Effects of the acute paralysis virus on honey bees (*Apis mellifera* l.) infested by *Nosema apis* z.

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ABSTRACT

For bees suffering from an acute *Nosema apis* infestation followed by an oral infection of Acute Paralysis Virus (APV), the worker bee mortality was caused mostly by Nosematosis. The additional APV infection did not show any effect and cannot be regarded as mortality factor with adult bees. The maintenance of the midgut barrier preventing access by agents is hypothesized. If the virus was injected into the abdomen of the honey bee, mimicing the supposed transfer of APV from the mite *Varroa destructor* Oud., an increase of the mortality rate was detected during the first days, due to the APV injection. The infestation by *Nosema apis* did not show any effect in this case.

The results did not show either a strengthening nor an antagonistic effect between both pathogen germs *Nosema apis* and APV.

***Keywords**: Acute Paralysis Virus,* Nosema apis, Varroa destructor, *antagonistic effect, midgut epithelium, peritrophic membrane*

INTRODUCTION

It is well known that, among others, viruses are responsible for the mortality in honey bees. Among the viruses the Acute Paralysis Virus (APV) shows a high pathogenicity towards adult bees and brood and can be regarded as ubiquitous (Allen & Ball, 1996). However, the viruses cannot penetrate through the intestinal epithelium to gain entry to the haemocoelium for replication. Thus the intestinal epithelium functions as natural barrier against APV, preventing an infection via the intestinal tract.

Nosema apis Z. is a protist belonging to the species of micro-sporida (Lucius & Frank, 1997). The infectious spores infest and destroy the midgut epithelium cells, especially in *Apis mellifera* worker bees.

In the cooler and especially in the moderate climatic zones Nosematosis is generally regarded as the most frequent cause for bee respectively colony losses. It affects nearly every colony (Ritter, 1996). Even in subtropical and tropical climatic zones *Nosema* can be detected (Matheson, 1993, 1995, Nixon, 1982), although in those areas it causes little mortality.

In this context, there is a potential possibility that both pathogens come together provoking a synergistic danger for the bee colony in case of a simultaneous infection.

Given the destruction of the midgut epithelium, APV could get access to the haemolymph. To investigate this situation, APVwas applied orally and subcutaneously to Nosema infected bees.

MATERIAL AND METHODS

BEE MATERIAL

During February to September 1998, three experiments were conducted. The bees for the experiments were *Apis mellifera* carnica (Pollmann, 1979). The were sampled at random from bees that were not infected by *Nosema*.

Test 1 was conducted during winter when there was no brood. For this purpose, bees were removed from the winter cluster. Feeding pollen was neglected because overwintering bees generally have more developed fat bodies thus providing larger protein reserves.

In test 2 and 3, sealed brood combs were removed from the colonies approximately 3 days before emergence. Until emergence, the brood combs were stored in the incubator (34°C; 50-60 % air humidity). The newly emerged bees were caged in groups of 120 at 28°C and 50-60 % air humidity and fed pollen and sugar water.

EXTRACTION OF NOSEMA SPORES

About 500 deep-frozen bees that had suffered from nosematosis were used as a source of spores. Their abdomen were removed and smashed in a mortar and thinned with aqua bidest. Afterwards, the mass was filtered (10 µl) and centrifuged at 3000 rotations per minute for 10 minutes. The supernatant was collected by a pipette and the number of spores was counted with a Neubauer haematozyt counter.

A treatment by cold did not significantly reduce the viability of the spores (Bailey, 1972; Moffet & Wilson, 1971). According to Fries (1993), the absorption of a single Nosema spore may initiate an infection. The average infection dose, however, is from 20 to 90 spores per bee (Bailey, 1972; Fries, 1988). Fries (1988) showed that different quantities of Nosema spores always lead to an infection, but at different times. Larger initial spore quantities resulted in a much earlier infection. Gross & Ruttner (1970) produced a moderate infection with a dosage of 2×10^6 spores per millimeter sugar solution fed to 50 test bees for 2.5 days.

ARTIFICIAL NOSEMA INFECTION

The Nosema spores were mixed into a sugar solution (Apiinvert®) within a period of 3 days. Because Nosema infection can cause a high mortality, two different preparations for infection were chosen:

Nosema initial infection (low): 5×10^4 spores/bee

Nosema initial infection (high): 5×10^5 spores/bee

The control group was only fed with Apiinvert®.

APV INFECTION OF NOSEMA INFECTED BEES

Eight days following the exposure by Nosema spores the initial infection was examined in five bees. For this purpose the midgut and the rectum was prepared and the number of spores was ascertained with a Neubauer haematozyt counter.

The control group and Nosema infected groups were divided and furthermore treated as follows:

1. Untreated group (untreated.): no further treatment measures.
2. Control – PPB (cont-PPB): injection of 2 µl of 0,001 molar potassium phosphate buffer (PPB) (solvent used for viruses) applied abdominally, laterally, inter-segmentally between the 5^{th} and the 6^{th} segment.
3. APV group (injection) (APV_{inj}): Injection of 2 µl APV dissolved in potassium phosphate buffer at a dosage of 10^{-5} ng/ml, at the same injection site as the PPB group.
4. APV group (feeding) (APV_{oral}): The bees were orally fed individually with 3 µl of sugar solution containing diluted APV 10^{-3} using a 20 µl pipette. This corresponds to the 100 fold virus quantity compared to the injected APV dose.

In order to prevent that exterior influences like e.g. the constitution play a role the individual tests were made at one day. Therefore, a scatter coefficient is not applicable.

The calculations for the Chi-square-test (χ^2) and the U-test were done by the software program "Win-Stat" (Version 3.0).

The APV for the experiments was extracted from bees of infected colonies according to the method of Bailey and Woods (1974). The virus concentration

(1 mg/ml) was determined by photometric measuring according to the method of Newman et al (1973) and Ball (1985).

During the test, the bees were kept in the incubator at 28°C and 50-60% air humidity. Dead bees were daily removed from the cages and deep-frozen at –18°C until further examinations.

For determining of the *Nosema* titer, the whole abdomen was removed and processed as described above.

RESULTS

VERIFICATION OF NOSEMA SPORES AT TIME ZERO

On the eigth day following exposure, *Nosema* spores were found in the midguts of all examnined bees.

However the control groups did not show typical clinical symptoms (see also Ritter, 1996). The untreated control groups were not at all infected. There were differences (U-test, Mann & Whitney) between the groups with a low initial infection now with an average of 2 – 4,5 x 10^6 spores/midgut and those with a large initial infection now with an average of 1,4 – 3,6 x 10^7 spores/midgut.

MORTALITY OF NOSEMA INFECTED BEES AFTER APV INJECTION

Two to four days after injection the APV solution, only the control group not infected by Nosema (i.e. Nosema free bees) showed significant differences ($P<0,01$) in the mortality rates compared to those bees having received injections of PPB (potassium phosphate buffer) and those infected by APV (see table 1). The groups infected by large and small *Nosema* doses did not differ significantly neither on the 2nd nor on the 4th day after the injection. In all test groups, compared to the PPB and APV injected groups, a lower significant percentage of mortality was observed in the corresponding control.

The large differences between the untreated und PPB rows indicates that the puncuture and solvent alow increased mortality. To resolve their individual effects an additional test was conducted with some bees receiving a puncture from the needle without injecting any material. They were treated under the same conditions like the bees of the PPB and APV_{inj} group.

The results in table 2 show that bees only punctured by a needle did not die more frequently compared to the control (χ^2, $P>0,1$). The highly Nosema infected group is the only exception compared to which the control with a deadfall of 7,7% remains considerably under the deadfall of the puncture group (29,8%) (χ^2, $P<0,001$).

Considerably higher deadfall rates are stated with Cont-PPB and APV_{inj} in comparison to the puncture group (χ^2, $0,001<P<0,01$). Whereas there is no major statistical difference between the deadfall percentage of the PPB and the APV_{inj} bees on the 2nd day, increasingly higher deadfalls occur on the 4th day of the test in the control group and high infected Nosema group (χ^2, $0,0001<P<0,05$).

MORTALITY OF NOSEMA INFECTED BEES ORALLY CONTAMINATED WITH APV

In test 1 and 2, until the 4th day, the control bees showed a higher deadfall than the bees fed with APV (see table 3). Even if in test 3 the mortality of the APV fed bees dominated a significance could not be proved.

During the proceeding test, similar deadfall percentages were received from the control groups and the orally APV infested bees of test 1 to 3 (see table 3). In the different seasons different mortality rates were found. At the end of winter (February) the highest mortality rate within the shortest period was observed.

An immune diffusion test (IDT) could not detect APV in the dead bees initially fed that virus.

DISCUSSION

The tests were conducted to investigate the effects on the honey bees simultaneously infected by Nosema and APV. The bees were randomly assigned to different groups. A control, those infected by a small dose of Nosema spores, and those infected by a large dose of Nosema spores. The different groups were afterwards infected by APV.

The groups having received an injection of APV as well as of potassium phosphate buffer in case of the control group showed a considerably higher mortality rate during the first days after the beginning of the test, compared to the untreated group.

A puncture treatment, where only a needle was inserted without infecting any material, was used as a particular control. This control indicated that the damaging effects were due to the introduction of the APV and potassium phosphate buffer.

Injected APV produces an encreased deadfall of worker bees independent of a Nosema infection or not, because the deadfall in the contol groups did not differ from the one of the Nosema infected bees. Moreover, it doesn't matter whether the bees are severely or only moderately infected because mortality rates did not differ.

In order to investigate whether APV can penetrate the haemolymph of a bee after Nosema infection of the intestinal epithelium, the virus was also applied orally, apart from the subcutaneous injection, but in 100 fold higher dosage.

A reason for the enhanced mortality rate of the Nosema infected control group in test 1 and 2, during the first 4 testing days, could have been a hyperactive behavior observed, whereas bees fed with APV sitting lethargically on the inserted comb foundation piece. During the further test process, there were no remarkable differences between the mortality rates of the corresponding test groups. By means of the immune diffusion test APV could not be proved in any of the worker bees.

It is known (Borchert, 1966; Fries, 1993) that Nosema infection destroys the epithelium cells of the midgut. Bährmann (1962) could show in his experiments that even in case of a an advanced Nosema infection the formation of the peritrophic membrane is not limited. Jacobs et al. (1990) conclude from their studies that Nosema spores cannot penetrate into the haemolymph if the midgut epithelium is infested, thus also demonstrating that a barrier is maintained.

Similar results to the those shown in this study were found by Bailey et al. (1983) in their work with nosematosis and the Filamentous Virus. That virus, however, is larger than APV. But in the experiments conducted here, a passage of APV into the intestine could not be shown.

The midgut epithelium functions as barrier probably in connection with the peritrophic membrane preventing the penetration of APV into the haemocoel.

It is most probable that the constant mortality of worker bees in the control group and the corresponding APV groups is mostly due to a Nosema infection and its interrupting food absorption.

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TABLES

Table 1: Deadfall of Nosema infested winter bees after APV injection

test no. 1 (%)	N	Control group		N	Nosema group low*		N	Nosema group high **	
days after injection		2.	4.		2.	4.		2.	4.
control	60	0,0	20,0	57	3,5	5,3	56	7,1	23,2
KPP	54	3,7	35,2	54	25,9	44,4	54	22,2	51,8
APV inj.	63	34,9	71,4	51	25,4	49,0	51	21,6	56,9

deadfall on the 2nd and the 4th day after APV injection; (low* = $5x10^4$ spores/bee; high** = $5x10^5$ spores/bee)

Table 2: Deadfall of Nosema infested winter bees after APV injection

test no. 2 (%)	N	Control group		N	Nosema group low*		N	Nosema group high **	
days after injection		2.	4.		2.	4.		2.	4.
control	48	2,0	4,2	60	1,7	8,3	52	5,8	7,7
stitch	41	2,4	4,9	56	3,6	12,5	47	14,9	29,8
KPP	65	53,8	58,5	56	48,2	62,5	50	44,0	50,0
APV inj.	53	52,8	77,3	57	61,4	75,4	50	50,0	72,0

deadfall on the 2th and the 4th day after KPP and APV injection in test no. 2; (low* = $5x10^4$ spores/bee; high** = $5x10^5$ spores/bee)

Table 3: Deadfall of severely Nosema infested control groups

(%)	**Test no. 1** (2/98)		**Test no. 2** (4/98)		**Test no. 3** (9/98)	
Test day	Control	APV fed	Control	APV fed	Control	APV fed
4	23,2	1,9	7,7	2,0	11,8	16,9
8	75,0	83,0	19,2	14,3	47,5	42,3
12	100	94,3	36,5	28,6	62,5	67,6
16	100		46,1	46,9	80,0	87,3
N	109		102		105	

Here the control and the groups fed orally with APV from the tests no. 1, 2 and 3 are compared