

Nosema disease in the honey bee (*Apis mellifera* L) infested with varroa mites in southern Spain

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Summary — Twenty-nine hives infested by *Varroa jacobsoni* were sampled over a 2-year period in order to find out their degree of infection by *Nosema apis*. The hives were situated in ten apiaries distributed throughout southern Spain. *N apis* has been found in 90% of the apiaries sampled and in 55.17% of the hives studied, but only 5.1% of the bees were infected. We have found a low correlation between the average number of spores per infected bee in the positive samples and the percentage of infected bees ($r^2 = 0.2438$; $P < 0.001$; $n = 33$), and between the average number spores in the composite samples of 60 bees and the percentage of infected bees ($r^2 = 0.4557$; $P < 0.001$, $n = 33$). Our results show that *N apis* and *V jacobsoni* could develop independently and that those samples which manifested a low, medium and high infestation by *V jacobsoni* had percentage infections with *N apis* of $22.6\% \pm 3.6\%$ vs $47.5\% \pm 16.2\%$ vs $16.7\% \pm 10.4\%$ respectively, without significant differences ($F = 0.2817$; $P = 0.7567$). A progressive increase in the number of spores per individual was detected with increasing levels of *V jacobsoni* infestation: 5.9×10^6 vs 9.1×10^6 vs 13.8×10^6 spores/bees, but no significant differences exist between them ($F = 0.6053$; $P = 0.5531$).

***Nosema apis* / infection level / *Varroa jacobsoni* / Spain**

INTRODUCTION

The microsporidian *Nosema apis* Zander is an endoparasitic protozoan of honey bees which causes considerable economic losses in the beekeeping industry. Detrimental

effects include the loss of adult bees in winter and a reduction in the hive's productivity (Farrar, 1947; L'Arrivée, 1966; Cantwell and Shimanuki, 1969; Fries et al, 1984). Some of these losses may be related to the three viruses associated with *N apis* (Bailey and Ball, 1991).

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Nosema apis is widely distributed throughout the world (Bradbear, 1988), but whilst its effects are not considered important in tropical and subtropical climates (Wilson and Nunamaker, 1983), this is not the case in countries with temperate climates. Differences in the seasonal incidence of infection depending on geographical location are well documented (Bailey and Ball, 1991; Fries, 1993).

In Spain, *N. apis* is well known, having been detected by many researchers. However, almost all the published studies have been limited to citing the presence of the microsporidia (Torrens Pastor, 1947; Habela et al, 1987; Gijón-Botella et al, 1987), whereas hardly any studies have been made of its seasonal incidence. Pajuelo and Fernández Arroyo (1979) report *N. apis* infection levels of 21% (number of samples and locations unknown), being most prevalent in the months of January to April.

The disease evolves without producing any visible signs, meaning that in many cases no treatment is given. Other factors, such as mite infestation, with a consequent weakening of the colony, may influence the subsequent development of *N. apis* infection levels.

The infection of bees by this protozoan of colonies infested with the *Varroa jacobsoni* Oudemans mite is poorly documented. Smirnov (1978) suggests that the mortality rate in these circumstances increases rapidly if no action is taken. In a study made in Poland, Romaniuk and Wawrzyniak (1991) concluded that the two parasites coexisted.

The aim of this research is to find out whether there is an interrelationship between these two pathologies in Spain. The prevalence and distribution of *N. apis* in our experimental apiaries, distributed throughout the southern half of the Iberian peninsula, are also reported.

MATERIALS AND METHODS

During the period October 1990 to October 1992, samples were taken monthly from 40 Layens beehives distributed among 12 apiaries in the south of the Iberian peninsula, to ascertain the dynamics of the *V. jacobsoni* population (Orantes Bermejo et al, 1994; García Fernández et al, 1995). One hundred and sixty-two (162) samples of bees from 29 of 40 beehives belonging to ten apiaries were analysed, to evaluate the extent of *N. apis* infection.

Adult worker bees were collected from brood nests, from mixed brood nests and honey storage areas, and a few were taken from the hive entrance. This provided us with a good representation of the hive's population (Calderone and Shimanuki, 1992) and minimised the effects occurring as a result of the infected bees found in particular locations within the hive (Moeller, 1956; L'Arrivée, 1963; Pickard and El-Shemy, 1989). The bees, whose age was unknown, were conserved in 70% ethanol.

Sixty bees were individually analysed from each sample. The abdomen of each bee was dissected and placed in an Eppendorf tube, homogenising it with a small piston in distilled water with a drop of 10% Nigrosin solution, finally adjusting the volume to 1 mL. A drop of this solution was placed in a haemocytometer to give a liquid depth of 0.1 mm under small grid areas of 0.0625 mm². The number of spores in 20 grid squares was counted, the total number being calculated by the expression: number of spores = 8000 × f; where f is the dilution factor used. This method is considered optimum for this calculation either for individuals or groups (Burnside and Revell, 1948; L'Arrivée, 1963; Doull 1961; Cantwell, 1970; van Laere et al, 1980; Wilson and Nunamaker, 1983; Pickard and El-Shemy, 1989) and its accuracy has been investigated by van Laere et al (1980).

Various mean analyses were made (Anova and Manova) and correlations between different parameters carried out. In order to make the variance homogenous in these mean analyses, the data were transformed by the expressions $y = \ln(x)$ for both the average number of spores per infected bee in the positive samples and the average number of spores in the composite samples of 60 bees; and $y = \arctan(x)$ for the percentage of infected bees in the positive samples. For all calculations we used SPSS. (Nie et al, 1983).

RESULTS

Nosema apis in southern Spain

Table I sets out the results of the sampling. *N apis* is widely distributed, being diagnosed in 90% of the apiaries, with 55.17% of the colonies monitored in our study being infected. In total, 9720 bees were analysed, of which 495 were infected, which represents 5.1%. These infected bees contained a mean of $7.6 \times 10^6 \pm 1.7 \times 10^6$ spores per bee (mean \pm SE).

As can be seen in table I, we found that *N apis* is most prevalent during spring, and in the colder months from November to January, except in the high mountainous regions (Lanjarón and Vadillo-Cazorla) where this occurred earlier, in September.

In our experiment, we have found a significant relationship between the average number of spores per infected bee in the positive samples and the percentage of infected bees, but with a low coefficient of correlation ($r^2 = 0.2438$; $P = 0.003$; $n = 33$) and between the average number of spores in the composite samples of 60 bees and the percentage of infected bees ($r^2 = 0.4557$; $P < 0.001$, $n = 33$).

N apis versus *V jacobsoni*

Table II shows the comparison between the number of samples infected by *N apis* and those infested with *V jacobsoni*: in 18.52% of the samples ($n = 162$) both parasites were found. It can be seen from table III how similar positive percentages were found in colonies with both low and high infestations by *V jacobsoni*, 22.8% vs 26.3%. Similarly there are no significant differences in the percentage of infected bees in the positive samples $22.6\% \pm 3.6\%$ vs $47.5\% \pm 16.2\%$ vs $16.7\% \pm 10.4\%$ ($F = 0.2817$; $P = 0.7567$). However, it was noted that the higher the percentage of parasitization by *V jacobsoni*,

the greater the number of spores, yet no significant differences existed in the means of the three different *V jacobsoni*-infested groups ($F = 0.6053$; $P = 0.5531$) nor was there a significant correlation between the two parameters (percentage of parasitization by *V jacobsoni* / number of *N apis* spores; ($r^2 = 0.2946$; $P = 0.102$).

DISCUSSION

N apis is an important pathogenic agent of hives which does not appear to have much effect in our climatic conditions, causing losses which are undetectable and insignificant to beekeepers (5.1% of bees analysed). However, it has a fairly high prevalence (55.17% of hives) and some bees develop large numbers of spores in their intestines (the maximum found in this study = 86.4×10^6 spores/bee).

We found that 20.37% of samples were infected by *N apis* ($n = 162$), a figure similar to the results of a previous study of samples from Spain where it was found to be 21% (Pajuelo and Fernández Arroyo, 1979). The previous study was made prior to the arrival of *V jacobsoni* on the Iberian Peninsula. This fact, together with the results set out in table III, lead us to believe that the two parasites coexist but develop independently of one another.

The hypothesis that a weakening of the colony caused by *V jacobsoni* infestation could alter internal factors in the bee, such as the permeability of the middle intestine walls, the effect of pteridines and the role of the intestinal microflora (Hartwig, 1976; Peroutka and Cihar, 1978), and thus provoke a greater multiplication of *N apis*, is still a matter of speculation. In this study, the colonies with the highest range of infestation by *V jacobsoni* had more spores per individual bee but, as stated in the results, there were no significant differences between the means of these three groups

Table 1. Results of survey of *Nosema apis* spores in adult worker honeybees from southern Spain carried out between October 1990 and October 1992 in 29 colonies sampled at different times of the year. (Month: month when *N apis* was detected.)

Locations	Altitude (m)	Month	No of samples analysed/ positive	No of bees analysed/ parasitized with %	% infected bees in the positive samples ($\bar{x} \pm SE$)	Average number of spores in the composite sample of 60 bees in the positive samples ($\bar{x} \pm SE$)	Average number of spores per infected bee in the positive samples ($\bar{x} \pm SE$)	No of apiaries sampled/ positive	No of hives sampled/ infected
Hornachuelos (Córdoba)	183	I, V	15/2	900/9 (1%)	7.5% \pm 4.1%	485649 \pm 227683	11805571 \pm 9594428	1/1	2/1
Trassierra (Córdoba)	111	XI, XII, IV, VII	16/6	960/87 (9.06%)	24.12% \pm 4.1%	3826711 \pm 1020081	14244744 \pm 3741120	1/1	2/2
Dos Hermanas (Sevilla)	92	-	10/0	600/0	-	-	-	1/0	2/0
Castellar de la Frontera (Cádiz)	248	V, VI, VIII, XI	18/5	1080/63 (5.83%)	21% \pm 6.3%	1547586 \pm 637318	5384576 \pm 2244807	1/1	2/2
Lepe (Huelva)	6	V, VI, VII	13/3	780/103 (13.2%)	57.22% \pm 7.7%	893564 \pm 454987	987987 \pm 155030	1/1	2/1
Cáceres	459	III, IV, V, VI	14/6	840/80 (9.52%)	22.22% \pm 11.4%	656766 \pm 404413	10837312 \pm 6827320	1/1	2/2
Lanjarón (Granada)	1100	IX, VII	20/3	1200/51 (4.25%)	28.33% \pm 15.5%	1379199 \pm 359057	9651501 \pm 6184644	1/1	4/2
Vadillo-Cazorla (Jaén)	1290	IX, X	18/2	1080/26 (2.41%)	21.61% \pm 16.67%	961283 \pm 958350	2533202 \pm 2474536	1/1	5/2
Maro-Nerja (Málaga)	21	XII, III, VII	22/3	1320/21 (1.59%)	11.67% \pm 4.4%	77793 \pm 70695	458888 \pm 328251	1/1	6/2
Murcia	57	I, II, VI	16/3	960/49 (5.1%)	27.22% \pm 23.09%	3224399 \pm 3223600	4414181 \pm 4387188	1/1	2/2
Total			162/33 (20.37%)	9720/495 (5.1%)	24.69% \pm 3.8%	1644167 \pm 395799	7655467 \pm 1748194	10/9 (90%)	29/16 (55.17%)

Table II. Results of diagnoses for *N apis* and *V jacobsoni* in the 162 samples considered in this study.

With neither <i>Nosema apis</i> nor <i>Varroa jacobsoni</i>	10 (6.17%)
<i>Varroa jacobsoni</i> only	119 (73.46%)
<i>Nosema apis</i> only	3 (1.85%)
Presence of both <i>Varroa jacobsoni</i> and <i>Nosema apis</i>	30 (18.52%)

Table III. *Varroa* infestation levels vs both *Nosema* spores and percentage of infected bees.

Range of parasitization rates by <i>Varroa jacobsoni</i>	<i>Nosema apis</i>		
	Negative	Positive	Mean no of spores in positive samples % of infected bees in positive samples
0.1% - 5% <i>n</i> = 92	71	21 (22.8%)	5941 239 ± 1680415* 22.6% ± 3.6% **
5.1% - 25% <i>n</i> = 38	34	4 (10.5%)	9139441 ± 4998860* 47.5% ± 16.2%**
25.1% - 100% <i>n</i> = 19	14	5 (26.3%)	13180095 ± 7859518* 16.7% ± 10.4% **

* No significant differences, $F = 0.6053$; $P = 0.5531$; ** no significant differences, $F = 0.2817$; $P = 0.7567$.

with different ranges of infestation by *V jacobsoni*.

Similar results were obtained in a study carried out in Poland (Romaniuk and Wawrzyniak, 1991) which point to a synergic effect between *V jacobsoni* and *N apis*. Sixty-one samples were found to be infected by *N apis* out of 209 samples with parasitization by *V jacobsoni* ranging from 0.1%-5% and a mean number of spores of 8.9×10^6 , whereas in 20 samples with parasitization by *V jacobsoni* ranging from 25.1%-100%, they found six samples infected with *N apis*, and the mean number of spores went up to between 1.8 and 27.2×10^6 .

The parasitic action of *V jacobsoni* on the honey bee causes a loss in the volume of haemolymph (Tewarson, 1983), a fall in the

number of haemocytes (Smirnov, 1978) and a blockage in the metabolism of proteins (Domatskaya, 1980), which may lead to a decrease in the resistance of the honey bee to *N apis*, as multiplication of *N apis* is favoured by the destruction of hemocytes (Gilliam and Shimanuki, 1967) and the acceleration of the catabolism of fats (Gontarski, 1952). Gontarski (1952) reported a relationship between the reserves of fats and proteins in the honey bee and its resistance to *N apis*.

Under the conditions of our experiment, we found significant correlations between the average number of spores per infected bee in the positive samples and the percentage of infected bees, but with a low coefficient of correlation ($r^2 = 0.2438$; $P = 0.003$; $n = 33$), the same occurs between

the variables, average number of spores in the composite samples of 60 bees and the percentage of infected bees ($r^2 = 0.4557$; $P < 0.001$, $n = 33$), contradicting the findings of L'Arrivée (1963), Bailey (1968), Flinger et al (1982) and Fries et al (1984).

With regard to the seasonality of *N apis*, only 5.1% of bees were found to be positive, making it difficult to represent the data or compare averages in the various climatic regions in which the hives were situated. We can only conclude that *N apis* is most prevalent during spring (May-July), except in high mountainous regions (September). Our station with the mildest climate (Maro, with a Mediterranean subtropical climate), where plentiful pollen and nectar exist throughout the year, is where the lowest parasitization rates and fewest spores in the bees analysed were found, probably due to the fact that the bees which developed nose-mosis defecate and die outside the hive.

A series of circumstances exist which may mean that this study would offer different results if it were repeated. For example, during the sampling period, Spain was suffering a severe drought, which had consequential limiting effects on flowering. Climatic conditions are important and have meant that many studies carried out in the same region give different results (Bailey and Ball, 1991).

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Résumé — La nosérose chez les abeilles (*Apis mellifera* L.) infestées par l'acarien *Varroa* dans le sud de l'Espagne.

Pour connaître l'incidence de *Nosema apis*, nous avons étudié 29 ruches infestées par l'acarien *Varroa jacobsoni* et ayant un taux d'infestation des abeilles adultes connu. L'échantillonnage a été réalisé d'octobre 1990 à octobre 1992, dans dix ruchers répartis dans le sud de l'Espagne (fig 1). Soixante abeilles ont été analysées individuellement par échantillon. L'abdomen de chaque abeille a été disséqué et mis à macérer dans de l'eau distillée additionnée d'une goutte de solution de Nigrosine à 10 % ; le volume final a été ajusté à 1 mL. Les spores ont été dénombrées à l'aide d'un hémocytomètre. *N apis* est largement répandu dans toute la région étudiée et présent dans 90 % des ruchers échantillonnés et dans 55.17 % des ruches étudiées. Son incidence est pourtant faible : 5.1 % ($n = 9720$) des abeilles analysées (tableau I). *N apis* présente une variation saisonnière avec deux maximums, de mai à juillet et de novembre à janvier, sauf dans les ruches situées en montagne où le deuxième maximum commence en septembre. Nous avons trouvé une relation significative entre les paramètres suivants : nombre moyen de spores par abeille infectée dans les échantillons positifs et pourcentage d'abeilles infectées, mais avec un faible coefficient de corrélation ($r^2 = 0.2438$; $P = 0.003$; $n = 33$). Cette relation est également valable pour le nombre moyen de spores d'abeilles infectées dans les échantillons composés de 60 abeilles et le pourcentage d'abeilles infectées ($r^2 = 0.4557$; $P < 0.001$, $n = 33$), ce qui contredit les résultats des études précédentes. Les deux parasites ont été trouvés dans 18,52 % des échantillons ($n = 162$) (tableau II). Nos résultats montrent que *V jacobsoni* et *N apis* peuvent se développer indépendamment l'un de l'autre. Les échantillons faiblement, moyennement et hautement infestés par *Varroa* présentaient un taux d'infestation par *N apis* de 22.6 % \pm 3.6 %, 47.5 % \pm

16.2% et 16.7% \pm 10.4% respectivement, sans différence significative entre ces trois moyennes ($F = 0.2817$; $p = 0.7567$) (tableau III). Une augmentation progressive du nombre de spores/individu a été observée parallèlement à l'augmentation du taux d'infestation par *V jacobsoni* ($5,9 \times 10^6$; $9,1 \times 10^6$; $13,8 \times 10^6$ spores/abeille respectivement pour un taux d'infestation faible, moyen et élevé), mais les différences ne sont pas significatives (tableau III).

Nosema apis / taux infestation / *Varroa jacobsoni* / Espagne

Zusammenfassung — Nosematose bei Honigbienen (*Apis mellifera* L.) bei Befall mit *Varroa* - Milben in Südspanien. Aus 29 Bienenvölkern mit einer bekannten Befallsrate von *Varroa jacobsoni* Oud wurden während einer Periode von 2 Jahren an verschiedenen Zeitpunkten des Jahres adulte Bienen entnommen und der Befallsgrad mit *Nosema apis* bestimmt. Insgesamt wurden 162 Bienenproben gesammelt. Die Bienenvölker waren auf 10 Bienenständen in ganz Südspanien verteilt (Tabelle I). Von jeder Probe wurden 60 Bienen entnommen und einzeln untersucht. Hierzu wurden zunächst die Abdomen in destilliertem Wasser mit Zusatz von einem Tropfen 10% Nigrosinlösung zerquetscht, das Volumen dann auf 1 mL justiert und die Sporen mit einem Haemocytometer (Zählkammer) ausgezählt. *N apis* ist in der untersuchten Gegend weit verbreitet und wurde in 90% der Bienenstände bei 55,2% der Bienenvölker nachgewiesen. Die Infektionsrate war mit 5,1% ($n = 9720$) befallenen Bienen jedoch gering (Tabelle 1). Die Nosematose folgt einem saisonalen Muster und kommt von Mai bis Juli und von November bis Januar häufig vor. Bei Bienenständen in den Bergen beginnt diese letzte Periode allerdings bereits im September. Wir haben eine signifikante Abhängigkeit zwischen folgenden Variablen gefunden: dem Mittelwert der Sporen

pro infizierter Biene in positiven Proben und dem Prozentsatz von infizierten Bienen. Allerdings ist der Korrelationskoeffizient mit $r^2 = 0,2438$; $p < 0,001$; $n = 33$) klein. Eine ähnliche Beziehung bestand zwischen den Variablen: Mittelwert der Sporenanzahl in den aus 60 Bienen zusammengesetzten Proben und dem Prozentsatz der infizierten Bienen. ($r^2 = 0,4557$; $p < 0,001$; $n = 33$), was den früheren Befunden widerspricht. In 18,52% der Proben ($N = 162$) wurden beide Parasiten, *V jacobsoni* und *N apis* nachgewiesen (Tabelle II). Unsere Ergebnisse zeigen, daß sich *N apis* und *V jacobsoni* unabhängig voneinander entwickeln und daß die Proben, die einen niedrigen, mittleren oder hohen Befall durch *V jacobsoni* aufwiesen zu 22,6% \pm 3,6% bzw 47,5% \pm 16,2% bzw 16,7% \pm 10,4% mit *N apis* infiziert waren. Es gibt demnach keinen signifikanten Unterschied ($F = 0,2817$; $p = 0,7567$) (Tabelle III). Allerdings wurde mit einem progressiven Anstieg in der Anzahl der Sporen pro Einzelbiene auch ein steigender Befallsgrad von *V jacobsoni* festgestellt ($5,9 \times 10^6$ Sporen / Biene bzw $9,1 \times 10^6$ Sporen / Biene bzw $13,8 \times 10^6$ Sporen / Biene), aber diese Unterschiede waren nicht signifikant ($F = 0,6053$; $p = 0,5531$) (Tabelle III).

Nosema apis, Infektionsgrad, *Varroa jacobsoni* Oud, Spanien

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