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Original article

Comparison of the responses of two races of honeybees to infection with *Nosema apis* Zander

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Abstract – Responses of New Zealand bees from Italian (*Apis mellifera ligustica*) and dark (*Apis mellifera mellifera*) races to infection with *Nosema apis* were compared. Newly emerged adults from five colonies of Italian bees and three colonies of dark bees were tagged, dosed individually with *N. apis* spores and placed in cages with undosed companion bees from the same colony. Dosed individuals were removed and examined at different times to measure progress of infection. At the end of the experiment, companion bees were examined to determine the spread of infection from dosed to undosed bees. There were no significant differences between the two races in either of these measures of response to *N. apis*. Bees from the different colonies were pooled according to race, and their responses (percentage infection and longevity of infected bees) to a range of doses of *N. apis* spores determined. These data also showed no significant differences due to race. © Inra/DIB/AGIB/Elsevier, Paris

***Apis mellifera ligustica* / *Apis mellifera mellifera* / nosema disease / spread of infection / dose response / tolerance**

1. INTRODUCTION

Nosema disease, caused by the microsporidium *Nosema apis* Zander, is found in adult honeybees (*Apis mellifera* L.) throughout the world, with a recent report listing its presence in 85 out of 90 countries surveyed [10]. While the biology of this microparasite and its effects on its host are well-understood in general terms [4], very little is known about whether bees have any genetically based variability in their susceptibility to *N. apis*, and nothing is known about genetic variability in the parasite itself. Breeding a nosema-resistant bee would be a desirable alternative to control-

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ling this disease with the antibiotic, fumagillin, or with labour-intensive hygienic measures [3]. In a study of queen bees from different North American locations, L'Arrivée [6] noted that some individuals could tolerate *N. apis* infection better than others and suggested that a line of bees with high tolerance to nosema disease could be developed. Studies of the effects of *N. apis* on the longevity of caged bees also suggest that such an approach would be feasible [12, 13, 16] and a recent comparison of ten colonies of Canadian and English bees has suggested that there may be genetic differences which influence the severity of *N. apis* infection in honeybees [15] as cited by Nelson [11]. However, our investigations of the responses of a range of different colonies, inbred lines and races of honeybees in New Zealand have failed thus far to demonstrate sufficient genetic variability to support such a control strategy [7–9]. This final study expands upon our previous examination of Italian and dark bees [9], with the inclusion of several colonies representing each race, and four different measures of susceptibility to *N. apis*. Since economically unacceptable levels of *N. apis* infection have not yet been defined, either in terms of individual bees (e.g. spores per bee) or colonies (e.g. percentage of infected bees), we have quantified the effects of this pathogen in the following ways: progress of infection in individual bees, spread of infection from dosed to undosed bees, and the responses of bees to a range of doses of *N. apis*, in terms of percentage of bees infected and the longevity of infected bees.

2. MATERIALS AND METHODS

A suspension of *N. apis* spores in water was prepared by crushing and filtering Italian bees, obtained from a naturally infected colony kept at Ponsonby, Auckland. The concentration of spores was determined by a haemacytometer count and the suspension kept at 4 °C. For bee dosing, suspensions of spores in 60 % (w/v) sucrose syrup were prepared from this by centrifugation and resuspension in a volume of syrup appropriate for the spore concentration required.

Honeybees from eight colonies, kept at a commercial bee breeder's apiary in the Far North region of New Zealand were used. Five of the colonies were headed by sister Italian queen bees (*Apis mellifera ligustica*) artificially inseminated with pooled semen (from about 100 Italian drones) and the other three colonies headed by dark sister queens (*Apis mellifera mellifera*) inseminated with pooled semen (between eight and ten dark drones). Frames containing capped brood from each colony were sent to the laboratory in Auckland as required.

To verify similarity to the progenitor subspecies, abdomen colour markings were noted and the cubital index [14] measured in 50 worker bees from each of the eight colonies.

Frames arriving in the laboratory were kept in paper bags in an incubator at 33 °C for up to 5 days to allow the bees to mature. For experiments, adult worker bees ready to emerge were taken from the frames by removing their cell cappings before they could ingest any wax. This was to ensure that they could not have become accidentally infected by ingesting *N. apis* spores contaminating the wax on these frames. The bees were then placed in a Petri dish for 1 h to ensure that they were hungry, marked on the thorax if necessary with a coloured, numbered queen bee tag, placed back in the dish for a few minutes, then dosed individually with *N. apis* spores. Only darker-coloured, mature bees were selected for dosing and each bee was force-fed with a 2-μL droplet of spore suspension in syrup containing a known number of spores. Bees that did not consume the entire droplet were discarded. Dosed bees were assigned to wooden cages with two mesh sides, measuring 9 × 8 × 6 cm (internal dimensions), and fitted with gravity feeders supplying water and 60 % (w/v) sucrose syrup and a small container of protein-based artificial food (sodium caseinate 0.12 parts, brewer's yeast 0.24 parts and sucrose 0.64 parts, mixed with water to a paste). These were then kept in darkness at 33 °C.

Two experiments were carried out. The first was designed to examine the progress of *N. apis* infection in bees from the eight different colonies and also its spread from these bees to a number of undosed bees accompanying them. The second experiment aimed at comparing the responses, in terms of bee longevity and percentage infection of Italian and dark bees to five different doses of *N. apis* spores.

For the first experiment, 15 bees from each of the eight colonies were tagged, dosed individually with 10^5 spores and then placed in cages containing 50 undosed, untagged bees taken from the same colony in the same manner. Three replicate cages and one control cage containing tagged bees 'dosed' with plain syrup were set up for each colony, i.e. 20 cages of Italian bees (5 colonies × (3 replicates + 1 control)) and 12 cages of dark bees (3 colonies × (3 replicates + 1 control)). The next day, the cages were checked for handling deaths and these bees removed, discarded and replaced with substitute bees from the appropriate colony dosed in the same manner. One tagged bee was then removed from each cage at 5, 12, 15, 18, 25 and 30 days after dosing. To do this, each cage was subjected to a flow of CO_2 until all the bees were anaesthetised and the tagged bee removed and placed at –20 °C for approx. 8 min. This was sufficient to kill the bee without freezing it solid. The midgut was dissected from each killed bee and stored frozen for a later spore count. All cages were inspected every 2 or 3 days and dead bees were noted, removed and stored whole at –20 °C for later microscopic examination for spores. After 30 days, all remaining bees were removed, killed by freezing and stored individually as above.

At the end of the experiment, the frozen midguts of the tagged bees were thawed and each was homogenised in 0.5 mL of water. Spores in these suspensions were then counted using a haemacytometer [1]. Where the spores were too numerous to count easily, an aliquot of the suspension was diluted with water and then counted. Sufficient spores for a reliable estimate of concentration (at least 100 spores) could usually be counted within 80 of the smallest haemacytometer squares. More squares were examined (up to the maximum of 400) if the original suspension was particularly dilute. Spores per bee were calculated in the following way: $= (x \times d \times 2 \times 10^6)/n$, where x = number of spores counted, d = dilution factor and n = number of smallest haemacytometer squares. The lowest number of spores able to be detected by this method is 5×10^5. In order to determine whether individual accompanying bees had become infected or not, a simplified method was used. The frozen cadavers of these untagged bees were thawed, homogenised individually in 0.5 mL of water, and a 20-mL aliquot examined for spores at a magnification of × 312 for 1 min. The lowest number of spores per bee that can be detected by this method is 2×10^3. For the purposes of this study, an 'infected bee' was defined as one in which any number of *N. apis* spores could be detected by either of the above methods.

Two sets of data were recorded from this experiment: mean spore loads of tagged bees removed at different time points from each cage, and the percentages of accompanying untagged bees that became infected after 30 days in these cages. Because of the skewed nature of the spore load data, these were log-transformed before analysis. Mean spore loads of bees from each race and over time were compared by analysis of variance (ANOVA). Undosed control bees in all categories had zero spore counts, except for one bee, taken at day 30 from dark colony 2, which must have been accidentally contaminated. The assumptions of ANOVA would not be met if the control bee zero data were included in the analysis and so it was considered more valid to separately calculate an LSD (0.05) for comparison of dosed and control mean spore loads. The percentage infection data obtained from the untagged bees were transformed by arcsin (square root (x)) before analysis of variance.

For the second experiment, bees from the five Italian colonies and the three dark colonies were pooled according to race in order to compare the responses of each race of bees to five different dosages of *N. apis* spores. Forty bees of each race, chosen at random from the different colonies, were individually dosed with 10, 100, 1 000, 10 000, or 100 000 spores each and placed together in cages. A control cage containing 40 bees 'dosed' with plain syrup was also set up. Every 2 or 3 days cages were checked for dead bees which were removed and stored frozen for later homogenisation and examination for spores (as described above) until all bees had died. Bees that died within 7 days of dosing were not examined. The entire experiment was replicated in full three times and partially for a 4th time (three treatments only, because of a shortage of bees). The replicate experiments were all conducted within a period of 21 days, i.e. 42 cages in total: 2 races × 6 treatments × 3 replicates = 36 cages plus 2 races × 3 treatments × 1 replicate = 6 cages. Percentages of infected bees were analysed as described above. Survival curves were plotted using data from each cage and compared using Mantel-Haenzel (log-rank) tests [5].

3. RESULTS AND DISCUSSION

The abdomen colour markings of the bees used in this study were as expected for

Italian and dark bees (see [14]). The mean cubital index for the Italian bees (2.50 ± 0.02; range: 1.75–3.57) was significantly higher than that of the dark bees (2.12 ± 0.02; range: 1.54–2.91) ($P < 0.0001$; ANOVA). These measurements fall within the ranges typical for the progenitor morphometric subspecies.

Figure 1 shows the development of *N. apis* infections in bees from each colony. The rapid increase in spore numbers to a relatively uniform maximum level at around 15 days post-emergence has previously been demonstrated by Fries [2], although in that study, the bees were dosed when they were 7 days old and each received only 6.25×10^3 spores. Fries showed that spore loads in infected bees reached a maximum level of around 5×10^7 spores per bee, regardless of the original dose, at about 2 weeks after dosing. Our results agree with this. Bees in the present study received a higher dose (10^5 spores) at an earlier age than those in Fries' study and reached their maximum loads (between 5×10^6 and 5×10^7 spores per bee) about 15 days from the time of dosing.

As the LSD shown in *figure 1* indicates, there were no significant differences among the mean spore counts recorded at each time point ($P \leq 0.05$) and no clear trends suggesting that the course of development of the disease differs in the Italian and dark bees tested. This result agrees with unpublished data obtained in an identical experiment conducted in our laboratory during the previous season, in which bees from five Italian colonies and five dark colonies were dosed and examined 5, 12, 19, 26 and 33 days after dosing. ANOVA of these data showed no significant differences between the two races except for bees examined on day 12, when the dark bees had significantly lower mean spore loads than the Italian bees ($P \leq 0.05$).

Table I shows the percentages of undosed 'companion' bees that became infected when caged with the tagged bees in experiment 1. Some bees caged with undosed control bees became infected, but at a significantly lower rate than those caged with dosed bees (ANOVA, $P < 0.001$). The most likely explanation for these infections is that they resulted from cross-cage contamination, all cages in this experiment being kept in close proximity within a single, fan-assisted incubator. Although 70.8–79 % of dark bees caged with dosed bees became infected, compared with 50.7–73.6 % of Italian bees, there was no significant difference between the two races (ANOVA, $P = 0.083$). This is similar to the findings of an earlier study in which the rate of spread of *N. apis* infection did not differ among caged bees taken from colonies originating from several locations throughout New Zealand [8].

The results of experiment 2 are shown in *tables II* and *III*. *Table II* lists the median survival times of Italian and dark bees that became infected after receiving five different doses of *N. apis* spores. Two sets of control data were used in the analysis. One set consisted of survival times of uninfected bees that had been given a 'zero dose' (plain sugar syrup) only and the other set consisted of these data combined with the survival times of any uninfected bees from the other cages ('new controls' in *table II*). There was no discernible relationship between bee longevity and spore dose for either race. While poor survival of control bees and apparently enhanced survival of some bees infected with ten spores contributed to this lack of a dose-dependent response, perhaps it is not surprising given the evidence that *N. apis* infections attain a uniform maximum spore load regardless of the dose of spores that the bee ingests (experiment 1 results and Fries [2]). χ^2 tests to compare the responses of Italian and dark bees to each treatment showed that there were no significant differences attributable to race in the survival of uninfected bees in either control group or of infected bees that had received 100 spore doses. There were significant differences due to race for the other

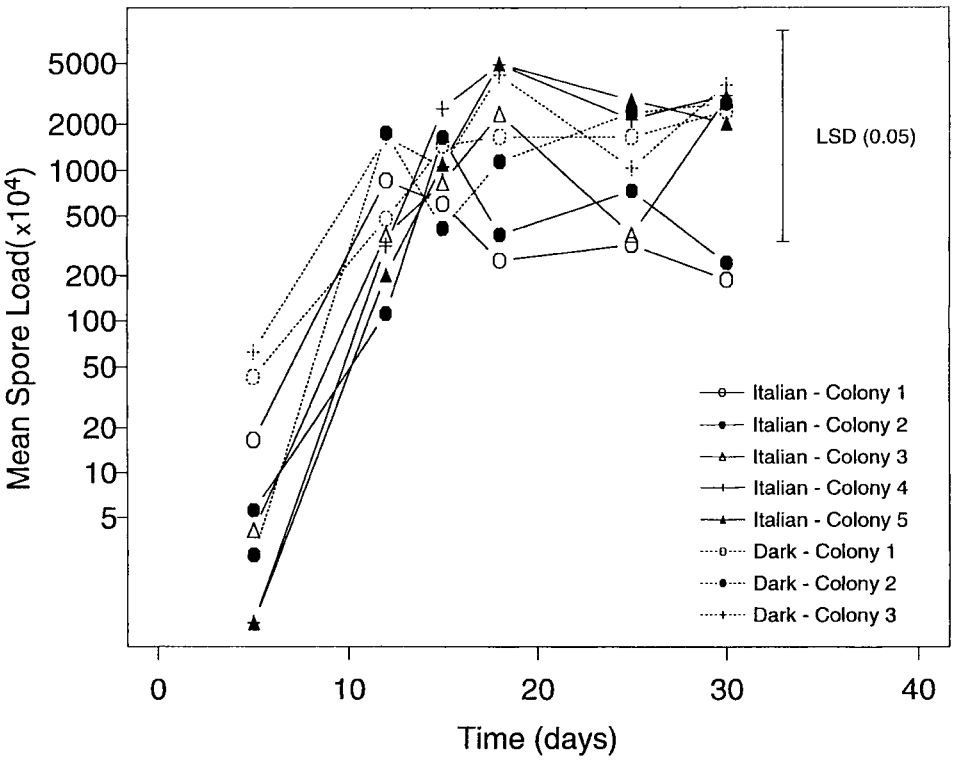

Figure 1. Mean loads of *Nosema apis* spores carried by bees taken from five Italian race colonies and three dark race colonies and dissected at 5, 12, 15, 18, 25 or 30 days after dosing. Spore loads are plotted on a log scale and the bar indicates the least significant difference at the 5 % level.

Table I. Percentages of undosed companion bees infected with *Nosema apis* when caged with individually dosed or control bees of the same colony and race.

Caged with:	Colony number					
	1	2	3	4	5	All pooled
Italian dosed bees (three replicates combined)	70.1 (144)	57.8 (139)	73.6 (144)	64.4 (126)	50.7 (144)	63.3 (697)
Dark dosed bees (three replicates combined)	70.8 (144)	78.5 (144)	79.0 (139)	– –	– –	76.1 (427)
Italian control bees (one replicate)	12.5 (48)	2.1 (47)	6.2 (48)	2.1 (48)	0 (48)	4.6 (239)
Dark control bees (one replicate)	0 (48)	4.2 (48)	2.1 (48)	– –	– –	2.1 (144)

Cross-cage contamination probably accounts for the infections in bees caged with undosed control bees and infection levels in these bees are significantly lower than for those caged with dosed bees. There are no significant differences due to race (ANOVA, $P = 0.083$). Total numbers of bees checked are given in parentheses.

Table II. Median survival times (days) of bees infected after receiving a range of doses of *Nosema apis* spores. Survival data are also given for control bees receiving a zero dose of spores and 'new controls' includes data from these bees and all bees that were dosed but did not become infected.

Dose (spores per bee)	Race		
	Italian	Dark	χ^2 P
0	18	20	0.943
New controls	18	21	0.427
10	54	18	0.027
100	39	25	0.320
1 000	22	25	0.049
10 000	22	26	0.025
100 000	23	18	< 0.001

Probability values are for χ^2 tests to compare the two races of bees for each treatment.

Table III. Percentages of bees infected after receiving a range of doses of *Nosema apis* spores, including control bees receiving a zero dose of spores.

Dose (spores per bee)	Race			
	Italian		Dark	
0	0.6 a	(117)	0 a	(127)
10	2.4 a	(118)	5.8 a	(116)
100	39.1 b	(101)	54.1 b	(114)
1 000	49.9 b	(141)	79.7 c	(137)
10 000	75.4 c	(113)	92.5 cd	(105)
100 000	97.4 d	(151)	94.9 cd	(126)

Values within columns without a letter in common differ at $P \leq 0.05$ (ANOVA). Total numbers of bees checked are given in parentheses.

treatments, but there was no clear pattern to these. Italian bees had better survival than dark bees after being infected with 10 or 100 000 spores, but the reverse applied to bees dosed with 1 000 or 10 000 spores.

Table III lists the percentages of bees that became infected after ingesting various doses of *N. apis* spores. Both Italian and dark bees responded in a dose-dependent way, with larger doses of spores resulting in significantly greater proportions of infected bees ($P \leq 0.05$). At every dose level, except the highest (100 000 spores), a greater percentage of dark bees became infected than Italian bees. However, this difference was significant only in the case of bees dosed with 1 000 spores ($P \leq 0.05$). Whether this difference in susceptibility had a physiological or behavioural basis was not clear.

In conclusion, these and earlier results [7–9] suggest that there are little or no useful, genetically based differences in the susceptibility or the responses of different races of New Zealand bees to infection with *N. apis*.

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Résumé – Comparaison de la réaction de deux races d'abeilles à l'infection par *Nosema apis* Zander. Les réactions à l'infection par le protozoaire *Nosema apis* Zander d'abeilles des races italienne (*Apis mellifera ligustica*) et noire (*A. m. mellifera*) ont été comparées dans deux expériences séparées. Dans la première, 15 abeilles fraîchement écloses de chacune des cinq colonies d'italiennes et de trois colonies d'abeilles noires (désignées ci-après par « race ») ont été marquées, infectées individuellement avec une dose de 10^5 spores de *N. apis* et encagées avec 50 abeilles accompagnatrices de la même colonie mais non traitées. Une abeille infectée et marquée a été prélevée et examinée à 5, 12, 15, 18, 25 et 30 j. après l'infection pour en mesurer la progression. Les spores ont été dénombrées à l'hémocytomètre. À la fin de l'expérience, on a également examiné les compagnes afin de déterminer le pourcentage de ces abeilles qui avaient contracté l'infection. Ceci a donné une mesure du taux de propagation de l'infection entre abeilles de la même cage infectées et non infectées. Il n'y a pas eu de différence significative entre les deux races (*figure 1, tableau I*). Pour la 2e expérience, les abeilles de différentes colonies ont été regroupées en fonction de la race et leur réponse à une série de doses de spores (10, 100, 1000, 10 000 ou 100 000 spores de *N. apis*) a été exprimée en termes de longévité des abeilles infectées (*tableau II*) et de taux d'infection (*tableau III*). Ces données ont elles aussi montré qu'il n'y a pas de différence significative entre les deux races. Les preuves sont donc insuffisantes pour dire qu'il existe en Nouvelle-Zélande des différences utiles d'origine génétique dans la sensibilité ou la réaction de différentes races d'abeilles à l'infection par *N. apis*. © Inra/DIB/AGIB/Elsevier, Paris

***Apis mellifera ligustica* / *Apis mellifera mellifera* /nosémose / propagation / sensibilité résistance / race**

Zusammenfassung – Vergleich von Reaktionen der beiden Honigbienenrassen von Neuseeland auf eine Infektion mit *Nosema apis* Zander. Die Reaktionen von entweder der Italiener Biene (*Apis mellifera ligustica)* oder der dunklen Biene (*Apis mellifera mellifera)* entstammenden Honigbienen auf eine Infektion mit *Nosema apis* wurde in 2 getrennten Versuchen miteinander verglichen. Im ersten Versuch wurden jeweils 15 frisch geschlüpfte Bienen aus jedem der 5 Völker mit italienischen und der 3 Völker mit dunklen Bienen (die im folgenden als Rassen bezeichnet werden) markiert, individuell mit einer Dosis von 10^5 *N. apis* Sporen infiziert und in Käfige mit 50 unbehandelten Begleitbienen desselben Volkes gesetzt. Jeweils eine behandelte Biene wurde 5, 12, 15, 18, 25 oder 30 Tage nach der Sporengabe untersucht, um den Befallsgrad zu bestimmen. Die Zahl der Sporen in jeder Biene wurde mit Hilfe einer Blutzählkammer bestimmt. Am Ende des Versuchs wurden auch die Begleitbienen untersucht, um den Prozentsatz der Ansteckung der Bienen zu bestimmen. Das gibt ein Maß für die Rate einer Verbreitung der Infektion von mit Sporen versehenen zu unbehandelten Bienen im selben Käfig. Es ergab sich in keinem Fall ein signifikanter Unterschied zwischen den beiden Bienenrassen in ihrer Reaktion auf *N. apis* (*Abbildung 1*, *Tabelle I*).
Im 2. Versuch wurden Bienen aus verschiedenen Völkern gepoolt und auf Grund ihrer Rasse wurde ihre Reaktion auf den Infektionsgrad (*Tabelle III*) und die Lebensdauer der infizierten Bienen (*Tabelle II*) mit 10, 100, 1 000, 10 000 oder 100 000 *N. apis*

Sporen pro Biene bestimmt. Auch diese Daten ergaben keinen signifikanten Unterschied zwischen den Rassen. Demnach gibt es praktisch keinen Hinweis auf nutzbare genetische Unterschiede in der Empfindlichkeit oder Reaktion der verschiedenen Rassen der Bienen in Neuseeland auf Infektionen mit *Nosema apis*.

Honigbienen / Nosemabefall / dunkle Biene / Italienerbiene / Verlauf und Verbreitung der Infektion / Dosisreaktion

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