



Literature review of chalkbrood

a fungal disease of honeybees

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Foreword

Chalkbrood of honeybees (*Apis mellifera*) is caused by the fungus *Ascosphaera apis*. It was first diagnosed in Australia in 1993 and is now endemic in most areas of Australia. Although chalkbrood is not usually fatal to honeybee colonies it can cause substantial production losses

The aim of this study was firstly to review the literature on chalkbrood so as to distil into one document the various control measures that have been used to reduce the economic losses caused by this disease. Secondly, the aim was to identify areas that could be investigated so as to expedite the development of control strategies to minimise the economic losses caused by chalkbrood.

Approximately, 160 papers, reviews and books were scanned in the preparation of this review. Fifty references were used in the preparation of this review and are listed in the reference section. Abstracts from some important papers have also been included. Those references identified with an asterisk in the text have their abstracts' included at the end of the document.

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Peter Core
Managing Director
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Executive Summary

Chalkbrood of honeybees (*Apis mellifera*) is caused by the fungus *Ascosphaera apis*. It was first identified in Australia from Queensland in January 1993 and since that time the disease has spread to all Australian states and the Northern Territory. The disease is endemic in most areas and although not usually fatal causes reduced honeybee colony productivity.

There are a number of control methods that have been reported to reduce the effects of chalkbrood. These include management techniques such as strengthening colonies with bees, hatching brood and enlarging colony entrances to aid ventilation. A broad range of chemicals has been used either in hives and/or in the laboratory to control chalkbrood but there have been no studies carried out to determine whether these chemicals produce residues when fed to honeybee colonies. No chemicals for the treatment of chalkbrood have been registered for use in the U.S.A or Australia.

There has been considerable interest in the development of chalkbrood resistant bees and it has been determined that such bees exist naturally in Australia. However, there has been little progress made in the large-scale production of such bees either overseas or in Australia. The thermal destruction (time/temperature) parameters have been determined for *A. apis* in honey and estimates made of the sensitivity of *A. apis* to gamma irradiation from Cobalt 60. Such treatments of honey or other bee products can reduce the spread of disease and are particularly useful in queen bee production operations.

Despite the broad range of experimental work that has been carried out to develop chalkbrood control strategies there is no specific strategy that has been universally adopted or accepted by beekeepers around the world. The fact that *A. apis* is so widespread makes the possibility of its eradication most unlikely. If the disease cannot be eradicated then any chemical that is considered for use against chalkbrood must first be demonstrated not to produce residues in honey or other bee products. Long term use of any chemical for disease control is likely to result in the development of resistance to that chemical. However, the use of a chemical that controls chalkbrood and does not produce residues in honeybee products would be of benefit to beekeepers even if in the long term *A. apis* did develop resistance. The development of antibiotic resistance by a honeybee pathogen has already occurred in the beekeeping industry in the U.S.A, Canada and Argentina where *Paenibacillus larvae* subspecies *larvae* (the cause of American foulbrood) has developed resistance to oxytetracycline hydrochloride.

Further work needs to be carried out to identify and test candidate chemicals for chalkbrood control. The propagation of chalkbrood resistant bees in the beekeeping industry needs to be further investigated. Beekeepers should also be made aware that they can carry out tests in their hives to determine whether their bees have resistance traits to this disease. These chalkbrood resistant bees could then be propagated provided they also have the necessary production capabilities. The effective control of chalkbrood will probably require a combination of control strategies.

1. Chalkbrood

1.1 INTRODUCTION

Chalkbrood of honeybees (*Apis mellifera*) has been recognised since the early 1900s and extensively studied over the years. The focus of this review is on the current knowledge of chalkbrood control. However, to place control strategies in perspective it is also important to understand the epidemiology of the disease. The author has not attempted to reinvent the wheel by reviewing the mass of literature dealing with chalkbrood epidemiology, which is presented in a number of excellent reviews and book Chapters. The author acknowledges the publications of Bailey and Ball (1991), Heath (1982a, b) and, Gilliam and Vandenberg (1990) whose work has been quoted extensively in this review.

1.2 CAUSE

Chalkbrood of honeybees (*Apis mellifera*) is a fungal disease, which affects their larvae. It is caused by *Ascosphaera apis* (Spiltoir, 1955; Spiltoir and Olive, 1955), a heterothallic organism that sporulates only when mycelia of opposite sex (designated + and -) come together. Spores form within dark brownish green fruiting bodies known as spore cysts or ascocarps. Spore cysts measure 47 to 140 µm in diameter. Spore balls enclosed within the cyst range from 9 to 19 µm in diameter, while individual spores are 3.0 to 4.0 µm by 1.4 to 2.0 µm. (Bailey & Ball, 1991). Feldlaufer *et al* (1993) have demonstrated that *A. apis* produces the fatty acid linoleic acid which has antimicrobial activity against bacterial honeybee pathogens.

1.3 OCCURRENCE

Chalkbrood was first reported in Australia in 1993 from south-east Queensland and by 1995 had spread to New South Wales, Victoria and South Australia. More recently it has been confirmed in Tasmania, Western Australia and the Northern Territory.

Chalkbrood is common in most beekeeping countries. It occurs widely in the temperate regions of the Northern Hemisphere. It has long been known in Europe, Scandinavia and Russia (Betts, 1932) and also in New Zealand (Seal, 1957). Not until about 1970 was it officially recognised in the U.S.A. and Canada, where it was found especially in the mid-western and western regions. Since then it has been detected in Argentina, Japan, the Philippines, central America and Mexico (Heath, 1985). It was said to be “the most widespread infectious disease” in Mexico (Wilson *et al.*, 1984); and to have been “most serious” in Norway in 1977 and “alarmingly on the increase” in Britain in 1932 (Heath, 1985); but there have been no data or subsequent events to support these affirmations.

There has been much discussion about its origins and spread between countries, but the sequence of its detection may be as a result of an increasing awareness and interest by investigators. There are suspicions that it was in North America during the 1920s but some investigators are convinced that the incidence of chalkbrood has increased in recent times (Heath, 1985). There are the usual suggestions of more susceptible strains of bees. However, a more likely change, if any has occurred, is that different strains of the fungus have become established, perhaps in solitary bees which have been cultivated and distributed on a large scale in the U.S.A. for many years, particularly for the pollination of alfalfa. They have suffered greatly from their own species of *Ascosphaera*, probably as a result of the increasingly industrial style of their management. *A. apis* has been isolated from some of them and there may be strains of increased virulence for honey bees among them. Newly emerging healthy adult leaf-cutter bees have to chew their way through any larvae killed by the fungus, or through contaminated nest material in the tunnel ahead of them; and each can then carry between 10^4 and 10^7 spores on their bodies (Vandenberg *et al.*, 1980). Some of these spores may find their way to honey bees foraging on alfalfa.

Although Heath (1985) found no cultural differences of any significance between American and British stains of *A. apis*, Glinski and Chmielewski (1982) reported up to 20-fold differences between the virulence of some of the 40 strains they tested on young honey bee larvae” (Bailey and Ball, 1991).

1.4 MULTIPLICATION AND SPREAD

“Larvae ingest spores of *A. apis* with their food. The spores germinate in the lumen of the gut, probably activated by CO₂ from the tissues (Heath and Gaze, 1987), and the mycelium begins to grow there, particularly at the hind end (Maurizio, 1934). The mycelium then penetrates the gut wall and eventually breaks out of the hind end of the larva’s body, often leaving the head unaffected. When they occur, fruiting bodies form on the outside of the dead larvae.

A. apis grows best in slightly chilled larvae as its optimal temperature for growth and formation of fruiting bodies is about 30°C (Maurizio, 1934). Experiments have shown that brood is most susceptible when chilled immediately after it has been capped (Bailey, 1967). The chilling need be only a slight reduction of temperature, from the normal 35°C, for a few hours; and it can easily occur, even in warm climates, in colonies that temporarily have insufficient adult bees to incubate their brood adequately. Larvae are most likely to be chilled in early summer when colonies are growing, and drone larvae often suffer most as they are generally on the periphery of brood nests. The smallest colonies are at the greatest risk of becoming chilled because they have the lowest capacity for heat and relatively large surface areas. Heath (1982a, b), in extensive reviews, quotes several observations that chalkbrood is aggravated when colonies are rapidly expanding in spring, i.e. when the ratio of brood to adult bees is high, or when it is increased experimentally; and that very small colonies used for mating virgin queens or in observation hives are very susceptible. Koenig *et al.* (1987) also noted that decreasing the ratio of adult bees to brood aggravated chalkbrood; and Pederson (1976) showed that artificially heating hives in spring diminished the incidence of the disease. Other non-lethal factors, such as slight infections by viruses or bacteria, or poisoning, or inadequate food from disease nurse bees may well cause the same effect as chilling by slowing the rate of development of larvae” (Bailey and Ball, 1991).

Heath (1982a, b) makes it evident that *A. apis* is widespread throughout Britain and states that in south-west England “it has been possible to detect *A. apis* in any colony of bees ... if the search is diligent enough”. Clearly then, spread of the disease within colonies is almost entirely suppressed spontaneously. The migratory nature of many commercial beekeepers within Australia is likely to have contributed to its rapid spread within the continent.

Each larva that is killed by chalkbrood and produces cysts forms about 10⁸-10⁹ spores. Most of these are ejected from the colony by the house-cleaning bees that remove dead larvae from their cells, but many will inevitably find their way to healthy larvae via mechanical contamination on nurse bees or become lodged in food stores, and especially in brood comb (Koenig *et al.*, 1986), where they stay infective for many years.

The spread of chalkbrood within colonies is probably much suppressed by the normal temperature of the brood-nest or the absence of possible predisposing factors; but some further limitation would seem to be imposed by the need for at least two spores of different strains to germinate and mate within a larva to form fruiting bodies. This limitation is indicated by the many chalky-white larvae and often very few larvae with fruiting bodies in typical outbreaks of disease. Indeed, combs have been found with all the dead larvae apparently infected with one strain of the fungus (Maurizio, 1934). This implies infection stemming from a single larva that had been infected with only one strain, and that larvae can become infected with mycelium as well as spores, which seems improbable. A more likely answer may lie in the observations of Christensen and Gilliam (1983) who isolated both strains from larvae that nevertheless appeared chalky-white. They suggested that unknown conditions within the gut sometimes favour the multiplication of one strain over the other. Consequently, larval remains may become overgrown and depleted by one strain before the other has time to mate and form cysts.

1.5 DIAGNOSIS

The diagnosis of chalkbrood is based on the recognition of signs of disease and the identification of *A. apis* in diseased material.

Clinical evidence of disease is characterised by the following signs.

- ◆ Larvae usually die of chalkbrood after their cells have been capped.
- ◆ Infection is more commonly found in worker and drone larvae than in queen larvae.
- ◆ Small perforations in otherwise normal cell cappings.
- ◆ When uncapped, dead larvae at first are somewhat fluffy white, swollen and sponge-like and may take on the hexagonal shape of the cell. later they become hard and chalk-like in appearance and are called “mummies” which will either remain white-ish or, if the fungus develops fruiting bodies, turn grey or black.
- ◆ The mummies remain white-ish if they are infected with only one strain of the fungus but will turn grey or black when infected with both strains of the fungus as a result of the production of fruiting bodies. By this stage the cappings have frequently been removed by the bees.

Several techniques are used in the laboratory diagnosis of chalkbrood.

1.5.1 Laboratory diagnosis (Hornitzky and Anderson, 2001)

The laboratory diagnosis is based on the demonstration of the causative agent (*A apis*) in diseased material. This is achieved by mounting some diseased material, preferably ‘mummies’ which have turned grey or black, on a microscope slide, adding water or a dye to the material and mixing thoroughly. The resultant suspension is then examined under the microscope. The presence of spore cysts is usually sufficient to make a diagnosis. These spore cysts, which are about 60 µm in diameter, contain smaller round bodies known as spore balls (average 12 µm in diameter) and it is in these spore balls that the spores (average 2.9 x 1.4 µm), the most infective stage of the fungus, are found (Gilliam, 1990).

In samples where only white ‘mummies’ have been submitted and spore producing bodies cannot be detected when examined under the microscope it may be necessary to grow the fungus on potato-dextrose agar or yeast-glucose-phosphate medium.

Rapid detection and identification of *A apis* can be performed by a polymerase chain reaction (PCR) using primers specific for *A apis* (see below).

1.5.2 Culture of *Ascosphaera apis*

Yeast-glucose-phosphate agar is comprised of 1% yeast extract (Oxoid), 0.1% cysteine or cystine (BDH), 1% glucose (Ajax), 1.35% KH₂PO₄ (Ajax) and 1% soluble starch (Ajax). The plates should be incubated at 37°C in an atmosphere containing 5 to 10% CO₂. Fungal colonies grow moderately slowly and are 5 to 7 cm in diameter after 10 days; they produce aerial mycelia deeply floccose or matted, white to pale buff and may become coral to pale reddish brown with age (Bissett, 1988). To confirm the identity as *A apis* it is necessary to mate it with one of two known mating strains of *A apis* (Anderson *et al*, 1998). This is achieved by inoculating the culture medium with the suspect *A apis* culture and inoculating the two known mating strains on either side of the test isolate about 1 cm away. The fungus is confirmed as *A apis* if it mates with one of the two mating strains by producing spore cysts. These appear as a brown line at the junction where the test fungus and control fungus meet and mate. Mating usually takes from 5 to 10 days, however, the production of spore balls and the spores within make take up to 15 days.

1.5.3 PCR for the detection and identification of *Ascospaera apis*

DNA is extracted from the suspect fungus by either a rapid or slow DNA extraction method. For the rapid method, suspect fungal spores or mycelia are placed in a PCR tube (a 0.5 mL eppendorf tube) with an equal volume of glass beads and 100 μ L distilled water. This mixture is vortexed for 30 s and centrifuged for 2 min at 6,000 g in an Eppendorf bench centrifuge. The supernatant is then removed to a clean PCR tube and used directly in the PCR reaction. For the slow extraction method, which produces cleaner DNA preparations than the rapid method, 0.4 to 0.5 g of compressed fungus mycelia are ground in liquid nitrogen to a fine powder in a pre-cooled mortar and pestle. Three millilitres of freshly prepared K buffer (2.0 mg/mL proteinase K, 0.1 M Tris-HCl pH 8.5, 0.05 M EDTA, 0.2 M NaCl and 1% SDS) is added, the mixture held in a water bath at 65°C for 1 h, then extracted with an equal volume of phenol saturated with 10 mM Tris-HCl, and 1 mM EDTA pH 8 (TE) at room temperature for 15 min. The phases are then separated by centrifugation at 2,000 g for 10 min, the supernatant removed to a clean centrifuge tube, extracted as above with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged to separate the phases. The supernatant is once again removed to a clean centrifuge tube where 2 volumes of cold ethanol are added to precipitate the DNA. The DNA is then pelleted by centrifuging at 6,000 g for 10 to 15 min at 4°C, resuspended in 75% ethanol, pelleted again at 6,000 g for 5 to 10 min, dried at room temperature, resuspended in 500 μ L TE containing 10 pg/mL RNase A and incubated at 37°C for 30 min. This solution is extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) as described above and centrifuged to separate the phases. The supernatant is removed to a clean centrifuge tube, 50 μ L of 3 M sodium acetate pH 6.0 added and the DNA precipitated with 2.5 volumes of cold ethanol. Precipitated DNA is pelleted, resuspended and dried as described above, resuspended in 200 μ L of distilled H₂O and used directly in the PCR reaction or frozen at -20°C until needed.

The nuclear rDNA region containing sequence of the *A. apis* internal transcribed spacer regions and 5.8S rDNA (ITS1-5.8S-ITS2) is amplified by PCR using either one or two forward primer 5'-GCTAGGTGCCCCTAAACAAGGC- Y(CBP1) or 5'-TTTGAGTTCCCCCTGGCTAGC- 3' (CBP2) in conjunction with the reverse primer 5'-ACTAGAGCGAAAGACAAAGCC- 3' (CBP3) using methods described by Anderson *et al.*, (1998). These primers were constructed from original sequence data generated by Anderson *et al.*, (1998) and are *A. apis*-specific. Each primer combination will generate a single PCR product of about 500 base pairs but the combination of CBP1 and CBP3 primers will generate a slightly larger product than the CBP2 and CBP3 primer combination. For PCR reactions, 2 to 20 ng of purified DNA are added to 5 μ L of 0.01 mM AB28 primer, 5.0 μ L 0.01 mM TW81 primer, 5.0 μ L 10 x PCR buffer (670 mM Tris- HQ pH 8.8, 166 mM ammonium sulphate, 2 mg/mL gelatin, 15 mM M9C12 and 4.5% Triton X-100), 50 μ M each of dATP, dCTP, dGTP and dTTP and 1 drop of oil. DNA is amplified using an automated thermal cycler (Corbett Research, Model FTS-1) and the following protocol: 5 min initial denaturation at 94°C, after which 2 U of Taq polymerase is added, then 30 cycles of 1 min at 94°C, 1.5 min annealing at 54 to 55°C and 2 min extension at 72°C. A final extension period of 5 min at 72°C completes the amplification. Five microlitres of PCR product are then added to 2 μ L of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400, Pharmacia) in water, and electrophoresed together with Lamda DNA-Hind 111 as a marker in a 1% agarose gel containing 10 μ g ethidium bromide per 100 mL TAE at 60 v for about 3 h. DNA bands are then visualised using an ultra-violet transilluminator.

1.6 CONTROL

No chemotherapeutic agents are registered in Australia for use against chalkbrood. However, there are a number of techniques that can be used to minimise the effects of chalkbrood and a range of chemicals both artificial and natural that are reported to have activity against *A. apis*.

1.6.1 Management techniques

- ◆ Strengthening badly diseased colonies by adding young adult bees, hatching brood, by feeding sugar syrup and by not allowing bees to winter in too large a brood chamber (Seal, 1957)
- ◆ Enlargement of colony entrance to aid ventilation (Gochnauer *et al*, 1975).
- ◆ For severe cases, destruction of affected combs has been recommended (Betts, 1951).
- ◆ New comb can reduce the incidence of chalkbrood (Nelson and Gochnauer, 1982; Koenig *et al*, 1986).
- ◆ Anderson *et al*, (1997)* have demonstrated that honey can be rendered free of viable *A. apis* spores by holding it in water baths at 65° for 8 h or at 70° for 2 h. This has implications for honey/candy used in the transport of queen bees.
- ◆ Gamma irradiation from Cobalt 60 can also be used to render hive materials and products free of viable chalkbrood spores (Katznelson and Robb, 1962) and Shimanuki *et al* (1984) used high-velocity electron beams for killing spores associated with chalkbrood mummies.
- ◆ Gochnauer and Margetts (1980) found that ethylene oxide fumigation killed *A. apis* in infected combs.
- ◆ Methyl bromide has been used to fumigate hive equipment but chemical residue was detected in wood and wax (Faucon *et al*, 1982)

1.6.2 Chalkbrood resistant bees

Bailey and Ball (1991) have reported that there is no doubt that bees can be selected with resistance that is greater than average towards disease as has been demonstrated by Rothenbuhler and his colleagues with American foulbrood. Unfortunately, resistance towards this disease, which is the most likely of all microbial diseases to kill a bee colony, is at least partly determined by recessive factors. This suggests that death of infected colonies has enabled the species to survive better than has natural selection for resistance. Whatever the reason, it makes the maintenance of resistant strains more difficult than if resistance were due to dominant genes.

There has been considerable interest in the development of disease resistant bees, which includes a significant body of work for the development of chalkbrood resistance, both overseas and in Australia. Rothenbuhler (1964) reported that the efficiency of the hygienic behaviour of adults in removing diseased larvae was further separable into a factor for uncapping the cells and a factor for removing the larvae. This behavioural characteristic has been further examined by Spivak and Gilliam (1993) who determined the effect of changing colony strength on hygienic behaviour, responses to freeze-killed and live brood of hygienic and non-hygienic bees, the affect of adding hygienic bees to non-hygienic colonies and the response of feeding *Ascospaera apis* (the cause of chalkbrood) to hygienic and non-hygienic bees.

Spivak and Downey (1998)* reported that the propagation of colonies that demonstrate resistance to chalkbrood and American foulbrood, and that remove pupae infested by *Varroa* mites is becoming increasingly important in apiculture. They evaluated 2 commonly used field assays used to screen colonies for hygienic behaviour: the freeze-killed brood and the pierced brood assays. Both involve determining the time required for worker bees to remove dead capped brood from a section of comb. Colonies in the experiment displayed a wide range of removal rates and were grouped as hygienic,

non-hygienic or intermediate. Their results indicated that bees from non-hygienic lines can be induced to express hygienic behaviour only if a sufficiently strong stimulus is present. Both hygienic and non-hygienic colonies removed significantly more pupae treated with haemolymph from a dead pupa than haemolymph from a live pupa, indicating that the cue that stimulates removal behaviour is stronger in dead pupae. It was concluded that the freeze-killed brood assay is the most conservative and reliable screening procedure for hygienic behaviour. When developing hygienic breeder stock, the hygienic colonies should be challenged with the pathogens of American foulbrood (*Paenibacillus larvae* subspecies *l. larvae*) or chalkbrood (*A. apis*) to ensure resistance.

Spivak and Reuter (1998)* compared colonies with naturally mated queens from a hygienic line of Italian honeybees to colonies from a commercial line of Italian bees not selected for hygienic behaviour. They compared: rate of removal of freeze-killed brood; amount of chalkbrood; incidence of American foulbrood (*P. l. larvae*); honey production; and the number of *Varroa jacobsoni* on adult bees. The hygienic colonies removed significantly more freeze-killed brood than the commercial colonies, had significantly less chalk-brood, had no American foulbrood, and produced significantly more honey than the commercial colonies.

Studies of the natural hygienic behaviour of Australian honey bees were carried out by Oldroyd (1996)*. Ten strains of Australian commercial honeybees were evaluated for hygienic behaviour. Dead pupae were inserted into the colonies. They were prepared by inserting small pieces of combs of sealed brood (5 cm² and containing about 100 pupae per side) which had been cut from combs using a sharp knife and a template. Comb sections were wrapped in absorbent paper and placed in a freezer at -20⁰ C until required. Once inserted into colonies they were checked after 3, 5 and 7 days for the number of pupae removed. Most colonies (80%) were non-hygienic, but 2 strains gave a good overall performance in the test and comprised 1 or 2 colonies that were highly hygienic. Colonies were evaluated 3 times, and the good performance of these colonies was repeatable across trials. The results suggest that hygienic behavioural morphs exist in Australia's commercial bee strains, and that selective breeding should be able to produce suitable genotypes (Oldroyd, 1996).

1.6.3 Chemical control

A broad range of chemicals has been tested either in honeybee colonies and/or on *A. apis* in culture for the control of chalkbrood. Heath (1982a) has produced a comprehensive list of chemicals (below) in his review of chalkbrood.

TABLE 1. Chemical treatments which under some conditions have given control of chalkbrood in honeybee colonies, or the inhibition of *A. apis* in culture.

Substances(s)	Concentration	Procedure
sorbic acid	0.05%	in culture
sorbic acid	0.1%	fed to colonies
sorbic acid	5%	aqueous soln on culture
sorbic acid & sodium propionate (N.B. pollen patties also contained oxytetracycline)	0.1%	fed to colonies in pollen cakes
sorbic acid & sodium propionate	0.2%	fed to colonies in 225 g pollen supplement
sorbic acid & sodium propionate	0.1%	fed to colonies in syrup
methyl parahydroxybenzoate	0.05%	in culture
methyl parahydroxybenzoate	0.5%	in aq. soln on culture for 5min in culture
thiabendazole	1%	in sucrose dusted over colonies
thiabendazole	0.2%	in 225 g pollen supplement
thiabendazole		
benomyl	0.25%	fed to colonies in syrup
benomyl	0.25%	as above & also sprayed over frames
dinocap	0.25%	fed to colonies in syrup
Acti-dione		
griseofulvin		in culture
Nipagin	2%	fed to colonies in syrup
Nipagin & potassium sorbate		fed to colonies in syrup
cycloheximide	0.0025%	in culture
cycloheximide	0.01%	fed to colonies in syrup
Amphotericin B		in culture
Nystatin		in culture
Mycostatin ⁴ (=Nystatin)	1 million i.u./l	fed to colonies in syrup
Mycostatin	0.05%	aq. soln on culture for 24 h
Mycocidin	100-150 g/col	sifted over colony
citral	5 µl/dish	vapour on culture
geraniol	10 µl/dish	vapour on culture
3P (polyfungine cholate)	40%	sprayed on brood in syrup
Amphotericin B		in culture
Lastanox (bis-tributyl-stannic oxide)	2.0%	aq. soln for 5 min on cultures
Nitrofungin (2-chlor-4-nitrophenol triethylene-glycolum)	2.0%	aq. soln for 24 h on cultures

TABLE 1. Chemical treatments which under some conditions have given control of chalkbrood in honeybee colonies, or the inhibition of *A. apis* in culture (continued).

Substances(s)	Concentration	Procedure
Chinosol	0.05%	sprayed over bees
Soloxin		fed to colonies in syrup
formalin	0.2%	aq. soln for 1 h on cultures
Fesia-form	4%	250 ml sprayed over combs of colony
allylithio-cyanate	1.0%	aq. soln for 1 h on cultures
boric acid	0.5%	aq. soln for 24 h on cultures
salicyclic acid	1.0%	aq. soln for 24 h on cultures
thymol	0.7%	in culture
thymol	0.7%	sprayed over combs
thymol	0.8%	sprayed on colonies
propolis	15%	alcoholic soln on culture for 24 h
copper sulphate	0.1%	fed to colonies in syrup
acetic acid	glacial	vapour on culture for 1 h

Heath (1982) (References for chemicals listed above can be found in Heath, (1982))

Since this review a number of other further studies demonstrated the activity of chemicals against chalkbrood have been published:

- ◆ Tanaka *et al* (1984) demonstrated that trichloroisocyanuric acid (TCA) in water placed in the hive controlled chalkbrood. However, researchers in Spain (Serrano et al, 1995)* reported that TCA which releases chlorine gas when exposed to air has a fungicidal effect on *A. apis* spores artificially placed in hives. The effect, however, was dependent on the number of spores and the degree of aggregation of spores in spore balls.
- ◆ Vapours of propionic acid proved efficacious (Kajikawa and Nakane, 1986)
- ◆ Herbert *et al*, (1985; 1986) have shown that certain alkyl amines not only stimulate the removal of chalkbrood cadavers but also inhibit the growth of the fungus *in vitro*.
- ◆ In the USA the management of honeybee colonies is becoming increasingly dependent on the use of chemotherapeutic agents. In addition to the use of oxytetracycline for the control of brood diseases, in the USA the recent introduction of two parasitic mites has resulted in the use of additional agents. Menthol is sometimes used for control of the tracheal mite, *A. woodi* and fluvalinate is used for the control of *V. jacobsoni*. This increasing dependence on the use chemotherapeutic agents has created several problems.
 - Profit margins of beekeepers have been reduced as expenditures for chemicals have risen.
 - Labor requirements have increased as the number of treatments increases
 - Beekeepers have become vulnerable to the evolution of resistance in the parasite and pathogen populations due to the limited number of chemotherapeutics available for control of each individual organism
 - toxicological hazards to beekeepers and bees have increased and
 - chemical residues have contaminated some honey crops.

These factors combined with the lack of any effective control agent for chalkbrood, have resulted in an increased interest in the investigation of alternative control strategies. This prompted Calderone, *et al* (1994)* to test 8 plant extracts for their activity against *P. l. larvae* (the cause of American foulbrood), *A. apis* and *Paenibacillus alvei* (a common secondary bacterial invader in European foulbrood infections). They found that cinnamon oil completely inhibited the growth of *A. apis* at 100 ppm for 168 h. Bay oil, citronellal, clove oil, origanum oil and thymol inhibited all growth at 1,000 ppm for 168 h. Camphor inhibited all growth at 10,000 ppm for 168 h, and alpha-terpinene inhibited all growth for 72 h at 10,000 ppm. They also pointed out that translating the results of their *in vitro* studies into a successful management strategy would require the resolution of several complex problems. These include (a) demonstration of *in vitro* activity at levels that are not toxic to honeybee larvae, pupae and adults and which do not affect the flavor of the honey; and (b) the development of an adequate delivery system. They reported that typically, the consumption of supplements containing extracts at concentrations above ½% was greatly reduced and that formulations must be competitive with naturally occurring nectar and pollen (Calderone, *et al*, 1994).

- ◆ Researchers in Spain (Jimenez *et al*, 1994) demonstrated that that IQB-863-f a new imidazole as well as other imidazole derivatives (miconazole, econazole, ketonazole, isoconazole and thioconazole) possessed appreciable antifungal activity against *A. apis*.
- ◆ Glinski and Chmielewski (1997)* demonstrated that the imidazole derivative Klotrimazole stimulates defence reactions of the honeybee. Phagocytosis and the level of innate and inducible haemolymph immune proteins increased in worker bees exposed to Klotrimazole.
- ◆ Jendrejsek and Kopernicky (1998)* evaluated the efficacy of 85% formic acid (Apiform) against *Varroa jacobsoni* and *A. apis*. The medication was applied thorough a wick in the centre of the honey chamber. They reported 100% effectiveness in 1994 and 87.9% in 1997 where affected combs were removed and bottom boards of hives were cleaned before treatment.

A wide range of chemicals has been tested for the control of chalkbrood. However, none has proved efficacious to the point where it has been universally accepted. A chemical which is effective against chalkbrood, does not produce residues in bee products and is not harmful to bees is yet to be found.

1.6.4 Banana fruit

There is some anecdotal evidence in Australia that banana fruit placed in hives is an effective control for chalkbrood. However, the author was unable to find any data on the effect of exposing chalkbrood infected hives to banana fruit.

“Any beneficial effect that banana fruit placed within the hive might have in controlling or reducing the severity of chalkbrood would most probably arise from volatiles evolved by the fruit. It is not inconceivable that the fruit might evolve one or more volatile compounds that are inhibitory to the spore germination or mycelial growth of the causal fungus *A. apis*. Such fungistatic or fungitoxic volatiles might be the normal products of fruit metabolism or the products of microbial degradation of the fruit as it senesces. The banana fruit contains at least 200 individual volatile components (Palmer, 1971). Many more volatile compounds, including fermentation products, could arise from microbial action (Sureh and Ethiraj, 1991). The fermentation products ethanol and acetaldehyde inhibited mould growth on oranges (Yuen *et al*, 1995)” (Wade, personal communication).

2. Discussion

Despite the broad range of experimental work that has been carried out to develop chalkbrood control strategies there is no specific strategy that has been universally adopted or accepted by beekeepers around the world. Management strategies, chemicals and the use of bees that show resistance to chalkbrood have all been shown to have some benefit although no individual control strategy will ensure a cure for the disease. The effective control of chalkbrood will probably require a combination of control strategies.

The fact that *A. apis* is so widespread makes the possibility of its eradication most unlikely. If the disease cannot be eradicated then any chemical that is considered for use against chalkbrood must first be demonstrated not to produce residues in honey or other bee products. Long term use of any chemical for disease control is likely to result in the development of resistance to that chemical. However, the use of a chemical that controls chalkbrood and does not produce residues in honeybee products would be of benefit to beekeepers even if in the long term *A. apis* did develop resistance. The development of antibiotic resistance by a honey bee pathogen has already occurred in the beekeeping industry in the U.S.A, Canada and Argentina where *P. l. larvae* (the cause of American foulbrood) has developed resistance to oxytetracycline hydrochloride.

3. Recommendations

Further work needs to be carried out to identify and test candidate chemicals for their ability to control chalkbrood and to determine whether they leave residues in honeybee products. The propagation of chalkbrood resistant bees in the beekeeping industry needs to be further investigated. Beekeepers should also be made aware that they can carry out tests with their colonies to determine whether their bees have chalkbrood resistance traits. These chalkbrood resistant bees could then be propagated, provided they also have the necessary production capabilities.

4. References

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5. Abstracts

- TI: Detection and thermal destruction of the chalkbrood fungus (*Ascosphaera apis*) in honey.
- AU: Anderson-D; Giacom-H; Gibson-N
- AD: CSIRO Division of Entomology, Black Mountain, P.O. Box 1700, Canberra, ACT 2601, Australia.
- SO: Journal-of-Apicultural-Research. 1997, 36: 3-4, 163-168; Bb.
- PY: 1997
- LA: English
- AB: A sensitive culture technique for detecting viable spores and mycelial elements of *A. apis* is described. The technique involves embedding honey or distilled water containing *A. apis* spores or mycelial elements in 15 ml of sterile liquid nutrient agar medium (10 g yeast, 10 g glucose, 13.5 g KH₂PO₄, 10 g soluble starch and 20 g agar) cooled to 60°C. This medium is then poured on a 7-ml layer of similar but solid agar medium in a standard 8.5-cm petri dish and allowed to solidify by cooling. The medium is incubated in an anaerobic environment at 37° for 24 h, and then incubated in an aerobic environment for 9 days at 37°. It is examined daily for *A. apis* growth. The technique facilitated the detection of viable *A. apis* in honey and was used to show that pre-packaged retail honeys contain viable *A. apis*. The technique was also used to show that honey may be rendered free of viable *A. apis* by holding it in water baths at 65° for 8 h or at 70° for 2 h.
- AU: Calderone,-N.W.; Shimanuki,-H.; Allen-Wardell,-G.
- TI: An in vitro evaluation of botanical compounds for the control of the honeybee pathogens *Bacillus larvae* and *Ascosphaera apis*, and the secondary invader *B. alvei*.
- SO: J-essent-oil-res. Carol Stream, Ill. : Allured Publishing Corporation. May/June 1994. v. 6 (3) p. 279-287.
- CN: DNAL SB298.J66
- PA: Other-US
- PY: 1994
- LA: English
- CP: Illinois; USA
- PT: Article
- AB: Bactericidal and fungicidal effects of eight plant extracts on the growth of two honeybee pathogens, *Bacillus larvae* (causative agent of American foulbrood) and *Ascosphaera apis* (causative agent of chalkbrood), and *Bacillus alvei* (a secondary invader in European foulbrood), were evaluated. Cinnamon oil completely inhibited the growth of *B. larvae* at 10 ppm for 72 h. Camphor and citronellal inhibited all growth at 100 ppm for 72 h. Bay oil, clove oil, origanum oil, and thymol inhibited all growth at 1,000 ppm for 72 h, and alpha-terpinene inhibited all growth at 10,000 ppm for 72 h. Cinnamon oil completely inhibited the growth of *A. apis* at 100 ppm for 168 h. Bay oil, citronellal, clove oil, oiiganum oil and thymol inhibited all growth at 1,000 ppm for 168 h. Camphor inhibited all growth at 10,000 ppm for 168 h, and alpha-terpinene inhibited all growth for 72 h at 10,000 ppm. Cinnamon oil and thymol completely inhibited the growth of *B. alvei* at 10 ppm for 1-2 h. Bay oil, camphor and origanum oil inhibited all growth at 100 ppm for 72 h. Clove oil and citronellal inhibited all growth at 1,000 ppm for 72 h, and alpha-terpinene inhibited growth at 10,000 ppm for 72 h. Several compounds reduced growth in a dose dependent manner at levels below their threshold values. These results suggest that plant extracts might play a significant role in the management of honeybee diseases.

Imidazole derivatives in control of the honey bee brood mycoses
Glinski, Z.; Chmielewski, M. (University of Agriculture, Lublin (Poland).
Faculty of Veterinary Medicine)

Journal: *Pszczelnicze Zeszyty Naukowe*, 1996, v. 40(2) p. 165-173

Notes: 5 fig., 14 ref. ISSN: 0552-4563 Notes: Printed 1997

Language: English Summary Language: English, Polish

Place of Publication: Poland

Document Type: Journal Article, Summary

Journal Announcement: 2506 Record input by Poland

The imidazole derivative, Klotrimazol (phenyl-2-chlorophenyl)-1-imidazolyl-methane), opens a very interesting opportunity for searching for effective and safe chemical uses in the control of chalkbrood and mixed fungal infections (*Ascosphaera apis* and *Aspergillus* sp.). Klotrimazole stimulates defense reactions of the honey bee. The value of phagocytosis, and level of innate and inducible hemolymph immune proteins increase in worker bees exposed to Klotrimazole.

Evaluation on therapeutic efficiency of the preparation Apiform for varroa and chalkbrood disease control (Zhodnotenie terapeutického ucinku pripravku apiform pri lieceni varroozy a zvapenatenia vcelieho plodu)

Jendrejak, R.; Kopernicky, J. (Research Institute of Animal Production, Nitra (Slovak Republic))

Journal: *Journal of farm Animal Sciences*, 1998, (no.31) p. 209-214

Notes: 3 tables; 8 ref. ISSN: 1335-3691

Language: Slovak Summary Language: English, Slovak

Place of Publication: Slovak Republic

Availability: Slovak Republic Center

Document Type: Journal Article, Summary, Nonconventional Literature

Journal Announcement: 2604 Record input by Slovak Republic

The therapeutic utilization of 85% formic acid, chemically pure (edible), against *Varroa jacobsoni* Oudem. and ascospaeriosis was evaluated in this work. The preparation Apiform ad us. vet. was used. It was developed in RIAP - the Institute of Bee-keeping. The acid was applied by means of evaporation from the cover through wick in the centre of honey chamber. One pack with 40 ml of acid is used in each honey chamber. It evaporates freely. The optimum period of evaporation are 4 - 5 days. This preparation was highly effective in the tests with both infections. The treatment of varroasis was effective to 99.2% with two applications and after the removal of a sealed drone brood. The efficiency in ascospaeriosis treatment was 100% in 1994, 87.9% in 1997. The affected combs were removed and bottom boards of hives were cleaned before the medicament was used. There were noticed no excessive disturbances or injury to colonies during the tests. The formic acid does not impair the hygienic parameters of honey in the applied amounts and therefore it can be used in colonies during the summer production period if the honey flow is not in progress.

TI: Evaluation of Australian commercial honey bees for hygienic behaviour, a critical character for tolerance to chalkbrood.
AU: Oldroyd-BP
AD: School of Genetics and Human Variation, La Trobe University, Bundoora, Vic. 3068, Australia.
SO: *Australian-Journal-of-Experimental-Agriculture*. 1996, 36: 6, 625-629; Bc.
PY: 1996
LA: English

AB: Chalk brood of honey bees (*Apis mellifera*) is new to Australia. The best prospects for control are likely to come from the use of bees with a strong genetic tendency to uncap and remove dead pupae, together with good beekeeping practice. Ten strains of Australian commercial honey bees were evaluated for hygienic behaviour. Dead pupae were inserted into the colonies and checked after 3, 5 and 7 days for the number of pupae removed. Most colonies (80%) were non-hygienic, but 2 strains gave a good overall performance in the test and comprised 1 or 2 colonies that were highly hygienic. Colonies were evaluated 3 times, and the good performance of these colonies was repeatable across trials. The results suggest that hygienic behavioural morphs exist in Australia's commercial bee strains, and that selective breeding should be able to produce suitable genotypes.

AUTHOR: Flores Serrano J M; Puerta Puerta F; Bustos Ruiz M; Padilla Alvarez F; Jimenez Jimenez A; Trocoli Garcia F

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JOURNAL: Revista Iberoamericana de Micologia 12 (2):p49-51 1995

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DOCUMENT TYPE: Article

RECORD TYPE: Abstract

LANGUAGE: Spanish; Non-English

SUMMARY LANGUAGE: Spanish; English

ABSTRACT: Quantified amounts of *Ascosphaera apis* spores, causative organism of chalkbrood disease, were cultured in vitro after being treated with trichloroisocyanuric acid (TCA) (Yukoluck 93% a.i) inside honeybee colonies. This gaseous substance is registered in several countries for the control of chalkbrood, a invasive mycosis affecting honeybee larvae. TCA acts, on the spores that are present inside the hive. The product was applied at the recommended dose (3 g/80 ml/hive) and the spore inocula were kept inside a commercial hive treated with TCA for 48 h before being cultured in vitro. Results show that efficacy of TCA is strongly linked with two main factors: Amount of spores and the degree of agglutination in sporeballs.

TI: Field assays for hygienic behaviour in honey bees (Hymenoptera: Apidae).

AU: Spivak-M; Downey-DL

AD: Department of Entomology, 219 Hodson Hall, University of Minnesota, St. Paul, MN 55108, USA.

SO: Journal-of-Economic-Entomology. 1998, 91: 1, 64-70; 24 ref.

PY: 1998

LA: English

AB: Honey bee (*Apis mellifera*) hygienic behaviour is a mechanism of disease resistance and a mode of defence against the parasitic mite *Varroa jacobsoni*. Hygienic bees uncap and remove diseased and parasitized brood from the nest. The propagation of colonies that demonstrate resistance to chalkbrood and American foulbrood and that remove pupae infested by *Varroa* mites is becoming increasingly important in apiculture. This study evaluates 2 commonly used field assays used to screen colonies for hygienic behaviour: the freeze-killed brood and the pierced brood assays. Both involve determining the time required for worker bees to remove dead capped brood from a section of comb. Colonies in the experiment displayed a wide range of removal rates and were grouped as hygienic, nonhygienic or intermediate. The results of experiments 1 and 2 indicated that neither the age nor the source of the frozen brood had a significant effect on the removal rate by hygienic colonies (i.e. those colonies that consistently uncapped and removed freeze-killed brood with 48 h). In

experiment 3, only a weak correlation was found between the removal of young freeze-killed and pierced pupae, but a significant correlation existed between the removal of pre-eclosion freeze-killed and pierced pupae. Experiment 4 examined cues that elicit removal behaviour by hygienic and nonhygienic colonies. When pupae were pierced with an insect pin through the base of the cell (without piercing the wax cell capping), there was no difference in the number of pupae removed by the hygienic and nonhygienic colonies. On average, 30% of all pierced pupae survived the treatment, which considerably diminished the accuracy and reproducibility of the test. When pupae were treated with haemolymph extracted from either a live or freeze-killed pupa, there was also no difference in the rate of removal by hygienic and nonhygienic colonies. These results indicated that bees from nonhygienic lines can be induced to express hygienic behaviour only if a sufficiently strong stimulus is present. Both hygienic and nonhygienic colonies removed significantly more pupae treated with haemolymph from a dead pupa than haemolymph from a live pupa, indicating that the cue that stimulates removal behaviour is stronger in dead pupae. It was concluded that the freeze-killed brood assay is the most conservative and reliable screening procedure for hygienic behaviour. The following procedures are recommended: Randomly selected comb sections (5 by 6 cm each) of capped brood should be cut from 1 healthy colony, frozen, and introduced into the test colonies. The assay should be repeated at least twice. Only colonies that remove >95% of freeze-killed brood within 48 h in both tests should be considered hygienic. When developing hygienic breeder stock, the hygienic colonies should be challenged with the pathogens of American foulbrood (*Paenibacillus larvae*) or chalkbrood (*Ascosphaera apis*) to ensure resistance.

TI: Performance of hygienic honey bee colonies in a commercial apiary in Wisconsin.

AU: Spivak-M; Reuter-GS

AD: Department of Entomology, 219 Hodson Hall, University of Minnesota, St. Paul, MN 55108, USA.

SO: Apidologie. 1998, 29: 3, 291-302; 24 ref.

PY: 1998

LA: English

LS: German, French

AB: Colonies with naturally mated queens from a hygienic line of Italian honey bees (*Apis mellifera ligustica*) maintained at the University of Minnesota, USA, were compared to colonies from a commercial line of Italian bees not selected for hygienic behaviour. The following characteristics were compared: rate of removal of freeze-killed brood; amount of chalkbrood; incidence of American foulbrood (*Paenibacillus larvae*); honey production; and the number of *Varroa jacobsoni* on adult bees. The hygienic colonies removed significantly more freeze-killed brood than the commercial colonies, had significantly less chalkbrood, had no American foulbrood, and produced significantly more honey than the commercial colonies. Estimates of the number of *Varroa* mites on adult bees indicated that the hygienic colonies had fewer mites than the commercial colonies in three of four apiaries. In previous studies on the relation between hygienic behaviour and resistance to diseases and mites, the test colonies contained instrumentally inseminated queens. This is the first study to evaluate hygienic stock in large field colonies with naturally mated queens.

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