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Original article

Can fatty acids and oxytetracycline protect artificially raised larvae from developing European foulbrood?*

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Abstract – A quantitative assay for the transmission of European foulbrood (EFB) in artificially raised larvae was developed. This assay was used to determine the concentration of oxytetracycline (OTC) required to prevent larvae from developing EFB and whether 8 fatty acids (undecanoic, lauric [dodecanoic], myristic, myristoleic, ricinoleic, ricinelaidic, homo-y-linolenic and 13,16,19-docosatrienoic acids) which had previously been demonstrated to inhibit the growth of *Melissococcus plutonius* cultures, could protect larvae from developing EFB. The larval assay involved grafting individual larva (less than 24 hours old) into a single well in a micro-titre plate. Each larva was fed 10 μL of basic larval diet (BLD) containing 500 000 *M. plutonius* organisms. After 3 days the larvae were also fed 60 000 *Paenibacillus alvei* spores (a common secondary invader associated with EFB) in 10 μL BLD. The combination of these two organisms was required to reliably produce symptoms typical of that seen in field cases of EFB. Most larvae infected using this protocol died from EFB. To determine the efficacy of OTC, EFB infected larvae were fed 0, 1, 2.5, 5 10 or 20 μg/mL of OTC. Treatment with 1 μg/mL lowered the mortality rate from 93.75% to 69.5%. Treatments with 2.5 μg/mL to 10 μg/mL reduced the mortality rate further whereas treatment with 20 μg/mL reduced the rate to the same as the negative control. Larvae fed 20 or 200 μg/mL of each of the eight fatty acids were not protected from developing EFB.

***Melissococcus plutonius* / European foulbrood treatment / oxytetracycline / *Paenibacillus alvei* / fatty acids**

1. INTRODUCTION

European foulbrood (EFB) is a relatively poorly understood bacterial disease when compared with American foulbrood (AFB), the other major bacterial disease of honey bees. Researchers have not commonly attempted to transmit EFB to honey bee colonies due to the unreliability in producing disease. This is probably because the transmission of EFB is hampered by uncontrollable environmental conditions and the ejection of infected larvae by adult bees from inoculated colonies (Bailey, 1960). Inoculated colonies often require artificial manipulation, such as the removal of foraging adult bees, to increase their susceptibility to EFB (Bailey, 1960).

Techniques for transmitting EFB to colonies have been developed to instigate and confirm the cause of the disease (Tarr, 1936; Bailey, 1963; Bailey and Lochner, 1968). However, only one reliable method for infecting colonies or laboratory raised larvae with *M. plutonius* has been published (McKee et al., 2004). This method was successful in transmitting disease by feeding *M. plutonius* from EFB diseased material using EFB diseased larval extracts. However, the dose required to produce disease could only be estimated as the amount of infective material consumed by the larvae could not be accurately determined. EFB could not be

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transmitted by feeding *M. plutonius* cultures grown on an artificial medium, using the McKee et al. (2004) assay.

Unlike AFB, in which only the causative agent is involved in the disease process, EFB is associated with a number of secondary invaders (Bailey, 1960). One of the most common secondary invaders is *Paenibacillus alvei*. The role of *P. alvei* in the disease process is unknown.

Oxytetracycline (OTC) is the antibiotic of choice for the treatment of EFB. However, there are still unanswered questions about EFB and its treatment with OTC that need further study. Although OTC has been used to treat EFB for decades, the concentration of OTC in honey bee larvae required to protect them from developing EFB is still unknown. This information would provide an indication as to how much OTC is required to protect honey bee larvae from EFB and how much OTC is required for colony treatment.

Alternative methods for the control of EFB are needed if problems with residues are to be eliminated. In 1992 Shimanuki et al. (1992) demonstrated that chalkbrood mummies contained an antimicrobial substance which in laboratory tests inhibited the growth of *P. larvae* and *M. plutonius.* Feldlaufer et al. (1993) subsequently identified this substance to be the fatty acid linoleic acid. They also demonstrated that linoleic acid was active against *P. larvae* but did not test its effect on *M. plutonius.* Hornitzky (2003) tested 28 fatty acids for their activity against *P. larvae* and *M. plutonius* cultures. Fifteen of the fatty acids showed antibacterial activity against *P. larvae* and eight fatty acids showed activity against *M. plutonius.* As control agents fatty acids would be safe and environmentally-sound (Feldlaufer et al., 1993). Not only are these agents non-toxic to man but are actual foods and in the case of unsaturated fatty acids are essential for growth, development and health (Kabara, 1978).

The aims of this study were to develop a quantitative assay for the transmission of EFB in artificially raised larvae and to determine whether *P. alvei* has a role in the EFB disease process. This study was also carried out to determine the concentration of OTC required to prevent the development of EFB in honey bee larvae and to determine whether any of the eight fatty acids previously shown to inhibit the growth of *M. plutonius* could prevent honey bee larvae from developing EFB (Hornitzky, 2003).

2. MATERIALS AND METHODS

2.1. Collection of larvae

Worker honey bee larvae (*Apis mellifera*) were obtained from 3 colonies of Italian strain bees maintained at the Elizabeth Macarthur Agricultural Institute, Menangle, NSW. Individual larvae < 24 hour old were grafted into single wells of sterile 96-well cell culture plates (Greiner cellstar U-shape) using a size 00 paintbrush.

2.2. Preparation of *M. plutonius* and *P. alvei* inocula

2.2.1. M. plutonius

One of two duplicate smears prepared from individual larvae exhibiting clinical signs of EFB was examined microscopically after carbol-fuchsin staining for bacterial populations (Hornitzky and Wilson, 1989). From larval smears that were only infected with *M. plutonius*, the second smear was reconstituted with water and streaked on EBN culture plates (Hornitzky and Smith, 1998). The plates were incubated at 37 °C under anaerobic conditions for 5 days. *M. plutonius* was subcultured from single colonies on EBN plates and incubated as described above.

The inoculum was prepared by suspending the bacterial growth in 250 μL sterile distilled water per plate. The suspension was washed by centrifugation for 20 s at 18 000 × *g* and resuspending the pellet in 1/5th of the washing volume. The concentration of bacteria was determined microscopically with a Neubauer haemocytometer. As most *M. plutonius* harvested from culture plates appear as short chains, each chain was counted as a single infective unit. The *M. plutonius* cell suspension was frozen in 50 μL aliquots and stored at −80 °C.

Table I. Feeding and monitoring schedule for all larval raising experiments.

Hpg	0	67	73	90	97	114	121	138	145	162	169
	*	*	*	*	*	*	*	*	*	*	*
	+	+	+	+	+	+	+	+	+	+	
	⇑	↑						^1			^2

hpg = hours post grafting
* = monitor larvae and assess viability
+ = feed larvae
^1 = prepare smears from dead larvae
^2 = prepare smears from all remaining larvae
⇑ = inoculation (feeding) with *M. plutonius*
↑ = inoculation (feeding) with *P. alvei*

2.2.2. P. alvei

Smears from larvae with clinical signs of EFB were examined microscopically to confirm the presence of *P. alvei*. Smears containing *P. alvei* spores were resuspended in a drop of sterile distilled water (SDW) and streaked onto 7% sheep blood plates. After incubation for 3 days at 37 °C under aerobic conditions, single colonies were subcultured. Subcultures were expanded on blood plates and incubated for 6 days so that *P. alvei* spores were prevalent. The spores were scraped off the plates with a cell scraper, suspended in SDW (3 mL/plate) and washed 4 times in SDW by repeated spinning (5 min at 6000 × *g*) and resuspending of the pellet. The spores were finally resuspended in 0.5 mL SDW/plate and counted in a Neubauer haemocytometer. *P. alvei* spores were frozen in 50 μL aliquots and stored at −80 °C.

2.3. Larval assay

Larvae were fed a modified basic larval diet (BLD) (Peng et al., 1992). BLD was prepared by mixing 4.2 g freeze dried royal jelly into a solution of 0.6 g D-glucose (Ajax, 1364), 0.6 g D-(-)-fructose (Sigma F-3510) and 0.2 g yeast extract (Bacto) dissolved in 14.4 mL sterile distilled water. The sugar/yeast solution was sterile filtered and stored at 4 °C. Fresh royal jelly was obtained from Natural Life (Australia), freeze dried in 20 g aliquots and stored at −20 °C until required for the preparation of BLD.

The required amount of complete BLD was prepared for each experiment and stored at 4 °C for no longer than 5 days. Before feeding bee larvae the BLD was pre-heated to 35 °C. To ensure homogeneous distribution of inocula containing *M. plutonius* and *P. alvei* the bacteria or spores were suspended in the liquid component of BLD before adding royal jelly.

Each well into which a larva was grafted contained 10 μL pre-warmed BLD or BLD containing inoculum and treatment as shown in Table I. Larvae were reared in an incubator (Labec, Australia) at 35 °C with a tray of SDW to maintain high humidity. From 67 hours post grafting the larvae were examined for mortality and fed twice a day. Six to 10 μL of the diet were deposited near the head of the larvae using a direct displacement multistepper pipette. Larvae that died during an experiment were smeared on slides and the infection rate (*M. plutonius* and *P. alvei*) was determined microscopically after carbol fuchsin staining. The rearing was terminated 169 hours post grafting and the number of live larvae and the infection status of all remaining larvae was determined.

2.4. Bacterial inocula and OTC treatment

To determine the optimum *M. plutonius* inoculum for the assay larvae were fed with increasing concentration of *M. plutonius* from 10^5 to 10^9 infective units/mL BLD (= 10^3–10^7 infective units/larva) at grafting. To determine the role of *P. alvei* larvae were also fed with *M. plutonius* from 10^5 to 10^9 infective units/mL BLD (= 10^3–10^7/larvae) at grafting and subsequently fed with *P. alvei* with a dose of 6×10^6 spores/mL (= 6×10^4/larvae) after 67 hours.

Test larvae were fed 500 000 *M. plutonius* organisms in 10 μL in the BLD which was placed in

Table II. OTC treatment regime.

Group	Treatment + BLD		
	M. plutonius	*P. alvei*	OTC
1. Uninfected control	0	0	0
2. Infected control	500 000	60 000	0
3. Infected + OTC	500 000	60 000	1 μg/mL
4. Infected + OTC	500 000	60 000	2.5 μg/mL
5. Infected + OTC	500 000	60 000	5 μg/mL
6. Infected + OTC	500 000	60 000	10 μg/mL
7. Infected + OTC	500 000	60 000	20 μg/mL

the base of the microtitre plate well prior to grafting. These larvae were also fed a single dose of 60 000 *P. alvei* organisms after 67 hours.

For each experiment larvae were grafted into two 96-well plates in 5 or 6 groups of 16 larvae/group/plate. Larvae fed OTC at the specified concentration at each feeding point (Tab. II).

2.5. Fatty acid testing

Eight fatty acids (undecanoic, lauric [dodecanoic], myristic, myristoleic, ricinoleic, ricinelaidic, homo-y-linolenic and 13,16,19-docosatrienoic acids) that have shown inhibitory effects against *M. plutonius* were used in this study (Hornitzky, 2003). Stock solutions and dilutions of 100 times the final concentrations were prepared in EtOH and stored at −20 °C. One percent of each fatty acid stock solutions was added to BLD to produce the required final concentration of 20 or 200 μg/mL.

To determine any toxicity of fatty acids or 1% ethanol healthy honey bee larvae were fed lauric acid at concentrations of 20, 200 and 2000 μg/mL with a final concentration of 1% ethanol. Four treatment groups of larvae were used for each assay. For each experiment larvae were grafted into two 96-well plates in 4 groups of 24 larvae/group/plate (Tab. III). A duplicate experiment was carried out on a separate occasion for each fatty acid.

2.6. Statistical analysis

The objective of this analysis was to compare treatments with untreated EFB infected honey bee larvae (positive control). Data from OTC treatment groups and from fatty acid groups were analysed separately. The larval mortality was observed regularly between 67 to 169 hr after grafting. Data (time when the larvae were found dead) were

Table III. Fatty acid treatment regime.

Group	Treatment + BLD		
	M. plutonius	*P. alvei*	Fatty acid
1. Uninfected control	0	0	0
2. Infected control	500 000	60 000	0
3. Infected + fatty acid	500 000	60 000	20 μg/mL
4. Infected + fatty acid	500 000	60 000	200 μg/mL

analysed using a parametric survival regression analysis (Kalbfleisch and Prentice, 1980). Time was subtracted by 60 hr to give a better fit to Weibull distribution on the accelerated failure time. The survivor function of larvae from each treatment group was considered as follows:

$$S(t) = \exp(-\lambda t^{\alpha})$$

with link function being

$$\log_e(\lambda) = \text{Treatment effect}$$

where λ is a constant used in Weibull hazard function and α is Weibull distribution shape parameter. All analyses were performed on GenStat version 11 (Payne et al., 2008).

3. RESULTS

3.1. Infection rate and mortality of larvae infected with *M. plutonius*

As the number of *M. plutonius* organisms fed to larvae increased the percentage of larvae dying from EFB also increased. There was also a trend which linked an increased dose of *M. plutonius* with an increased infection rate. However, it was not as obvious as the mortality trend. The group fed 10^6 organisms/larvae had less infected individuals than the group infected with 10^5 organisms (Fig. 1). To obtain a 100% infection rate 10^7 *M. plutonius* organisms were required. None of the negative control larvae developed EFB.

3.2. Infection rate and mortality of larvae infected with both *M. plutonius* and *P. alvei*

There was an increase in the mortality and infection rate of larvae which were fed both *M. plutonius* and *P. alvei* compared to those

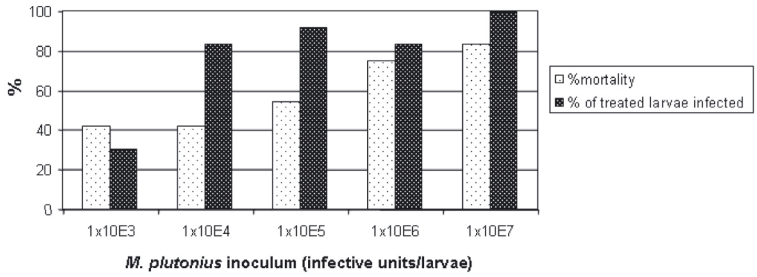

Figure 1. Mortality of honey bee larvae at different infection levels of *M. plutonius*.

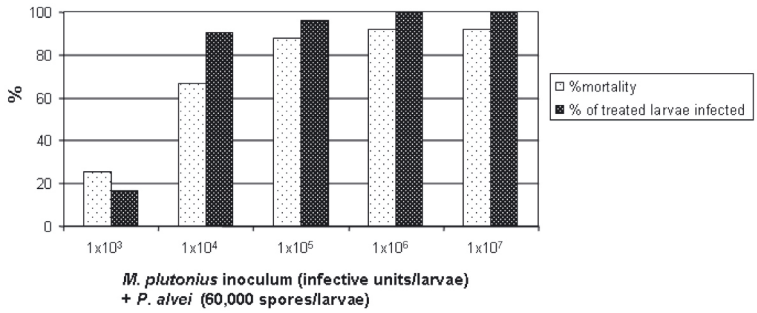

Figure 2. Mortality of honey bee larvae at different infection levels of *M. plutonius* and secondary infection with *P. alvei*.

Table IV. Survival regression coefficients, standard error and hourly mortality rate (λ) from t = 60 hr for treatment groups.

Doses/groups	Parameter	SE	Coefficient	λ	Total dead %
Negative control	–8.225	0.171	–8.225	0.00027e[†]	31.25
Positive control	2.093	0.195	–6.132	0.00217a	93.75
OTC 1 μg/mL	1.320	0.203	–6.905	0.00100b	69.53
OTC 2.5 μg/mL	0.679	0.250	–7.546	0.00053cd	46.87
OTC 5 μg/mL	0.394	0.224	–7.831	0.00040de	40.63
OTC 10 μg/mL	0.906	0.241	–7.319	0.00066c	54.69
OTC 20 μg/mL	–0.104	0.309	–8.329	0.00024e	28.12

[†] λ values followed by the same letter are not significantly different at P = 0.05.

only fed with *M. plutonius* except for the lowest dose of 1×10^3 (Fig. 2). None of the negative control larvae developed EFB.

3.3. Larval mortalities following OTC treatment of EFB infected larvae

Feeding larvae with OTC in a concentration range from 1 to 20 μg/mL protected larvae from developing EFB. The coefficient of the survivor regression was highest for the positive control group and this corresponds to the highest mortality rate of the infected larvae. An OTC treatment at 1 μg/mL lowered the mortality rate to 69.2% compared to the untreated positive mortality of 93.8%. OTC treatments at concentrations of 2.5 to 10 μg/mL reduced the mortality rate further whereas OTC feeding at 20 μg/mL reduced the rate to the same as the negative control (Tab. IV). The predicted survivor function is presented in Figure 3.

Table V. Survival regression coefficients, standard error and scale parameter (λ) from t = 60 hr for fatty acid treatments; Weibull scale α = 1.84; λ = exp(Coefficient).

Fatty Acid/doses	Parameter	SE	Coefficient	λ	Student t value	Significance	Total dead %
Positive	–7.323	0.036	–7.323	0.00066	*	*	93.16
LA 20 μg/mL	0.403	0.150	–6.920	0.00099	2.68	ns	97.92
LA 200 μg/mL	0.605	0.150	–6.718	0.00121	4.04	ns	97.92
UDA 20 μg/mL	0.030	0.115	–7.293	0.00068	0.26	ns	95.45
UDA 200 μg/mL	0.197	0.110	–7.126	0.00080	1.8	ns	96.87
MA 20 μg/mL	–0.129	0.111	–7.452	0.00058	–1.17	ns	94.79
MA 200 μg/mL	–0.002	0.110	–7.325	0.00066	–0.02	ns	95.83
MLA 20 μg/mL	0.110	0.111	–7.213	0.00074	0.99	ns	93.75
MLA 200 μg/mL	–0.018	0.111	–7.341	0.00065	–0.16	ns	93.75
RLA 20 μg/mL	0.006	0.112	–7.317	0.00066	0.05	ns	92.71
RLA 200 μg/mL	–0.116	0.114	–7.439	0.00059	–1.02	ns	88.54
RLD 20 μg/mL	–0.151	0.112	–7.474	0.00057	–1.34	ns	91.67
RLD 200 μg/mL	–0.152	0.112	–7.475	0.00057	–1.36	ns	93.68
HLA 20 μg/mL	–0.026	0.110	–7.349	0.00064	–0.23	ns	95.83
HLA 200 μg/mL	0.082	0.110	–7.241	0.00072	0.74	ns	95.83
DTA 20 μg/mL	–0.165	0.111	–7.488	0.00056	–1.49	ns	93.75
DTA 200 μg/mL	–0.091	0.112	–7.414	0.00060	–0.81	ns	92.71
Negative	–1.647	0.065	–8.970	0.00013	–25.21	< 0.001	39.24

† ns = not significance at 5% level obtained by one-sided student t test against positive control.

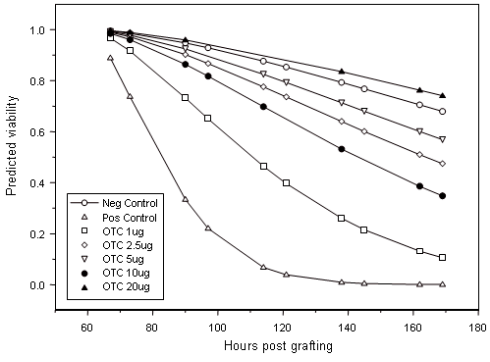

Figure 3. Predicted survivor function of EFB infected larvae treated with different OTC concentrations.

3.4. Fatty acid testing

None of the eight fatty acids protected larvae from developing EFB. There was no significant difference between the mortality of the infected control larvae and infected larvae which were also treated with each of the fatty acids. The percentage mortality using the fatty acids ranged from 88.54% to 97.92% while the percentage positive control mortality was 93.16%. The negative control mortality was 39.24% (Tab. V).

Lauric acid was not toxic to honey bee larvae when fed to healthy larvae at concentrations of 20 and 200 μg/mL. A concentration of 2000 μg/mL LA caused a 20% reduction in viability. The ethanol used to dissolve the fatty acids did not affect the viability of larvae at a concentration of 1%.

3.5. Control larvae

Although none of the control larvae in this study developed EFB there was considerable variation in the survival of control larvae. In the spring months an average of 48.5% of control larvae per month survived. In summer the average survival per month increased to 73.6%.

4. DISCUSSION

The objective in developing the EFB larval assay was to establish a feeding regime which provides a high rate of infected larvae and also

induced clinical symptoms typical of EFB. In initial experiments to transmit EFB to larvae it was necessary to feed a high dose of *M. plutonius* over the whole period of the experiment to achieve high infection rates and high mortality in larvae. Even then, most larvae died only towards the end of the larval rearing period. Often the dead larvae did not exhibit the typical clinical symptoms for EFB. The colour changed to a greyish brown rather than a yellowish colour and the gut content of infected larvae was watery rather than pasty.

There was a clear relationship between the percentage of larvae dying from EFB and the infective dose of *M.*plutonius (Fig. 1). There was also a trend which linked an increased dose of *M. plutonius* with an increased infection rate which was not as obvious as the mortality trend. The group fed 10^6 organisms/larvae had less infected individuals than the group infected with 10^5 organisms (Fig. 1). However, this anomaly was not seen when both *M. plutonius* and *P. alvei* were used to infect larvae indicating the importance of both organisms in the development of EFB.

The combination of both *M. plutonius* and *P. alvei* also produced symptoms typical of that seen in field cases of EFB. It was considered important to select an inoculum feeding regime which would cause EFB in most treated larvae if the larval assay was to be useful in testing OTC and fatty acids. Using a feeding regime which used very high concentrations of *M. plutonius* was considered unsuitable; as such a regime would not reflect infection under natural condition and hence not give a realistic appraisal as to the effects of OTC and the fatty acids on EFB treatment.

A feeding regime of 500 000 *M. plutonius* organisms in 10 μL of BLD that was placed in the microtitre plate well prior to grafting of the larva, and then feeding 60 000 *P. alvei* spores in 10 μL of BLD after 67 hours was chosen. This feeding regime was the mid-point between 1×10^7 and 1×10^8 *M. plutonius* organisms/mL (Fig. 2) i.e. 5×10^7 mL which equals 500 000 organisms in 10 μL.

An added advantage of rearing larvae in individual wells was that they were less likely to be injured compared to previously reported methods where multiple larvae were grafted in larger cell culture dishes and later transferred into single wells (McKee et al., 2004). The rearing of individual larvae in 96-well plates also reduced the BLD required in the assay compared with the larger cell volume dishes used by McKee et al. (2004) and allowed for an accurate determination of infectious doses as larvae consumed all of the BLD containing *M. plutonius* or *P. alvei*.

Another important factor in raising larvae was the quality of the royal jelly used for preparing the BLD. In developing the larval assay there was considerable variation in the viability of the larvae associated with different batches of royal jelly used to prepare BLD. We selected batches of royal jelly that supported high larval viability.

There was considerable variation in the survival of the non-infected control larvae in this study. This varied from about 88% to 34%. The survival of negative control larvae was highest in summer (December–February) where the average control larvae survival per month was 73.6%. The survival of negative control larvae in spring (September–November) was, on average, 48.5% per month. This may have been a function of the reduced availability of nectar and pollen in spring compared with summer. However, despite these differences the data clearly demonstrated that fatty acids could not protect larvae from EFB despite the conditions under which larvae were taken from hives for the EFB larval assay.

In this study the mortality and infection rate of larvae was similar in larvae fed with *M. plutonius* prepared directly from infected bee larvae smears, from primary cultures and from subcultures derived from the original smear.

OTC has been the antibiotic of choice for the treatment of EFB for more than 50 years. There have only been two studies carried out to determine the sensitivity of *M. plutonius*. Hornitzky and Smith (1999) reported that there were no OTC resistant strains of *M. plutonius* in Australia and that all 104 isolates tested were sensitive to between 1 and 2 μg/mL of OTC. Waite et al. (2003) tested 80 isolates from the UK and demonstrated that all isolates were sensitive to an average concentration of 3.9 μg/mL. Studies have also been carried out to determine what

concentration of OTC is reached in larvae following treatment with OTC. Hornitzky et al. (1988) demonstrated that significant levels of OTC reached the larvae when fed a range of treatments. The period of detectable OTC (≥ 5 μg/g) ranged from 1 to 9 days. The longest periods were achieved with a treatment of 1 g of OTC in 100 g of castor sugar which usually lasted five days or more. This is a recommended treatment for EFB in Australia. The peak OTC larval concentration in colonies following treatment varied from 10 to 184 μg/g of larval material. However, the larval OTC concentrations diminished until no OTC activity could be detected.

In this study as little as 1 μg/mL reduced the mortality of larvae from 93.75 (positive control) to 69.53%. However, protection of such a small percentage (24.22%) of larvae is unlikely to eliminate clinical signs of disease in a colony. A concentration of 20 μg/mL provided a level of protection equivalent to the negative control (Tab. IV). However, a concentration this high is unlikely to be necessary to effectively treat EFB in a honey bee colony. Lower concentrations coupled with the natural hygienic behaviour of bees would be likely to eliminate clinical evidence of disease in colonies with EFB. Hence, concentration of 2.5–10 μg/mL may be sufficient to provide an effective treatment for EFB in honey bee colonies.

None of the fatty acids provided any protection to larvae. This was clearly indicated by the fact that the number of dead larvae following treatment with the fatty acids was very similar to the number of deaths in the untreated positive controls. The fact that even high doses of 200 μg/mL of fatty acids were unable to protect any larvae whereas very small concentrations of OTC (1 and 2.5 μg/mL) protected larvae reinforces the unsuitability of fatty acids as a treatment option.

This study clearly shows the importance of testing treatments by using both laboratory cultures of *M. plutonius* (in vitro methodology) and the EFB larval assay (in vivo methodology). The most likely reason for the discrepancy between the activity of fatty acids against *M. plutonius* cultures and the complete lack of protection of larvae infected with *M. plutonius* and *P. alvei* is that the fatty acids were in some way inactivated by the BLD or the gut environment of the larvae.

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Les larves d'abeilles élevées artificiellement peuvent-elles être protégées de la loque européenne par les acides gras et l'oxytetracycline ?

***Apis mellifera* / *Melissococcus plutonius* / traitement contre la loque européenne / oxytétracycline / *Paenibacillus alvei* / acides gras**

Zusammenfassung – Können Fettsäuren und Oxytetrazykline künstlich aufgezogene Larven vor einem Befall mit Europäischer Faulbrut schützen? Die Europäische Faulbrut (EFB) ist im Vergleich zur Amerikanischen Faulbrut (AFB) eine relativ schlecht untersuchte Bakterienerkrankung. Anders als bei AFB, bei der lediglich ein Erreger in den Krankheitsprozess involviert ist, sind bei EFB eine ganze Reihe von sekundären Eindringlingen bekannt, von denen *Paenibacillus alvei* einer der häufigsten ist (Bailey, 1960). Da die Übertragung von EFB durch unkontrollierbare äußere Bedingungen sowie durch das Ausräumen von infizierten Larven durch Adultbienen behindert wird (Bailey, 1960), ist es schwierig, eine EFB-Erkrankung in Honigbienenvölker verlässlich durch künstliche Infektion zu erzeugen. Um die Bedeutung von *Melissococcus plutonius* und *Paenibacillus alvei* für die Entwicklung von EFB zu untersuchen und um die unterschiedlichen Behandlungsmethoden ohne Störung durch Adultbienen beurteilen zu können, entwickelten wir einen quantitativen Biotest für die Übertragung von EFB auf künstlich aufgezogene Larven.
Dieser Larventest beinhaltete die Übertragung individueller Larven (jünger als 24 h) in eine Vertiefung einer Mikrotiter-Platte. Jede Larve wurde mit einer Basisdiät gefüttert, die 500 000 *M. plutonius*-Organismen enthielt. Nach 3 Tagen wurden die Larven zusätzlich mit 60 000 Sporen von *P. alvei* gefüttert, um den Verlauf von EFB besser zu simulieren. *P. alvei* ist ein verbreiteter sekundärer Eindringling im Zusammenhang mit EFB und eine Beimpfung mit *M. plutonius* allein ruft nicht die typischen klinischen Symptome einer EFB hervor.
Oxytetrazyklin (OTC) ist derzeit das Antibiotikum der Wahl bei der Bekämpfung von EFB. Obwohl OTC seit Jahrzehnten zur Bekämpfung von EFB benutzt wird, ist nach wie vor unbekannt, welche

Konzentration von OTC in einer Bienenlarve benötigt wird, um sie vor dem Ausbruch von EFB zu schützen. Um die Wirksamkeit von OTC zu bestimmen, wurden EFB-infizierte Larven mit 0, 1, 2,5, 5, 10 oder 20 μg/mL OTC gefüttert. Eine Behandlung mit lediglich 1 μg/mL reduzierte die Mortalität der Larven von 93,75 (Positivkontrolle) auf 69,53 %. Eine Konzentration von 20 μg/mL bot bereits einen Schutz, der vergleichbar mit der negativen Kontrolle war. Allerdings ist es unwahrscheinlich, dass eine solch hohe Konzentration benötigt wird, um EFB effektiv im Bienenvolk zu bekämpfen. Geringere Konzentrationen in Verbindung mit dem natürlichen Hygieneverhalten der Bienen würden vermutlich ausreichen, um klinische Symptome in Völkern mit EFB auszuschließen. Daher sollten Konzentrationen zwischen 2,5–10 μg/mL, für eine effektive Behandlung von EFB im Bienenvolk ausreichen.

Falls Rückstandsprobleme vermieden werden müssen, sind alternative Methoden zur EFB-Kontrolle notwendig. Acht Fettsäuren (Undecan-, Laurin-, Myristin-, Myristolein-, Rizinol-, Ricinalaidin-, Linolen- und 13,16,19-Docosatrienoic-Säure), für die bereits eine Hemmung des Wachstums von *M. plutonius*- und *P. larvae*-Kulturen nachgewiesen wurden (Hornitzky, 2003), wurden im Larventest beurteilt. Keine der Fettsäuren bot einen Schutz der Larven gegenüber EFB. So war die Anzahl an toten Larven nach der Behandlung mit den Fettsäuren ähnlich hoch wie bei der unbehandelten Kontrolle. Die Tatsache, dass selbst hohe Dosen von 200 μg/mL an Fettsäuren keinen Schutz der Larven boten, während sehr kleine Konzentrationen von OTC (1 and 2,5 μg/mL) bereits die Larven schützte, untermauert die Unzweckmäßigkeit von Fettsäuren für die Bekämpfung.

Diese Untersuchung zeigt klar, dass für den Test von Behandlungen sowohl *in-vitro*-Methoden (Laborkultur von *M. plutonius*) als auch *in-vivo*-Methoden (EFB-Larventest) verwendet werden sollten.

***Melissococcus plutonius* / Europäische Faulbrut / Bekämpfung / Oxytetrazyklin / *Paenibacillus alvei* / Fettsäuren**

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