General Enquiries on the form should be made to:
Defra, Procurements and Commercial Function (Evidence Procurement Team)
E-mail: research.competitions@defra.gsi.gov.uk

Evidence Project Final Report

- **Note**
  In line with the Freedom of Information Act 2000, Defra aims to place the results of its completed research projects in the public domain wherever possible.
  The Evidence Project Final Report is designed to capture the information on the results and outputs of Defra-funded research in a format that is easily publishable through the Defra website
  An Evidence Project Final Report must be completed for all projects.
- This form is in Word format and the boxes may be expanded, as appropriate.
- **ACCESS TO INFORMATION**
  The information collected on this form will be stored electronically and may be sent to any part of Defra, or to individual researchers or organisations outside Defra for the purposes of reviewing the project. Defra may also disclose the information to any outside organisation acting as an agent authorised by Defra to process final research reports on its behalf. Defra intends to publish this form on its website, unless there are strong reasons not to, which fully comply with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.
  Defra may be required to release information, including personal data and commercial information, on request under the Environmental Information Regulations or the Freedom of Information Act 2000. However, Defra will not permit any unwarranted breach of confidentiality or act in contravention of its obligations under the Data Protection Act 1998. Defra or its appointed agents may use the name, address or other details on your form to contact you in connection with occasional customer research aimed at improving the processes through which Defra works with its contractors.

Project identification

1. Defra Project code: VM0139

2. Project title:
   Evaluating the field efficacy of the fungicide Enilconazole (Imazalil) as a potential alternative chemical treatment for the control of *Nosema* spp

3. Contractor organisation(s):
   National Bee Unit
   The Food and Environment Research Agency
   Sand Hutton
   York
   YO41 1LZ
   Tel: 01904 462503
   Fax 01904 462240

4. Total Defra project costs (agreed fixed price): £ 45,529

5. Project: start date 01/09/2009
   end date 31/07/2011

6. It is Defra's intention to publish this form.

   Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

   (a) When preparing Evidence Project Final Reports contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.

   Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the Evidence Project Final Report can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.

   In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

   (b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Background

There is a need to protect UK honey bee stocks from the threats posed by the microsporidial fungal pathogens, *Nosema apis* and *N. ceranae*. The antibiotic Fumidil-B (Fumagillin/ bicyclohexylammonium, marketed by Ceva Ltd.) is proven to be effective in controlling Nosemosis caused by both *N. apis* and Asian variant *N. ceranae,* but this product is no longer licensed for the treatment of *Nosema* diseases in the majority of EU member states. Although Fumidil-B is currently still available for use in the UK as the sole approved chemical treatment for *Nosema* infection, it is possible that this product will be withdrawn from sale in the UK in the near future following reports of genotoxic effects of fumagillin in cytogenetic tests both *in-vitro* and *in-vivo* (Stanimirović 2006). This work has been reviewed by the Veterinary Medicines Directorate (VMD) and it was recommended that an independent opinion should be sought on interpretation of the results, to establish if there is a potential risk to consumer safety. A suitable alternative treatment for this honey bee disease is therefore desirable.

Objectives and Methods

The objective of this study was to:

- evaluate the field efficacy of the fungicide Enilconazole sulphate (trade name Imazalil sulphate) as a potential alternative chemical treatment for the control of *Nosema* spp when applied to naturally infected honey bee colonies.

Findings

No detrimental effects on colony performance or acute mortality of adult bees were observed throughout the test period following treatment with the test item. In terms of *Nosema* control results showed no consistent evidence to suggest that there were any treatment related effects on levels of infection when treating colonies of *Apis mellifera* naturally infected with *Nosema spp*.

There was an overall reduction in the proportion of colonies testing positive for *Nosema* infections, however, this occurred similarly across all groups including the controls. This decline over time was probably due the seasonal nature of the pathogen.

Whilst *Nosema* spp. infections on their own may not be responsible for large honey bee colony losses in the UK when coupled with the other potential stressors found to impact upon honey bee colony performance such as the parasitic mite *Varroa destructor* and associated virus infections, other pest and pathogens and also general management practices there has been cause for concern that *Nosema* infections may have an impact. Following on from this, with the probable withdrawal of the last remaining available Nosema treatment Fumidil-B the need for a potential new treatment remains high on the agenda.

Future options

Large scale field testing is obviously needed for the development of new veterinary medicines for use within apiculture; however, the large scale means that there are substantial financial costs involved at this level of testing. Therefore a more cost effective and efficient method of early stage screening allowing for a more informed selection of potential candidate treatments before moving to the large scale field is very appealing. Within the last two years there have been significant advances in the development of *in vitro* honey bee techniques. A new and more reliable *in vitro* assay has been developed which now makes it possible to carry out laboratory infection of individual bees with *Nosema* spp at known doses (spore numbers) and to monitor the effects of various treatments on infection levels at known times post infection/treatment. The benefits of this system mean that it would be possible to screen a wide range of potential active ingredients or products within the confines of the laboratory at relatively low cost compared to the running of larger field studies. This is something which could be could be developed further and exploited in the future.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Exchange).

1. Background

Two species of a highly specialised microsporidial fungal pathogen, *Nosema* spp., have been identified in honey bees: *N. apis* and the Asian variant *N. ceranae.* Both species invade the lining of the mid-gut of adult bees where they multiply rapidly, packing the cells with spores. When the host cell ruptures, spores are shed into the gut where they accumulate in masses, to be later excreted by the bees. If spores from the excreta are picked up and swallowed by another bee, they can germinate and once more become active, starting another round of infection and multiplication. *N. apis* has traditionally been found in the UK and is responsible for 'Spring Dwindling' and loss of colonies. *N. ceranae* was identified in the UK more recently (Budge 2008), where its impact on beekeeping has yet to be determined. There have been particular concerns over increased losses of honey bee colonies in the USA, and there is a general upward trend of bee loss across Europe (Kluser *et al*. 2010, Van Englestorp *et al* 2009, Potts *et al* 2010). Many of the losses recorded in Spain and Portugal have been attributed directly to infections of *N. ceranae* (Higes *et al* 2008 and 2009). Although similar declines observed throughout the rest of Europe may reflect poor husbandry and/or inadequate *Varroa* control, *N. ceranae* may act as additional stressor resulting in the loss or decline of already weakened or damaged colonies.

Fumidil-B (Fumagillin/bicyclohexylammonium, marketed by Ceva Ltd.) has long been used for the treatment of *Nosema* diseases in honey bees, and has demonstrable efficacy against both *Nosema* species pathogenic to honey bees. Whilst Fumidil-B is no longer licensed for the treatment of *Nosema* diseases in the majority of EU member states, it is currently still available for use in the UK as the sole approved chemical treatment for infections of *Nosema*, however, it is possible that this product will be withdrawn from sale in the UK in the near future it is possible that this product will be withdrawn from sale in the UK in the near future following reports of genotoxic effects of fumagillin in cytogenetic tests both *in-vitro* and *in-vivo* (Stanimirović 2006). These reports have been reviewed by Veterinary Medicines Directorate (VMD) and it was recommended that an independent opinion should be sought on interpretation of the results, to establish if there is potential risk to consumer safety. A suitable alternative treatment for this honey bee disease is therefore desirable.

Aims and Objectives The original proposal of this study was to evaluate the field efficacy of the fungicide Enilconazole (trade name Imazalil) as a potential alternative chemical treatment for the control of *Nosema* spp infections in honey bees (*Apis mellifera*). This substance was initially selected as a possible candidate treatment as it had previously been used in honey bee colonies to control another fungal pathogen of honey bees – chalk brood (*Ascophaera apis*) and also because of its low mammalian toxicity (Sulimanovic 1987). After consultation with technical staff at Ceva Ltd (producers of the active ingredient) it was decided that the sulphate form of the compound (Enilconazole sulphate/Imazalil sulphate) would be a more suitable choice as its activity is similar to that of Enilconazole, but it has a higher solubility in water and would thus be easier to administer to colonies in aqueous sucrose solution.

This was carried out by selecting colonies of honey bees which had been identified to be infected with *Nosema* spp. Three groups (n=20) of infected colonies were used for the study, two test item treatment groups using two dose rates, the third group remained untreated as a control. The colonies within the treatment groups were dosed with a solution of enilconazole sulphate, made up in sucrose solution and fed to the bees in a rapid tray feeder. Control colonies remained undosed but were fed 50% sugar syrup. Samples of adult bees were collected from all colonies throughout the study; one pre dosing and then a further 8 time-points. All samples were screened for the presence of *Nosema* spp. using both traditional microscopic techniques and molecular diagnostic methods (Real-time PCR). Nosema levels were assessed both qualitatively and quantitatively. The levels of infection in treated and untreated colonies were measured and the interactions between time, treatment, infection levels pre/post treatment were investigated to allow the efficacy of enilconazole sulphate as a fungicidal treatment for *Nosema* spp in honey bee colonies to be assessed.

2. Materials and Methods

2.1 Test Item Details

Test Item

NBU Number:	GC500
Test item name:	Imazalil Sulphate
a.i.:	Imazalil Sulphate
Purity:	75% w/w
Product category:	Fungicide
CAS registration no.:	58594-72-2
Batch No.:	SC027180EXA007
Appearance:	Off White/Cream Crystalline Powder
Received:	On receipt at Fera, the test item was booked in according to SOPs and stored according to the data sheets provided by the producer.
Storage conditions:	Room temperature
Stability:	Expires 25/12/2012
Safety:	The test item was handled with routine procedures to maintain both human and environmental safety.

2.2 Test System

2.2.1Test organism

Sixty field colonies of the European honey bee (*Apis mellifera* L.) naturally infected with *Nosema* spp were selected from the Fera, National Bee Unit managed apiaries. All of the NBUs 150 colonies were pre-screened for the presence of both *Nosema* spp using molecular diagnostic methods as outlined below. The sixty infected study colonies were selected from colonies identified by the screening process. Where possible colonies chosen were headed by queens of a similar age and origin. Selected colonies had a low incidence of minor brood disease (chalk brood, sac brood and bald brood) and the absence of clinical symptoms of American foul brood (causative agent *Paenibacillus larvae*) and European foul brood (causative agent *Melissococcus plutonius*). The test colonies were queen right with a normal brood pattern. Each colony was housed in a wooden, double brood chambered Smith hive. Each hive was uniquely numbered and labelled with the study number and treatment group.

Any honey harvested from colonies within the treatment groups within the study was disposed of by incineration. All of the colonies from the treatment groups were shook swarmed onto new foundation after completion of the practical phase of the study.

2.2.2 Study Sites

The apiaries selected to site the colonies for the treatment and monitoring during the study were National Bee Unit Apiaries, sited at a sufficiently large sufficient distance to each other to prevent any cross contamination by drifting/robbing.

2.3 Experimental Procedures

2.3.1 Experimental groups

The study consisted of two test groups and a control group, each group comprising 20 colonies;
Group 1 (TG1): Test item dosed colonies 500mg a.i. per colony.
Group 2 (TG2): Test item dosed colonies 200 mg a.i. per colony.
Group 3 (Controls): Un-dosed, (sugar syrup fed colonies).

2.3.2 Apiary set-up

Three NBU apiary sites selected for the study; Block 25 (control) Kissthorne (TG1) and Mildred's Farm (TG2) Twenty test colonies were placed at each of the test apiaries at least two days before the day of treatment to avoid disturbance to the colonies. The apiary sites were all located at least 3.5 km apart to exclude the possibility of drifting between treatments.

2.3.3 Colony development assessments

The colony development was measured by carrying out colony full assessments. At each assessment point the number of adult bees was assessed by the area of the comb covered by bees. This was expressed as total numbers of occupied frames of bees (British Standard Frames 14" x 8.5" or 355mm x 216mm). Each frame was then removed and visually examined by the assessor, and the percentage coverage of total brood area (sealed brood, and unsealed brood) on each frame was recorded as percentage coverage per frame. To maintain continuity during the assessments the same team carried out the colony assessments. Colony assessments were carried out once prior to treatment, once approximately one month after treatment and a final assessment carried out in the spring following treatment.

2.3.4 Treatments

TG1 colonies were dosed with 500 mg a.i. imazalil sulphate per colony made up in 10 litres 50% w/v aqueous sucrose solution. TG2 colonies were dosed with 200 mg a.i. test item/colony made up in 10 litres 50% w/v sucrose solution. The 20 control colonies were established in parallel. The reason for the staggered dosing was to allow for evaluation of any possible acute toxic effects upon the colonies – if there were any detrimental effects then the study could have been terminated. Test feed and control feed solutions were administered to each colony by using an Ashforth type rapid tray feeder.

2.3.5 Sample collection

Adult bee samples: At designated sample points (Pre-treatment, 1, 2, 4, 8 and 12 weeks post treatment and at the end of the beekeeping season) a sample of approximately 100 adult worker bees was collected from each treatment group hive for disease analysis. The original plan stated that this would be carried out at 8 time points post dosing, this was an error, as the 3-4 day post treatment time point should not have been included within the adult sampling programme. The samples collected were flying bees taken where possible from the supers to ensure that nurse bees were not sampled (these are young bees and are less likely to carry *Nosema* infections).The bees were collected into tubes of 70% ethanol, labelled with hive number, treatment group, the study number and dated and initialled. The samples were returned to the laboratory and stored at -20°C. The samples were then sub-sampled, thirty adult workers were removed from each sample and placed into labelled extraction bottles This sample size provided a 95% probability of pathogen detection in colonies containing at least 10% prevalence. Sub-samples were stored at -20°C until required. Remaining material was disposed of by incineration at the end of the study. Samples were also collected from the control colonies at the same time intervals.

Honey/nectar samples: On the day of treatment a honey sample (minimum 100g) nectar/honey was taken from each colony. Honey/nectar was then sampled 4 days post treatment and 1, 2, 4, 8 and 12 weeks after treatment; a final additional sample was taken at the end of the beekeeping season. The samples were taken from four points within the hive: two from the brood chamber and two from the super combs. Each sample consisted of a section of comb cut out, which was placed in a labelled plastic bag. In all cases, the location from which the samples were taken was recorded. These were bulked from super sources and brood chamber sources, respectively, where there were multiple supers a bulked sample was collected for each one. On return to the laboratory the samples were

stored at -20°C until required. The honey was extracted by filtering the comb sample through cloth into a clean labelled container. All honey/nectar samples were stored at -20°C for possible residue analysis at a later date.

2.3.6 *Nosema* diagnosis

Qualitative Analysis

i) Molecular diagnosis: DNA was extracted from each sub-sample. This was carried out using an automated magnetic particle extraction system using established protocols. Using a Kingfisher 96 in conjunction with a Wizard® Magnetic DNA Purification System (Promega, FF3751) using manufacturer's protocols. The quality of this extract was assessed by the use of an 'internal housekeeping gene' known to be present in samples of honey bees (EF1). The pathogens were screened for by performing a specific one step real-time RT-PCR (TaqMan® real-time PCR). Each sample was tested in duplicate and analysed using sequence detection software (Applied Biosystems). This software provided a numerical value in the form of a cycle threshold value known as a Ct value. These values were used to qualitatively score the samples. Ct values of < 40 were defined as positive.

ii) Microscopic diagnosis: A sub-sample of the suspension used for DNA extraction was returned to the NBU for microscopic diagnosis, based on methods described in the OIE Terrestrial Manual. It would have been possible for a trained technician to distinguish between *N. apis* and *N. ceranae, but* for the purpose of this study a simple positive/negative diagnosis was given for *Nosema* spp. ie the presence or absence of spores.

Quantitative Analysis

iii) Molecular Diagnosis: Standard curve dilutions of both *N. apis* and *N. ceranae* spores were prepared by performing 1:10 serial dilution of; $1x10^6$ spores/ml through 5 levels for *N. apis* and $1x10^7$ spores/ml through 6 levels for *N.* ceranae DNA extractions were performed as previously described and TaqMan® analysis run. The amount of each Nosema species was estimated in experimental samples by comparing against the standard curve.

iv) Microscopic diagnosis: The concentration, of spores per sample (expressed as spores/ml) was calculated for any sample which showed visible signs of infection (ie presence of *Nosema* spores). The spore counts were performed using disposable counting chambers (FastRead 102).

2.4 Statistical Analysis

Qualitative data from visual spore assessments and real-time PCR were analysed using a Generalized Linear Model (GLM) with a Bernoulli error structure and a logit link function and the effects of time, treatment and pre/post treatment application levels were investigated, together with their interaction.

Quantitative data (spore counts), were analysed with repeated measures (due to the additional available information (counts instead of presence/absence), using a mixed model to account for any possible autocorrelation between consecutive observations. Further, because they were very skewed, the data were log-transformed prior to being analysed. Again the effects of time, location and pre/post treatment application levels were investigated, together with their interaction.

All statistics were performed using Genstat 13.1

3. Results

3.1.1 Apiary Set-up

The three apiary sites were successfully established, the *Nosema* positive identified test colonies were moved onto site at least two days prior to the treatment. The majority (42) of study colonies had dual infections of both *N. apis and N. ceranae,* 17 had single infections of *N. ceranae* and 1 colony only had a single infection of *N. apis* only. Table 1 below shows the distribution of these colonies across the treatment groups.

Table 1. Distribution of colonies across treatment groups by *Nosema spp.* present

	Pre-Treatment *Nosema* spp. Infections Identified		
Treatment Group	***N. apis + N. ceranae***	***N. ceranae***	***N. apis***
Control (Block 25)	**13**	**7**	**0**
TG1 (Kissthorne)	**13**	**6**	**1**
TG2 (Mildred's Farm)	**16**	**4**	**0**

3.1.2 Treatment

TG1 colonies (500 mg a.i. imazalil sulphate per colony made up in 10 litres 50% w/v aqueous sucrose solution) and the control group colonies were dosed on 10/05/2010. TG2 (200 mg a.i. imazalil sulphate per colony made up in 10 litres 50% w/v aqueous sucrose) solution were treated one week later on the 17/05/2010.

3.1.3 Colony Assessments

At the initial pre-treatment assessment all colonies were queen right with egg laying queens with the exception of a single colony within the control group which had a virgin queen present. All colonies were of an acceptable size for the time of season, the smallest colony had 6.5 frames of bees and the largest had 30 frames of bees. At the second colony assessment point, 1 month post treatment all colonies within all treatment groups were queen right with laying queens, all colonies were increasing in size as would be expected with good brood development and colony expansion. There appeared to be no visible negative effects of treatment upon the colonies. No abnormal behaviour was observed or abnormal numbers of dead bees seen at the hive entrance of any test colonies. A single colony was lost TG1 during the autumn, it is thought that this was due to robbing by other honey bees. Following over-wintering at the final colony assessment point a total of 7 colonies had been lost out of the initial 60 colonies, 1 in the control group and 3 each within the two treatment groups. At the final assessment the remaining colonies within the control group and TG1 were all queen right with laying queens and colony size ranged from 10-20 frames of bees in the control group and 3-30 frames of bees per colony in TG1. However the colonies within TG2 were in a much poorer condition, there were 3 colonies with failing queens and two with virgin queens and the mean colony size was smaller at 9.8 frames per colony (ranging from 1-20 frames/colony). It should be noted that in the autumn of 2010 that the colonies at this apiary location were subject to severe robbing by wasps. No such problems were experienced at the other two test apiaries. The results for the colony assessments (i.e. colony size and brood nest size) pre and post treatment can be seen below in table 2.

Table 2. Colony Performance Indicators - Colony Assessment Data

Treatment Group	Pre-Treatment		Colony Assessment Point 1 Month Post Treatment		Post Winter	
	Mean number of frames of bees per colony (± SD)	Mean number of frames of brood per colony (± SD)	Mean number of frames of bees per colony (± SD)	Mean number of frames of brood per colony (± SD)	Mean number of frames of bees per colony (± SD)	Mean number of frames of brood per colony (± SD)
Control	16.8 (6.20)	7.00 (2.49)	21.2 (6.49)	6.9 (1.70)	16.8* (3.5)	6.03* (1.59)
TG1	13.8 (5.3)	6.13 (2.91)	18.1 (6.15)	6.1 (2.40)	12.9** (8.5)	5.08** (2.14)
TG2	18.5 (6.9)	6.78 (2.60)	22.5 (7.9)	6.52 (1.40)	9.8** (5.9)	2.8** (2.62)

* One Colony lost over Winter (n=19)

** Two Colonies lost over winter (n=17)

3.1.4 Sampling

Adult bee samples and honey/nectar samples were collected on the following dates.

Table 3. Sampling Points

	Sampling Date		
Time Point (sample number)	Control Group	Treatment Group 1 (500mg/hive)	Treatment Group 2 (200mg/hive)
Pre-Dosing (1)	10/05/2010	11/05/2010	17/05/2010
Day 3-4*	14/05/2010	14/05/2010	21/05/2010
Week 1 (2)	17/05/2010	17/05/2010	26/05/2010
Week 2 (3)	25/05/2010	24/05/2010	02/06/2010
Week 4 (4)	09/06/2010	09/06/2010	16/06/2010
Week 8 (5)	08/07/2010	07/07/2010	13/07/2010
Week 12 (6)	02/08/2010	03/08/2010	11/08/2010
End of Season (7)	20/09/2010	27/09/2010	23/09/2010
* Honey Sample Only			

3.1.5 Nosema Diagnosis

Qualitative Analysis

i)<u>Molecular diagnosis:</u>

a) Nosema apis

An initial increase in *N. apis* prevalence was followed by a general decrease over the sampling period (Figure 1). Regression analysis showed that when comparing the proportion of colonies which tested positive for *N. apis* there was no overall difference between pre and post treatment levels (not taking time post treatment into account) (p=0.81), nor was there any overall difference seen in the proportions of positive colonies between treatment (p=0.40). However, there was evidence of an effect of time post-treatment (ie there is a significant change in the proportion of positive colonies over time) (p<0.001) and there was also evidence that this change in positives is different between treatments (p=0.02).

b) Nosema ceranae

All Control and TG1 groups tested positive for *N. ceranae* pre-treatment, levels of infection were also high at 95 % in TG2. Levels within TG1 and the controls dropped through time, there was a less clearly defined change in the numbers showing positive for *N. ceranae* within TG2 with a drop in only 10% over the last two time points (Figure 2). Regression analysis showed there was some evidence of an overall difference between pre and post treatment levels (not looking at time after treatment) (p=0.04) and there was some statistical evidence of an overall difference in the proportion of samples testing positive for *N. ceranae* between treatments (p<0.001). As with *N. apis* there was evidence that the change in the proportion of samples over time post treatment was statistically significant as a whole (p<0.001), however, there was no statistical difference seen in that change between the treatment groups, (p=0.20).

<u>ii) Microscopic diagnosis</u>

As with the qualitative molecular diagnosis for *N. apis* there was an initial increase in the proportion of colonies seen testing positive followed by a general decline (Figure 3). Statistical analysis showed that there was evidence of an overall difference between pre and post treatment (ie the overall proportion of colonies testing positive for *Nosema* spp across all treatments after treatment was significantly different to the overall proportion pre-treatment) (p=0.004). However, there was no evidence to

suggest the overall proportion of positives differed between treatment groups (p=0.08). As observed when looking at the TaqMan® qualitative data for both *N. apis* and *N. ceranae* there was evidence of an effect of time post-treatment (ie the proportion of positive colonies changed over time post treatment) (p<0.001) but there was no evidence of any treatment related effects as there were no differences observed in the post-treatment changes between treatment groups. (p=0.16).

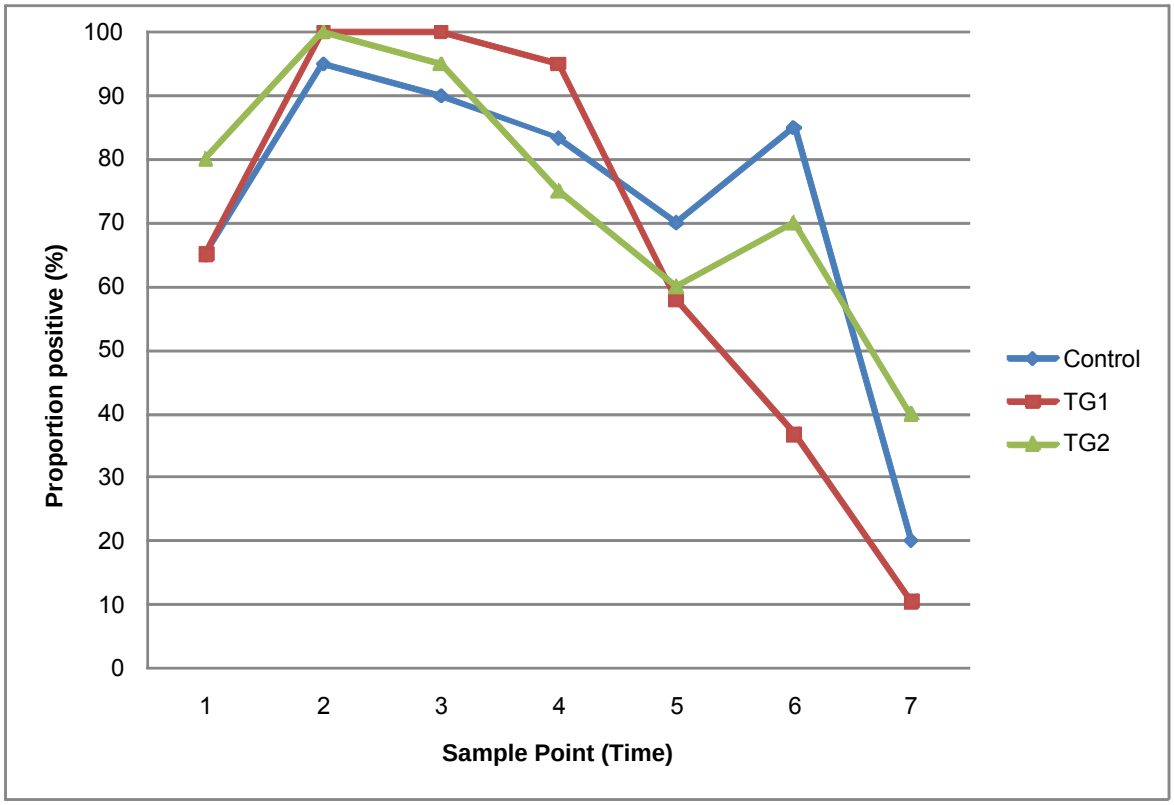

Figure 1. Proportion of colonies testing positive (%) over time for *Nosema apis* using TaqMan® PCR

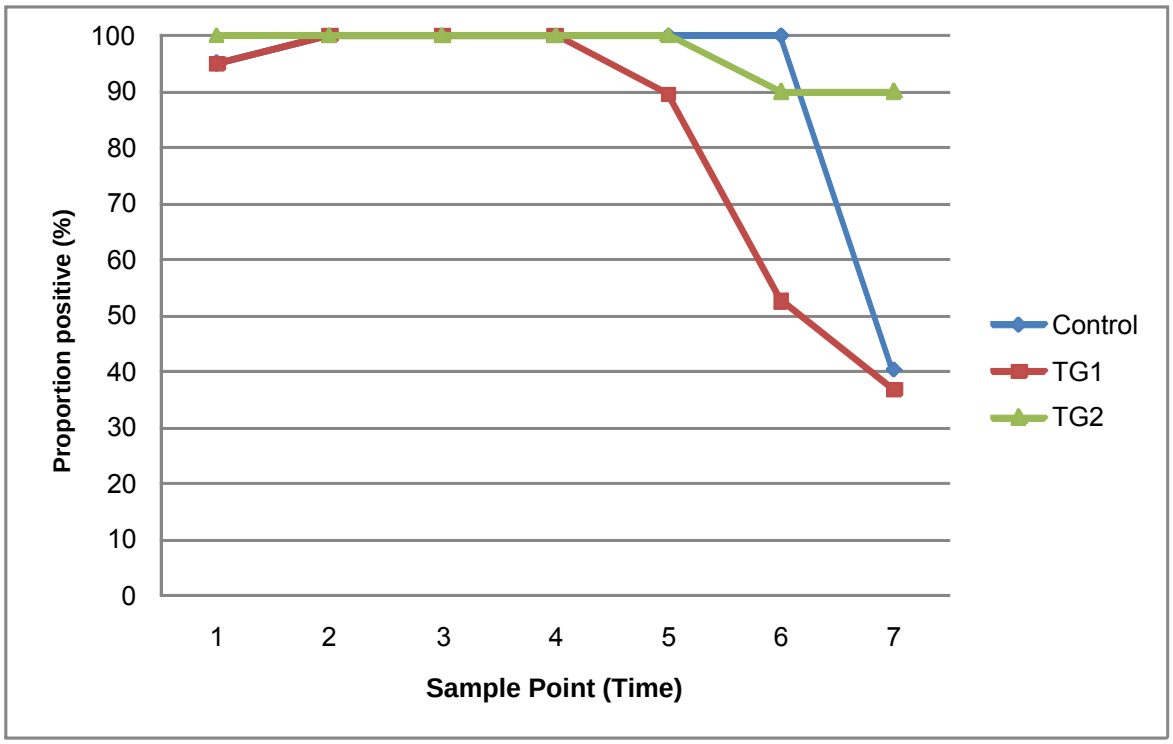

Figure 2. Proportion of colonies testing positive (%) over time for *Nosema ceranae* using TaqMan® PCR

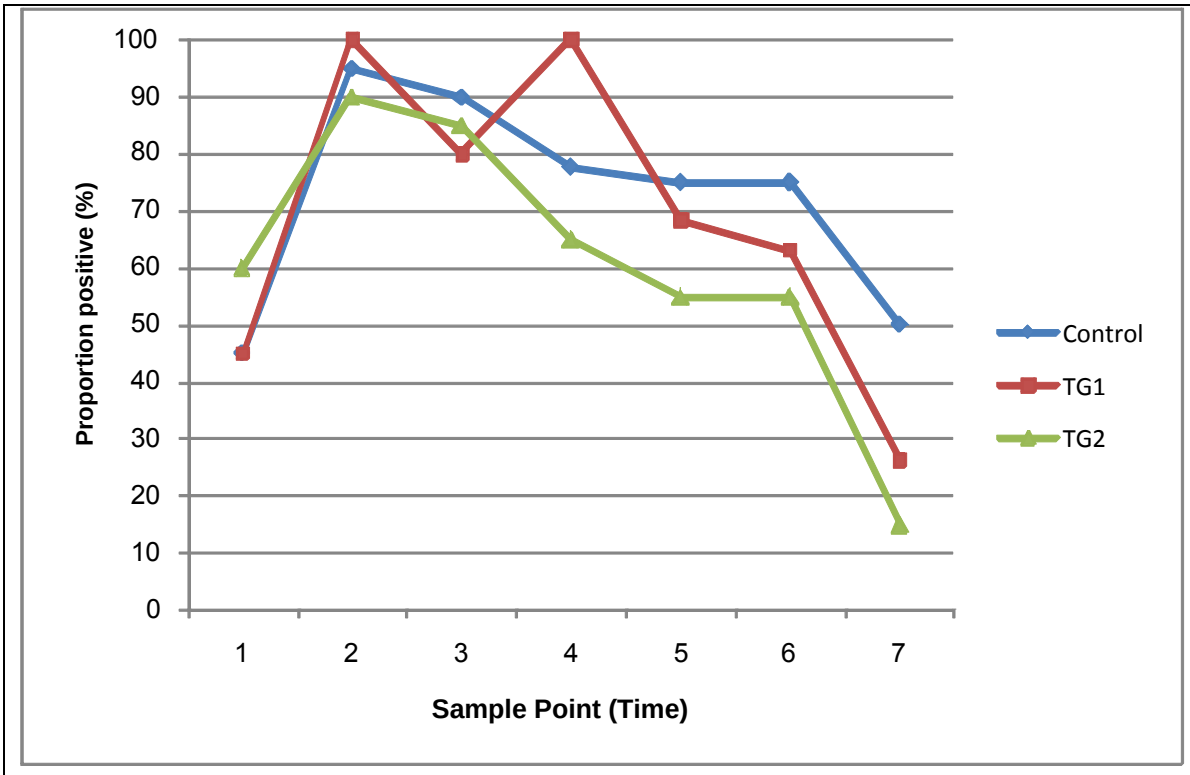

Figure 3. Proportion of colonies testing positive (%) over time for *Nosema spp* using routine laboratory microscopic diagnosis

Time points 1-7 refer to Pre-treatment, 1, 2, 4, 8 &12 weeks after treatment and end of season sample points respectively

Quantitative

Quantification of Ct Values obtained from TaqMan® Results:

Standards prepared from *N. apis* and *N. ceranae* spore dilutions demonstrated a linear response with decreasing spore concentration (Figures 4 *N. apis* & 5 *N. ceranae*).

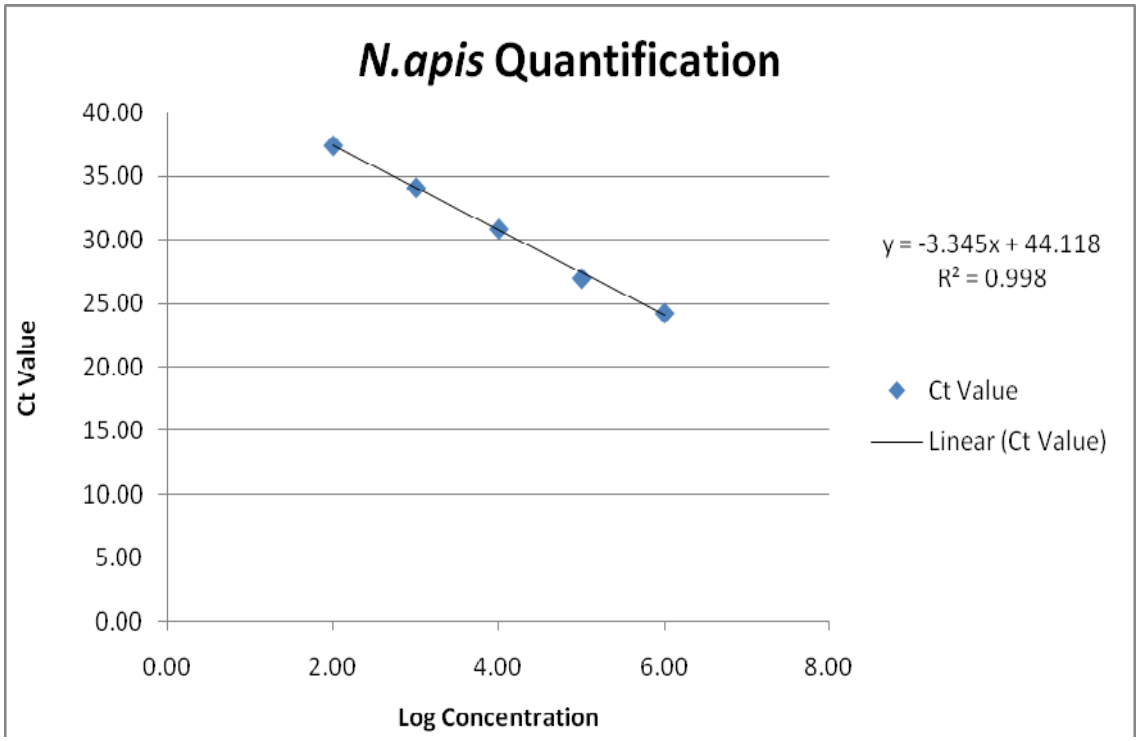

Figure 4. Quantification of Ct values obtained for Standard Dilutions of *N apis* spores

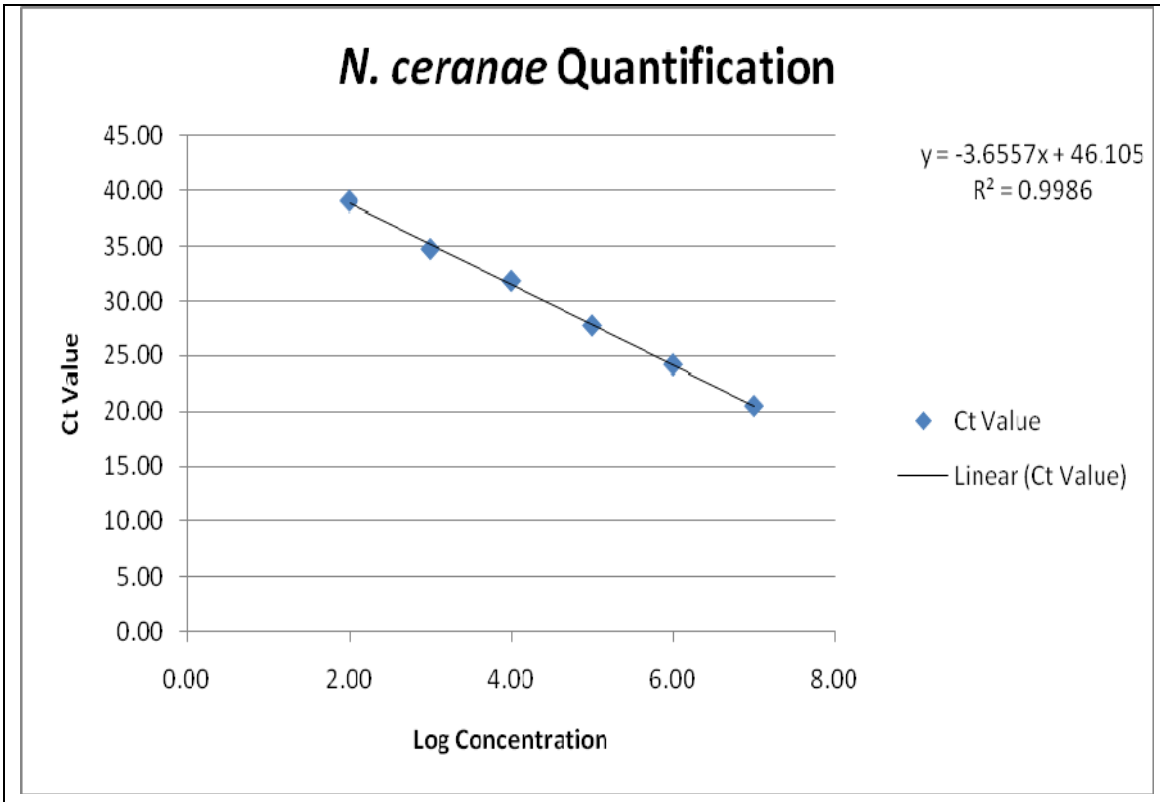

Figure 5. Quantification of Ct values obtained for Standard Dilutions of *N. ceranae* spores

i) <u>Molecular diagnosis:</u>

a) *Nosema apis*

There was an initial rise in the quantity of Nosema detected followed by a decline throughout the study. Repeated measures mixed model analysis showed that there was no evidence of any overall difference in the levels of *N. apis* between pre and post-treatment across all treatments (p=0.45), but that there was some evidence of an overall difference between treatments (p=0.03). There was also evidence of an effect of time post-treatment (ie the numbers of spores changing over time post-treatment) (p<0.001) but there was no evidence of any treatment related effects (ie differences in the post-treatment changes between treatments) (p=0.43).

b) *Nosema ceranae*

When looking at TaqMan positive (for *N. ceranae*) apiaries only, the analysis showed that there was some evidence of an overall difference in the overall levels of *N. ceranae* between pre and post-treatment (p<0.001), as well as between apiaries (p=0.004). There was also evidence of an effect of time post-treatment (p<0.001) and there was some 'borderline' evidence that this effect differed between treatments (p=0.04).

ii) Microscopic diagnosis

Analysis showed that there was some evidence of an overall difference in the numbers of spores between pre and post-treatment (p=0.02), but that there was no evidence that the numbers of spores differed significantly between treatments (p=0.23). As found with all analyses there was clear evidence of an effect of time post-treatment (p=0.002) but was no evidence that this effect differed between treatments (p=0.80).

Table 6. Summary of mean spore counts per sample per time point

Treatment	Number of *N. apis* positive colonies	Mean *N. apis* spores per sample	Number of *N. ceranae* positive colonies	Mean *N. ceranae* spores per sample	Mean number Spores/ml (lab-diagnosis)	Sample Point
Control	13	1.85×10^6	19	1.73×10^6	1.04×10^6	1
	19	2.41×10^7	20	6.6×10^8	2.16×10^6	2
	18	9.48×10^7	20	9.17×10^8	2.56×10^6	3
	17	5.57×10^7	20	7.55×10^8	2.92×10^6	4
	14	1.36×10^7	20	8.27×10^7	2.00×10^6	5
	17	1.08×10^7	20	2.71×10^8	1.03×10^6	6
	4	8.07×10^6	8	6.14×10^7	8.60×10^5	7
TG1	13	3.72×10^6	19	6.76×10^6	3.75×10^6	1
	20	5.05×10^7	20	1.3×10^{10}	6.4×10^6	2
	20	1.26×10^8	20	6.93×10^8	3.29×10^6	3
	19	2.00×10^8	20	2.17×10^9	9.49×10^6	4
	12	5.00×10^6	18	5.58×10^8	2.71×10^6	5
	7	2.75×10^6	10	5.04×10^7	1.02×10^6	6
	2	7.43×10^4	7	6.00×10^7	6.07×10^5	7
TG2	16	2.33×10^7	20	1.26×10^7	4.56×10^6	1
	20	9.79×10^7	20	6.35×10^8	1.01×10^6	2
	19	2.35×10^7	20	1.63×10^9	2.88×10^6	3
	15	8.37×10^6	20	5.86×10^9	1.14×10^6	4
	12	1.18×10^7	20	1.13×10^9	1.54×10^6	5
	14	6.07×10^6	18	3.38×10^8	1.03×10^6	6
	8	1.24×10^7	18	6.08×10^7	1.94×10^6	7

Checking whether TaqMan® tests are positives when visual spore diagnoses are positive.

Table 7. TaqMan® diagnostic results vs Microscopy diagnostic results

	Visual Identification	
TaqMan® Identification	Negative	Positive
Negative	26	13
Positive	108	267

4. Conclusions and Recommendations

Colony health assessments made during this experiment clearly demonstrated that there were no adverse affects on honey bee colonies when enilconazole sulphate was fed at rates of 200mg a.i./colony and 500mg a.i./colony. Whilst some differences were evident in over winter performance between the treatments ie poor overwintering within TG2. It was considered that this poor performance was unlikely to have been treatment related particularly due to the fact that similar effects were not observed within the colonies receiving the higher dose within TG1. It is more likely that this was an effect of apiary; it was within the TG2 apiary where there was considerable robbing by wasps. It is thought that this had the effect of weakening the colonies before wintering and had a knock-on effect upon the survival rate and strength of the colonies when they emerged from over-wintering. This issue of apiary effect could have been reduced by locating all of the study colonies on a single site, however,

this could have implications, such as there would be potential for robbing to occur between colonies and thus subsequent transfer of test item between different treatment groups. Another option for reducing apiary effect would have been to use three apiary sites with colonies from each treatment group spread equally across the sites would have been, however, this would also have posed potential problems relating to robbing and exchange of test item between treatment groups already mentioned.

This study found no consistent evidence that treatment with enilconazole sulphate was effective at causing a long term reduction in either the prevalence or amount of *Nosema* in naturally infected honey bee colonies. The fact that over time there was a significant drop in both the proportion of colonies testing positive for *Nosema* and also levels of spores found over time across all treatments was not to be unexpected. It has been generally accepted that *Nosema apis* levels within colonies will decline throughout the summer after a spring peak. This was the probable reason for the overall decline in the proportion of colonies testing positive throughout the course of the study. The exception to this which can be was seen within the qualitative data for *N. ceranae*, where the proportion testing positive remained relatively high throughout the study. However within the context of this study this difference in behaviour between the two species was not necessarily important because the objective was to investigate any treatment related effects and for a treatment to be considered successful then it would be expected that that there would be a clearly marked treatment response when compared to untreated controls, which was not seen within this study.

This study clearly identified a substantial seasonal change of *Nosema* prevalence. Such fluctuations could cause variation in the ability to detect *Nosema* within 30 bee sub-samples. If the level of infection within the colony was close to the 10% threshold of detection small changes in infection levels between sampling points could mean that positive colonies were missed within the diagnostic frame. However, to have increased confidence levels for detection, at lower infection levels an increased sample size would have been needed to be analysed, which would have been cost prohibitive. Quantitative data for this study showed a large degree of variation between spore counts obtained between colonies within treatment groups. Due to the fact that analysis was carried out on a bulked 30 bee sample and not individual bees the interpretation of the quantitative data was made more difficult, as a high spore count may have been have been derived from many bees within the sample with a low level of infection or from a low number of bees with very high levels of *Nosema* infection.

It should be noted that within the study none of experimental colonies exhibited symptoms of *Nosemosis*. The identification of the pathogen within the test colonies does not necessarily indicate that the colony will express clinical symptoms of the disease. Interpretation of the results would also depend upon whether a candidate medicine was to be used as a prophylactic treatment or to treat clinical signs of disease. It would have been beneficial to have compared the treatment with the known effects of a positive control, such as Fumidil-B which is known to have an effect on both *N. apis* and *N. ceranae*. However, at the time of study initiation this was not a cost effective option.

The impact of *Nosema* spp. infections on their own may not be completely understood, but when coupled with the other potential stressors found within honey bee hives such as the parasitic mite *Varroa destructor* and associated virus infections there could be possible compromises placed on colony health. Following on from this, with the probable withdrawal of the only currently approved *Nosema* treatment available, the need for potential new treatment should remain high on the agenda. Large scale field testing is a requirement for the development of new veterinary medicines for use within apiculture; however, this large scale means that there are substantial financial costs involved at this level of testing. Therefore, a more cost effective and efficient method of early stage screening allowing for a more informed selection of potential candidate treatments before moving to the field test stage would be an extremely useful tool. Within the last two years there have been significant advances in the development of *in vitro* honey bee experimental techniques. A new and more reliable *in vitro* assay has been developed making it possible to carry out laboratory infection of individual honey bees with *Nosema* spp at known doses (spore numbers) and then to monitor the effects of various treatments on subsequent infection levels at known times post infection/treatment. The benefits of this system mean that it would be possible to screen a wide range of potential active ingredients or products within the confines of the laboratory at relatively low cost and is an area which could be exploited in the future.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Budge G. (2008) *Nosema ceranae* – What is *Nosema ceranae* and how do you test for it? Bee Craft, January 2008, 23-24.

Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV, Barrios L, Del Nozal MJ, Bernal JL, Jiménez JJ, Palencia PG, Meana A. (2008) How natural infection by Nosema ceranae causes honeybee colony collapse. Environmental Microbiology Reports Oct;10(10):2659-69. Epub 2008 Jul 18

Higes, M; Martin-Hernandez, R; Garr 521 Ido-Baillon, E; Gonzalez-Porto, A V; Garcia-Palencia, P; Meana, A; Nozal Del, M J; Mayo, R; Bernal, J L (2009). Honeybee Colony Collapse Due To Nosema Ceranae In Professional Apiaries. Environmental Microbiology Reports, Doi:10.1111/J.1758-2229.2009.00014.X

Kluser, S; Neumann, P; Chauzat M P; Pettis, J S (2010). Global honey bee colony disorders and other threats to insect pollinators. UNEP Emerging Issues. http://www.unep.org/dewa/Portals/67/pdf/Global_Bee_Colony_Disorder_and_Threats_insect_pollinators.pdf

Potts, S G; Roberts, S P M; Dean, R; Marris, G; Brown, M A; Jones R; Neumann, P; Settele, J (2010). Declines Of Managed Honey Bees And Beekeepers In Europe. Journal Of Apicultural Research 49(1): 15-22. Doi 10.3896/Ibra.1.49.1.02

Stanimirović, Z; Stevanović Jevrosima; Kulić, M and Stojic V (2006) Frequency of chromosomal aberrations in the evaluation of genotoxic Potential of dicyclohexylamine (fumagillin) *in vivo.* Acta Veterinaria (Beograd), Vol. 56, No. 4, 353-366, doi: 10.2298/avb0604353s

Sulimanovic, D; Paroutka, M and Krpan, Z. (1987) Field trial of Enilconazole efficacy against chalk brood and tolerability to bee brood, adult bees and queens. 31st International Congress of Apimondia (Warszawa 19-25 VIII. 1987)

Van Engelsdorp, D; Evans, J D; Saegerman, C; Mullin, C; Haubruge, E; Nguyen, B K; Frazier, M; Frazier, J; Coxfoster,D; Chen, Y; Underwood, R; Tarpy, D R; Pettis, J S (2009). Colony Collapse Disorder: A Descriptive Study. Plos One 4:E6481. Doi: 10.1371/Journal. Pone.0006481 http://www.plosone.org/article/info:doi/10.1371/journal.pone.0006481