of the low credibility of such results a postponed infestation experiment was started in cca 1st to 3rd instar (three pseudocolonies). The brood development was not injured in such cases. Therefore, the next infestation was postponed to the next oviposition and the brood were infested praemergent. The brood of such infested eggs has partly died. These observations are tentative indicating only the next possibilities.

Experiment No. 3. Most parameters characterising a successful pseudocolony development were observed just in the control group. The results obtained were not significant because some colonies could not be tested (mould, flowed out drinking tubes etc.). The total number of tested pseudocolonies (number of ele-
ments in sampling populations) decreased under the optimum level of statistic tests. The influence of *B. thuringiensis* should be better tested in more pseudoccolonies to enable statistically significant evaluation of pseudoccolonies.

VANDENBERG (1990) tested safety of *Bacillus thuringiensis* var. *tenebrionis* and *B. sphaericus* on the caged workers of the adult honey bees. None pathological effects were confirmed in spite of concentrated inoculum. The reduction of the adult honey bee longevity was significant only in case of *B. thuringiensis*. The adults of the honey bees are equally large, therefore, their mixocoel tissues can be divided from tissues of alimentary canal and analysed apart in contrast to the present and the previous studies (PRIDAL et al., 1997; PRIDAL, 2001). Therefore, presence of the bacteria could be confirmed only from the whole body. The bacterium *Bacillus sphaericus* has been isolated also from the dead bumble bee larvae (PRIDAL, 2001).

MALONE et. al. (2001) also did not confirm any effects on the longevity or flight activity of the adults of the honey bee workers fed with a *Bacillus thuringiensis* toxin.

The analysis of dead larvae was carried out only in mixed samples without sorting according to pseudoccolonies because of financial reasons and previous experiences. The presence of *B. fusiformis* in dead larvae of the control group is characteristic of larvae from poorly developing colonies (PRIDAL et al., 1997) and of dead larvae generally (PRIDAL, 2001). Therefore, the higher larval mortality in the control group during the 3rd experiment can be explained by presence of this bacterium in the dead larval bodies.

These results came into existence under experimental condition of so-called pseudoccolonies. These conditions are not the same as in normal bumble bee colonies (i.e. queen right, with more number of workers etc.). Perhaps, any tests with normal colonies would result in more reliable answer. On the other hand, the present tests were carried out on haploid organisms (male larvae), which are commonly susceptible to infection. For example, this phenomenon is known in case of the chalkbrood in honey bees.

**SUMMARY**

It has been found in previous papers that certain species of bacteria are observed in the dead larvae repeatedly (syndrome of so-called „blackening“ of larvae). The aim of this study was to verify the potential pathogenicity of the most frequent isolates of *Paenibacillus glaucanolyticus*, *Bacillus pumilus* and *B. thuringiensis* by means of experimental infestation.

On the basis of both infested and non-infested pseudoccolonies the bacteria *Paenibacillus glaucanolyticus* and *Bacillus pumilus* can be classified as weak pathogens and their importance increase with the increasing of the stressing factors. The bumble bee larvae infested by the spores of these bacteria were dying in irregular numbers. Although a sudden blackening of all larvae (so-called absolute collapse) was observed in some colonies, there were also such pseudoccolonies with only average (normal) larvae mortality and having its larvae until the end of the experiment in spite of the repeated experimental infestation.

Even lesser injury was observed in case of the generally entomopathogenic agents *Bacillus thuringiensis*. The differences in the development success between the control group and the infested group did not exceed the level of significance. If *Bacillus thuringiensis* is pathogenic agents then one belongs to the class of the weak pathogens being not the primary pathogen with the blackening of larvae syndrome.

It seems with reference to the results in pseudoccolonies that the tested bacteria do not primarily cause the syndrome of larvae blackening and they are only weak pathogens. Probably, their pathogenity can manifest oneself only in connection with any stressing factor. The quality of nutrition appears to be this factor according to common experiences from laboratory rearings of bumble bees.

**SOUHRN**

Vlivy tří druhů bakterií na samčí larvy čmeláků (*Bombus terrestris*) v laboratorních podmínkách

Již z výsledků dřívějších prací bylo zjištěno, že v uhynulých larvách (tzw. syndrom černání larv) se opakovaně vyskytují určité druhy bakterií. Cílem této práce bylo ověřit potenciální patogenitu nejvíce se opakujících izolátů druhů *Paenibacillus glaucanolyticus*, *Bacillus pumilus* a *B. thuringiensis* pro čmeláči larvy pomocí experimentálních infekcí.

Na základě pozorování vývoje neoinfikovaných a infikovaných pseudokolonii bylo zjištěno, že *P. glaucanolyticus* a *B. pumilus* mohou být označení maximálně za slabé patogenní bakterie, jejichž škodlivost je přímo uměrná stresovým faktorům. Larvy infikované sporami těchto bakterií hynuly v nepravidelných množstvích. I když v některých pseudokolonii došlo během pokusu k náhlemu totálnímu zčernání všech larv (tzw. zhroucení), existovaly vedle těchto pseudokolonií i takové, které plodily až do konce pokusu s přiměrným úhynem larv.
Ještě menší škodlivost byla pozorována u obecně entomopatogenního agenta *B. thuringiensis*. Rozdíly v úspěšnosti rozvoje kontrolní a infikované skupiny jsou pod hranici statistické přikaznosti. Pokud je *B. thuringiensis* vůbec patogenní, patří maximálně do skupiny slabých patogenů a ani tato bakterie není primárním patogenem u těchto syndromu černání larev.

Zdá se tedy, že syndrom černání larev není primárně způsobován bakteriemi, ale některým ze stresových faktorů. Z dosavadních výsledků laboratorního chovu čmeláků se jeví být tímto faktorem výziva. Testování druhů bakterií lze tedy na základě výsledků považovat nanejvýš za patogeny slabé.

*Bombus terrestris*, čmelák, laboratorní chov, bakterie, *Bacillus thuringiensis*

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