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Impact of ethanol on Nosema spp. infected bees

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Summary

Nosema spp. spores are extremely resistant to external stress factors and can survive several years without losing the ability for further infection within the insect body. For this reason, combating nosemosis is difficult. Some beekeepers add ethanol to the sucrose solution before the winter to prevent nosemosis infection and to cure already infected colonies. Others feed infected colonies with herbal ethanol extracts. Therefore the aim of this study was to evaluate the ethanol impact on bees infected with Nosema spp.

Four groups of uninfected and Nosema spp. infected bees were fed with sugar-water syrup (1:1) supplemented with ethanol at the following concentrations: 10%, 5% and 2.5% and 0% as a control (only a sucrose syrup).

Generally, bees consumed 10% EtOH solution in an amount even 50% lower than in other concentrations. The impact of EtOH on the increase of bees' mortality was observed at a 10% EtOH concentration for healthy bees and even from 5% EtOH concentration for Nosema spp. infected bees. In our study the highest number of Nosema spp. infestation was noticed for bees fed with 5% EtOH and the lowest pH level was also measured for this group of bees. Therefore, a clear correlation was observed between the feeding bees with EtOH, which resulted in the acidification of bees, and the degree of Nosema spp. infestation.

A synergistic effect of the ethanol and nosemosis on the rise of the mortality of bees has been observed. The addition of ethanol to sucrose syrup facilitates conditions for the development of nosemosis in honey bees. The strongest effect of ethanol on the level of Noseama spp. infection was observed for the 5% ethanol solutions. Moreover, ethanol at 10% concentration in sugar syrup exerts severe toxic effects even on healthy bees. All these factors induced immune-suppression in bees and enhanced the level of Nosema spp. infestation.

Keywords: Apis mellifera, nosemosis, ethanol, toxicity, pH level

Nosemosis is a panzootic bee disease caused by two species of the genus *Nosema* (Fungi: Microsporidia). This disease was described for the first time in honeybees (*Apis mellifera*) 100 years ago (34). Initially, it was believed that nosemosis was caused solely by one species: *Nosema api*s. At the end of the twentieth century a new species of this microsporidium, *Nosema ceranae* (9), attacking the eastern honey bee (*Apis cerana*), was described in Asia. The first description of this parasitic fungus was reported in bees living in hot and humid climates (16, 17). It is probable that infections caused by *N. ceranae* in temperate climates contribute to the Colony Collapse Disorder (CCD) (12, 13, 32).

The first infection of *Apis mellifera* by *N. ceranae* was recorded in Taiwan in 2005 (17), and soon after that it was recorded in Europe (11, 26), in the United States (5), in China and Vietnam (20). Research results suggest that *N. ceranae* infects bumblebees, which are under species protection. This has caused a serious epidemiological threat to their further existence (27). At present, there has also been a widespread nosemosis infection (18) in Poland (32).

Nosema spp. develop as obligate intracellular organisms and exert severe energetic stress on their hosts cells (21). The implications of energetic stress on bees are particularly important for foragers. Such an energetic stress can strongly affect the energy balance of the entire bee colony and consequently can be the reason for the colony's collapse.

Nosema spp. spores are extremely resistant to external stress factors and can survive several years without losing their ability for further infection within an insect body. For this reason combating nosemosis is difficult. Until now no effective cure for nosemosis has been discovered that would be harmless to bees and not contaminate honey. Some beekeepers add ethanol to the sucrose solution before the winter to prevent nosemosis infection and to cure already infested colonies. Others feed infected colonies with herbal ethanol

extracts. Therefore the aim of this study was to evaluate the ethanol impact on bees suffering from nosemosis.

Material and methods

During two-year study (2011, 2012) healthy Buckfast bees were collected from a stationary apiary in the northern part of the Lublin region. Three experiments were conducted during June, July and August 2011 and 2012. Bees of one age (gray and sluggish) were individually hand-capturing from the hive. After that they were immediately inserted into wooden cages (12 × 12 × 4 cm) with glass front screens, ventilating and feeding slots equipped with 5 ml syringes. Every cage had 40 bees. Cages with bees were randomly divided in two groups: first group – uninfected (control) bees and a second group, in which bees were fed with a suspension of *Nosema* spp. spores.

The suspension of spores were obtained by crushing *Nosema* spp. infected bees in sterile water, filtering the suspension using sterile meshes and centrifuging for 3 minutes at 2000 × g to obtain spore suspensions of 1 000 000/ml. Bees from the second group were fed during the first day of each experiment with fresh *Nosema* spp. spores inoculums added to sugar-water syrup. After the groups were formed, the cages were transferred into an air-conditioned chamber. The chamber was kept at a constant temperature of 26°C and 65% relative humidity.

Starting from the second day of each experiment, uninfected and *Nosema* spp. infected bees were fed with sugar-water syrup (1:1) supplemented with ethanol (EtOH) at the following concentration: 10%; 5%; 2.5% and 0% as a control (only a sucrose syrup). Each day fresh concentrations of EtOH in the sucrose syrup were prepared and given bees in the 5 ml syringes. Every day, from the 3rd experimental day, the amount of consumed sucrose syrup was calculated on the number of milliliters less sucrose syrup in the syringes within 24 hours divided by the number of bees left in the cage.

From the 3rd experimental day, the number of dead bees was also counted every 24 h. Then the data were divided by the number of 10 experimental days to obtain the average number of dead bees per day.

Estimation of *Nosema* spp. spores number. On the 12th infection day the number of *Nosema* spp. spores was checked. From each group of healthy and *Nosema* spp. infested bees, 10 whole specimens were ground up in 10 ml of sterile, distilled water. The homogenate was placed under the cover glass of the Bürker chamber. Every sample was left about 3 minutes to allow the spores (if they were present in the homogenate) to settle and was checked immediately after that time, before the sample began to dry in the chamber. Two independent samples (per 10 dead bees) for each experimental group were conducted. Estimation of *Nosema* spp. spores per bee was accomplished using a Olympus BX 61 light microscope (4, 14).

pH value measurement. On the 12th infection day, in homogenates, prepared to estimation of *Nosema* spp. spores number, the level of pH was measured using the SP300 Slandi® Pehametr. Homogenates were prepared from 10 whole specimens grounded up in 10 ml of sterile, distilled water. Two independent samples (per 10 dead bees) for each experimental group were conducted. Therefore, 2 estimates of pH value were measured for each group.

Statistical analysis. The SAS software (29) using the ANOVA (the group effect was the experimental factor) and the Tukey's HSD (honestly significant difference) test were used to prepare the statistical analysis of the obtained data. This post hoc test (or multiple comparison test) can be used to determine the significant differences between group means in an analysis of variance setting. The Tukey's HSD test is generally more conservative than the Fisher LSD test, but less conservative than Scheffe's test, therefore it best suited to the analysis of the obtained data (for a detailed discussion of different post hoc tests, see 33). This test was used to estimate differences among: the amount of consumed syrup in relation to the EtOH concentration (Tab. 1) and the value of pH in bee homogenates from experimental groups (Tab. 3).

Results

Uninfected honey bees readily consumed 2.5% and 5% EtOH solutions in an amount comparable to that of the pure sucrose syrup eaten by the control group. 10% EtOH was eaten reluctantly, in an amount even 50% lower than in other concentrations (Tab. 1). Moreover, 10% EtOH substantially shortened the life-span of the caged bees and exhibited unequivocal toxicity (Tab. 2).

Tab. 1. The amount of syrup consumed by bees in relation to the ethanol concentration

Group of bees	The concentration of ethanol in the sugar syrup	Amount of consumed syrup (µl/bee)			
		Mean	SE	Min.	Max.
Uninfected bees	0.0%	42	0.20	34	56
	2.5%	40	0.15	33	47
	5.0%	31	0.11	28	39
	10.0%	24	0.03	15	28
Nosema spp. infected bees	0.0%	65	0.18	58	72
	2.5%	62	0.11	58	71
	5.0%	42	0.08	38	49
	10.0%	35	0.06	29	41

Explanations: SE – standard error; Min. – minimum value of the features; Max. – maximum value of the features, differences in groups are significant at $p \leq 0.05$ (ANOVA; Tukey's test), $F = 11.14$

Tab. 2. Daily mortality of bees in infected and uninfected bees in relation to the ethanol concentration

Group of bees	The concentration of ethanol in the sugar syrup	Mean
Uninfected bees	0.0%	0.2
	2.5%	0.3
	5.0%	0.5
	10.0%	6.8
Nosema spp. infected bees	0.0%	1.3
	2.5%	1.3
	5.0%	3.7
	10.0%	9.2

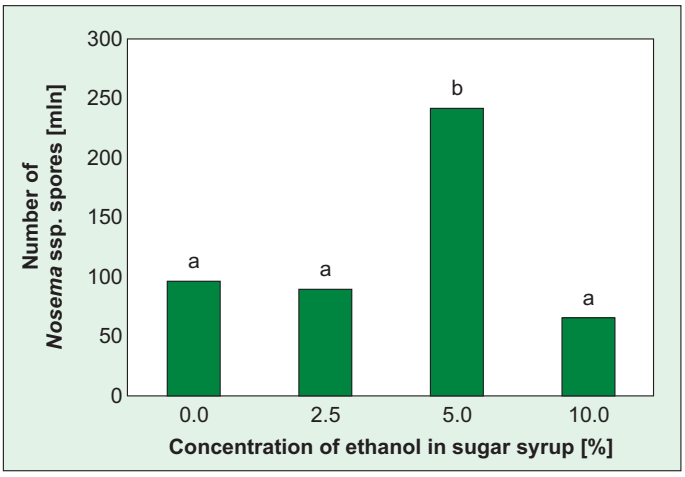

Fig. 1. The effect of ethanol concentration in sugar syrup on the level of *Nosema* spp. infection
Explanations: a, b – differences among groups are significant at $p \leq 0.05$ (ANOVA; Tukey's test, F = 10.96)

Generally, due to higher energetic demand *Nosema* spp. infected bees connected with infestation consumed a higher amount of sugar syrups, both without EtOH and with different EtOH concentrations. The 2.5% EtOH concentration was consumed in an amount comparable to that of the pure sucrose syrup, similarly to that in healthy bees group. But, 5.0% EtOH was eaten in significantly fewer quantities and 10% was consumed in an amount 53.8% lower compared to the volume of pure sucrose syrup eaten by *Nosema* spp. infected bees (Tab. 1).

Nosema spp. infected bees which consumed the 5% EtOH concentration had an increase in the degree of *Nosema* spp. infestation up to 242 mln. spores per bee, compared to the bees fed the pure sucrose syrup with an infestation on the level of 96 mln. spores per bee (Fig. 1). Other EtOH concentrations had no impact on the level of *Nosema* spp. infestation and differences among obtained data were not statistically significant. The 5% EtOH concentration was consumed in a higher amount than that of the 10% EtOH concentration, therefore the impact of the 5% EtOH concentration was more significant regarding the degree of *Nosema* spp. infestation and, moreover, increased bees' mortality (Tab. 2). However, the 10% EtOH concentration caused a much greater increase in bees' mortality than that of 5% EtOH concentration, both in *Nosema* spp. infected and uninfected bees (Tab. 2).

Generally, *Nosema* spp. infected bees had lower pH values than uninfected bees. The addition of EtOH also had an impact on the pH value of bees' homogenates (Tab. 3). The 2.5% and 5% EtOH concentrations were consumed in a comparable amount to that of the pure sucrose syrup, with 10% EtOH addition significantly lower. Therefore, the strongest effect of EtOH on pH value of bees' homogenates was observed for the 5% EtOH solutions (Tab. 3). Such an acidification of bees was correlated with the increased level of *Nosema* spp. infection (Fig. 1).

Tab. 3. pH value of bees homogenates in relation to the ethanol concentration

Group of bees	The concentration of ethanol in the sugar syrup	Mean	SE	Min.	Max.
Uninfected bees	0.0%	6.36^{b}	0.04	6.13	6.63
	2.5%	6.21^{b*}	0.05	5.98	6.62
	5.0%	5.96^{a}	0.20	5.32	6.70
	10.0%	6.10^{b*}	0.11	5.34	6.74
Nosema spp. infected bees	0.0%	6.25^{b}	0.03	6.07	6.57
	2.5%	5.95^{ab*}	0.06	5.57	6.21
	5.0%	5.65^{a*}	0.17	5.07	6.08
	10.0%	5.92^{a}	0.06	5.40	6.24

Explanations: a, b – differences in groups are significant at $p \leq 0.05$ (ANOVA; Tukey's test), F = 9.09, SE – standard error; Min. – minimum value of the features; Max. – maximum value of the features

Bees consumed sucrose syrup with EtOH reluctantly, in a lower amount than pure sucrose syrup (Tab. 1). Only 2.5% EtOH concentration was consumed in an amount comparable with pure sucrose syrup and had no impact on bees' mortality. But even such a low EtOH concentration influenced the pH value of bees' homogenates (Tab. 3), especially after *Nosema* spp. infestation. For this reason feeding bees even with a low EtOH concentration (2.5%) can have a long-term negative impact on the bees.

Discussion

Research conducted by Abramson et al. (1, 2) revealed that the exposure of honey bees to EtOH influences the behavior of foragers in a similar manner to that observed in experiments with vertebrates. Consumption of 10% and 20% EtOH solutions decreases locomotion and influences stinging behavior in harnessed foragers. During their experiments honey bees readily consumed 1%, 5%, 10%, 20% and even 95% EtOH, the last solution was consumed as long as the antennae did not make contact with the solution. In the study of Mixson et al. (25) it was observed that a low dose of EtOH (2.5% w/v EtOH in 1.5 M sucrose solution) is sufficient to disrupt both social and non-social behaviors in honey bees. In other studies, conducted on free flying bees, 5% EtOH sucrose solution had a large impact on bees' behavior (31).

All the above mentioned studies used bees as models for human EtOH intoxication and conducted tests were returned to changes in a bee's nervous system. In our study it was also noticed that bees fed with 10% EtOH solution were drowsy, reluctant to fly, laid longer on their dorsal thorax after inverting upside down, did not try to escape from their cages when the other bees were collected for samples (as did the bees in the control group or fed lower concentrations of EtOH). In the above mentioned studies, bees willingly ate 20% EtOH concentration, surprisingly in our study, bees consumed

10% EtOH solution reluctantly in an amount even 50% lower than pure sucrose syrup.

Effects of EtOH toxicity related to ours results were received by Cakmak et al. (3) in their study of the impact of EtOH on the queen honey bee. After consuming 20 µl of a 10% EtOH solution the bee queen laid fewer eggs, appeared in poor health and as a result the queen was superseded. EtOH intoxication was also observed in studies conducted by Maze et al. (24) on worker honey bees (*Apis mellifera*). Mortality of bees increased almost twofold after feeding with 9 µl of a 1.0 M sucrose solution containing 50% EtOH. It should be emphasized that in the above mentioned studies the bees were fed only once with EtOH and have had time for detoxification and recovery. In our study, different EtOH concentrations were administered for 10 experimental days. Consequently, the impact of EtOH on increase of bees' mortality have been observed at lower EtOH concentrations than in the above mentioned studies, i.e.: feeding bees with 10% EtOH concentration in the group of uninfected bees and even from 5% EtOH concentration for *Nosema* spp. infected bees, significantly raised bees mortality.

After consuming even one dose of EtOH, changes in bees' behavior (1, 2, 24, 25) and physiology (3, 24) had been observed. The impact of EtOH on free-flying bees is not so great because of the availability of other food and water. Exposure to ethanol influenced honey bees similarly to effects observed in experiments with vertebrates. Consequently, one dose of EtOH can have only a temporary effect and if the concentration of EtOH dose is not mortally poisonous bees have time for detoxification and recovery. In our study, bees were fed entirely with various concentrations of EtOH, therefore the impact of EtOH on those bees was very strong and can be described as chronic toxicity; similar to the impact of EtOH on the health of man who drinks alcohol on a regular basis. Summer bees have a very short life expectancy in comparison to humans or other vertebrates and live about 36 days. Therefore, when bees were fed with EtOH for 10 days, they were fed during almost one third of their life. Consequently, such a diet had a great impact on their behavior and physiology.

In bees and other insects, like in vertebrates, EtOH is metabolized by alcohol dehydrogenase (ADH) to acetaldehyde (22). Acetaldehyde is then converted to acetate by aldehyde dehydrogenase (ALDH) (6, 24, 28), which acidify intestinal contents (Tab. 3). Acidification of intestinal contents appeared to facilitate the infection of epithelial cells and created favorable conditions for the germination of *Nosema* spp. spores, which can be stimulated by an acidic medium (10). In our study the highest level of *Nosema* spp. infestation was noticed for bees fed with 5% EtOH, and the lowest pH value was also measured for this group of bees. But even 2.5% EtOH concentrations in bees' diets influenced the pH level of bee homogenates, especially after *Nosema* spp. infestation. In comparison to the infected bees fed only the pure sucrose syrup, *Nosema* spp. infested bees that consumed 5% EtOH concentration had an increased number of observed *Nosema* spp. spores in the homogenates. Therefore, feeding bees with a low EtOH concentration can have long-term negative impact on the bees.

The effect of faster *Nosema* spp. proliferation as well as higher mortality among infected workers was observed after exposure to CO_2 spores used for bees' inoculation (7). After dissolving in water, carbon dioxide produced carbonic acid; therefore, spores solution after exposure to CO_2 became acidic. Consequently, results of this (7) and our studies, in the mean of the influence of the intestinal contents acidification on the level of *Nosema* spp. infection can be compared. Acidification of the midgut is probably the reason for declining or loss of the function of regenerative crypts in the midguts (15). This has a significant impact on increasing the level of *Nosema* spp. Infection, which further reduces the functions of the intestine, and reduces the absorption of nutrients and is the cause of the malnutrition of bees and, consequently, increased mortality.

On the other hand, some studies have indicated that acidification of bees food has no impact on the course of nosemosis. In a study conducted by Forsgren and Fries (8) the impact of feeding bees with acetic acid and benzoic acid on the level of *Nosema* spp. infestation was examined. During their experiments, bees were fed with sugar solution with 0.2%, 0.4% acetic acid and 0.03% benzoic acid. In laboratory experiments bees were once fed with 10 µl of such solutions, while during the field studies free-flying bees were treated with these solutions added to their winter feeding. Such acidification of the food had no influence on nosemosis prevalence or development. But the addition of acetic and benzoic acids could have no impact on pH value of bees intestines because research conducted by Kluge et al. (19) had shown that supplementation of piglet diets with benzoic acid did not influence the pH value in the gastrointestinal tract. In our study, it was observed that a clear correlation between feeding bees with EtOH exists, resulting in the decrease of the pH level, and increasing the degree of *Nosema* spp. infestation.

During the conducted studies, *Nosema* spp. infected bees consumed more sugar syrup than healthy bees due to energetic stress connected with the disease, but addition of the EtOH decreased the amount of the consumed sugar syrup in infested bees in comparison to the amount of sucrose syrup eaten without EtOH in the same bee group. Similarly, healthy bees consumed EtOH sucrose solutions in lower amounts (even 57% lower for the highest EtOH concentration). All this can lead to bees' malnutrition, and in combination with

intestinal lesions caused by acidification can be the reason of higher mortality among bees fed with EtOH solutions. Therefore, the addition of EtOH to bees' diets has a strong negative impact on the bees.

Like all parasites *Nosema* spp. depend on their hosts for energy and consequently exert an energetic stress on them. Moreover, honeybees need to expend energy for mounting an immunological response after infection, which is an energetically expensive process (30). With their high metabolic demand due to flight foragers are especially prone to an energetic stress when they are infected (23). Therefore, *Nosema* spp. infected bees consumed more sugar syrup even with the addition of EtOH. Consequently, the strongest effects of EtOH toxicity were observed for bees suffering from nosemosis.

Conclusion

Synergistic effect of the ethanol and nosemosis on the rise of bees mortality had been observed. Addition of ethanol to sucrose syrup facilitates conditions for the development of nosemosis in honey bee. Moreover, ethanol at 10% concentration in sugar syrup exerts severe toxic effects on bees, both healthy and *Nosema* spp. infected. That all induced immune-suppression in bees and enhanced the level of *Nosema spp*. infestation. Therefore we strongly recommended not to use ethanol to bees treatment.

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