

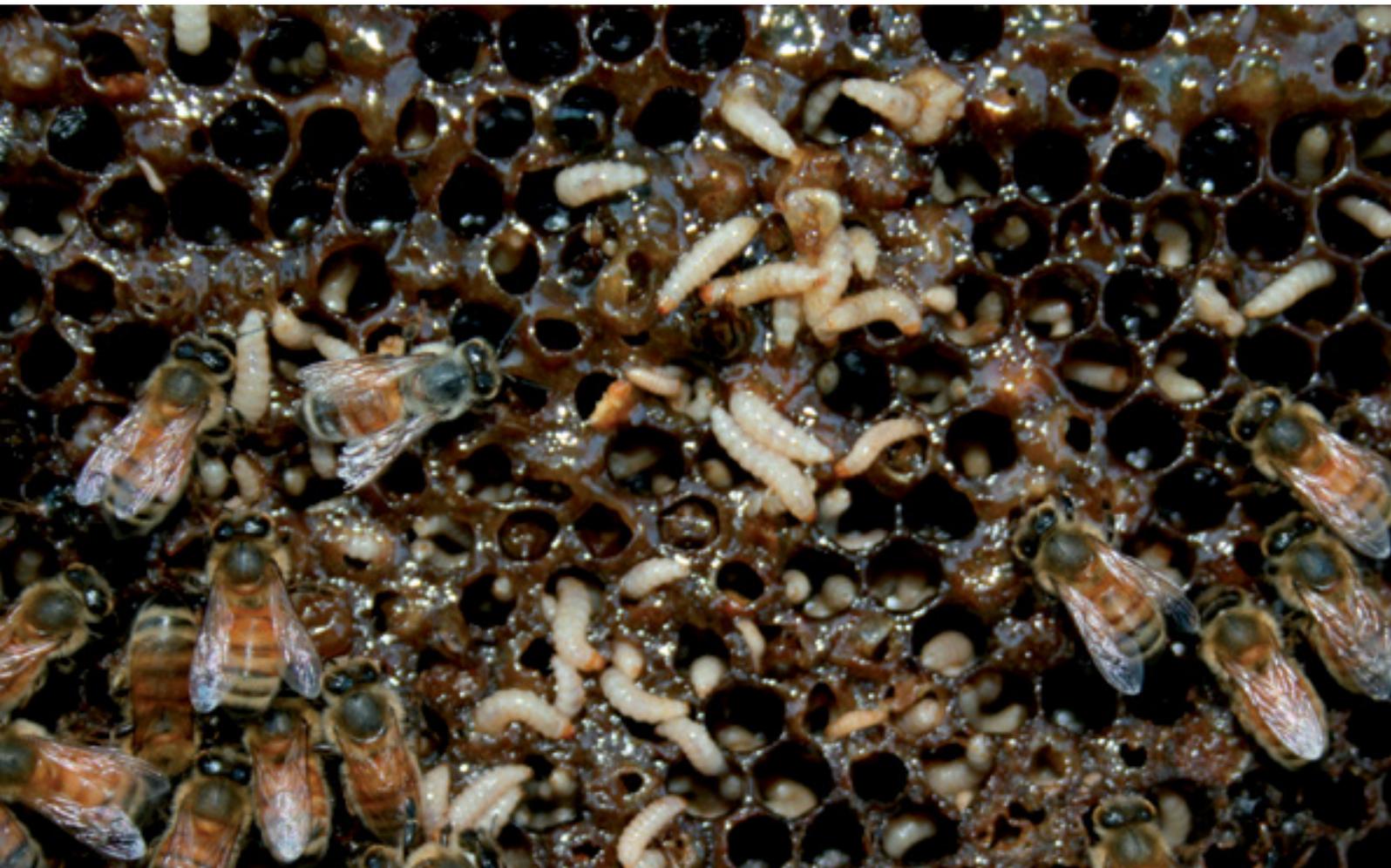


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In-hive Fungal Biocontrol of Small Hive Beetle



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Development Corporation**

In-hive Fungal Biocontrol of Small Hive Beetle

by Diana Leemon

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Researcher Contact Details

Diana Leemon
Agri-Science Queensland
Department of Employment Economic Development and Innovation
Ecosciences Precinct, Level 2A West
GPO Box 267
BRISBANE QLD 4001

Email: Diana.Leemon@deedi.qld.gov.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600

PO Box 4776
KINGSTON ACT 2604

Phone: 02 6271 4100
Fax: 02 6271 4199
Email: rirdc@rirdc.gov.au
Web: <http://www.rirdc.gov.au>

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Foreword

The Small Hive Beetle (SHB) was first reported in Richmond, New South Wales, Australia in 2002. It has now spread throughout eastern Australia to Mareeba in the north and the Melbourne CBD in the south. The minimum value of losses reported by Queensland beekeepers surveyed over three consecutive summers was estimated at \$8 million. When conditions are suitable, beetles lay their eggs on honeycomb and brood within hives and honey sheds. The eggs rapidly hatch to larvae which feed rapaciously on brood, stored pollen and honeycomb. Honey quickly becomes contaminated and begins to ferment, rendering it useless for extraction. Extreme infestations lead to a total collapse of the hive with a subsequent meltdown of the hive products as they are turned to a mass of strongly odorous slime in which thousands of SHB larvae develop. De-contaminating hives is a costly and time-consuming exercise with potential health risks from the yeasts in the slime.

Many control strategies have been implemented to minimise the impact of SHB although there is a need to investigate more options, especially non-chemical controls.

Beekeepers and future researchers will benefit from the findings in this research. These findings provide information for the safe management of larval infestations and lay the foundation for future investigations into a range of non-chemical control strategies for small hive beetle infestations of apiaries.

This research obtained a proof of concept for the control of SHB larvae exiting hives using the fungus *Metarhizium*. The research also identified several isolates of the fungus *Beauveria* able to kill adult SHB and reduce the fecundity of surviving beetles through exposure to spores in refuges. Laboratory assays and electron microscopy studies also provided support for the use of diatomaceous earth rather than oil in SHB traps deployed within hives. Extensive studies on the yeast *Kodamaea ohmeri*, known to be associated with SHB overseas, found it to be present in SHB populations in Australia. The findings in these studies also suggest this yeast to be a symbiont conferring benefit to the SHB. Moreover it was also shown that precautions should be undertaken when handling ‘slimed up’ hives destroyed by SHB larval infestations. Strains of *K. ohmeri* found in the slime and in SHB in Australia are genetically very similar to pathogenic strains which have been isolated from immunocompromised patients overseas. Studies on the volatiles emanating from both *K. ohmeri* and the slime associated with collapsed hives confirmed their attractiveness to adult SHB and identified key chemical components common to both.

This project was funded by the RIRDC Honeybee R&D program and co-funded by the State of Queensland acting through the Department of Employment, Economic Development and Innovation.

This report is an addition to RIRDC’s diverse range of over 2000 research publications and it forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry through the organisation, funding and management of a research, development and extension program that is both stakeholder and market focussed.

Most of RIRDC’s publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns

Managing Director

Rural Industries Research and Development Corporation

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Abbreviations

ARI – Animal Research Institute

CER – Controlled environment room

DE – Diatomaceous earth

DEEDI – Department of Employment Economic Development and Innovation

ESP – Ecosciences Precinct

GC-MS – Gas Chromatograph Mass Spectrometry

ITS – Internal Transcribed Spacer

NSW – New South Wales

PCR – Polymerase Chain Reaction

Qld – Queensland

RIRDC – Rural Industries Research and Development Corporation

SDA – Sabouraud's Dextrose Agar

SHB – Small hive beetle (*Aethina tumida*. Murray)

UQ – University of Queensland

UWS – University of Western Sydney

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Executive Summary

What the report is about

The small hive beetle (SHB) (*Aethina tumida*), is a native scavenger of bee hives in South Africa where it is regarded as a minor pest. It was discovered in Australia in 2002. Since this time SHB populations have increased in number and range in the eastern states of Australia where in some areas they are causing significant losses. There is concern for even greater damage from this pest to the apiary industry in the warm moist regions of Australia if management practices to check its spread are not developed and implemented.

Who is the report targeted at?

This report targets the beekeeping industry in Australia, particularly beekeepers in the warmer regions affected by the small hive beetle, and extension staff in advisory roles. It is intended to provide information on non-chemical control options for the small hive beetle as well as important information about the yeasts associated with this pest which aid in the destruction of bee hives and stored comb and potential health risks.

Where are the relevant industries located in Australia?

Currently the SHB is found in apiaries in Queensland, New South Wales, Victoria, Australian Capital Territory and a small population in the Kimberley region of Western Australia. SHB have been recorded from Mareeba in the north to the Melbourne CBD in the south. However SHB is having the greatest impact in areas where the climatic conditions suit the survival and rapid breeding of this apiary pest; particularly areas with moderate winters and warm moist summers such as those along the coastal regions of Queensland and northern NSW.

Background

Anecdotal evidence suggests SHB in Australia are causing major damage to bee hives after a return to wet summers in recent years following years of drought in the eastern states. Control options for the adult and larval stages of SHB have been investigated both overseas and in Australia. A range of traps designed around a beetle refuge principle are showing promise including one recently available trap deploying a powerful insecticide. Chemical soil drenches also appear effective against larval SHB. The use of chemicals in and around hives is limited because of toxicity to bees, and the potential for the development of resistance. The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* are being considered for in-hive beetle control because of their lower toxicity to bees. Previous research (RIRDC PRJ-000037) demonstrated that endemic isolates of these fungi can kill adult and larval SHB. Investigations first concentrated on *Metarhizium* isolates which were found to be highly effective against larvae but less pathogenic to adult beetles, although they did lower the fecundity of surviving adult beetles. *Beauveria* isolates were later found to be very effective against adult SHB in the laboratory. It was concluded from the results of PRJ-000037 that in hive control of SHB may be feasible but more research with *Beauveria* isolates was needed. Researchers in the US isolated the yeast *Kodamaea ohmeri* from adult and larval SHB, and reported that it produces a range of volatiles including bee “alarm pheromones” which were highly attractive to SHB. The use of such SHB attractants in traps might increase the uptake of fungal spores by SHB.

A *Metarhizium* based fungal biopesticide has the potential to control larval SHB outside of the hive, while *Beauveria* used with an attractant in a refuge trap has the potential for controlling adult SHB in the hive through limiting their reproductive capacity. Some beekeepers report using

diatomaceous earth (DE) in traps inside hives as an alternative to chemicals or oil, however little empirical data exists on the efficacy or mode of action of DE on SHB.

Aims/objectives

This research aims to provide information on non-chemical control options for larval and adult SHB as well as a better understanding of the yeast *Kodamaea ohmeri* and its relationship to the SHB. In particular this research focuses on:

1. Characterising and screening a range of *Beauveria bassiana* isolates from Queensland to determine their virulence towards adult SHB, then using a practical method that could translate to in hive testing to expose beetles to spores of the most virulent isolates
2. Determining the effect on SHB fecundity of sub-lethal doses of spores of the most virulent isolates of *B. bassiana*
3. Investigating the effect of diatomaceous earth on adult SHB
4. Developing a formulation and application method for applying *Metarhizium anisopliae* spores to the soil around a hive to inhibit the pupation of larval SHB
5. Conducting an investigation into the volatiles associated with *K. ohmeri* and SHB affected hive products, including a chemical analysis of the primary chemical components and the attractiveness of these materials to SHB
6. Exploring the practicality of using *K. ohmeri* associated attractants for trapping adult SHB
7. Confirming the presence of the beetle vectored yeast *Kodamaea ohmeri* in adult SHB collected from at least 10 different locations throughout Eastern Australia
8. Determining if the yeast *K. ohmeri* is present in all stages of the SHB life cycle, including where it might be carried inside adult SHB and any role(s) it might have in the life cycle of the SHB
9. Using molecular techniques to identify and compare isolates of *K. ohmeri* taken from Australian collected SHB to published records of *K. ohmeri* including isolates taken from immunocompromised human patients
10. Conducting a preliminary investigation into the virulence of SHB derived isolates of *K. ohmeri* to mammals.

Methods used

Isolates of the fungi *Beauveria* and *Metarhizium* were obtained from either soil samples or dead insects, including SHB, in Queensland. Laboratory assays and field trials were used to investigate the potential of these fungi for the control of larval and adult SHB. Laboratory assays were used to assess the efficacy of Diatomaceous earth (DE) in SHB traps; subsequently electron microscopy was used to help understand the uptake by and action on SHB of DE.

Extensive studies were undertaken to understand the yeast *Kodamaea ohmeri* associated with SHB using microscopy, molecular investigations, gas chromatograph mass spectrometry (GC-MS) investigations of volatiles, insect behavioural studies and mouse virulence studies.

Results/key findings

This research showed that a *Metarhizium* based control added to soil can infect and kill a large proportion of larvae entering the soil to pupate, thus preventing the build up in SHB numbers around hives. The negative impact of predation and weather on pupating SHB was also highlighted. Several isolates of *Beauveria bassiana* highly virulent to adult SHB with the potential for in hive testing were

identified; furthermore it was shown that sub-lethal doses of these fungi will significantly reduce the fecundity of surviving beetles.

The ability of DE to kill adult SHB when exposed to it in traps was confirmed, while electron microscopy showed that DE particles adhere to and coat the sensilla of adult SHB rather than scratching the surface as has been postulated.

Extensive studies into yeasts and the SHB found that *K. ohmeri* was present in all samples of adult SHB collected throughout NSW and Qld, furthermore this yeast was also found in all stages of the SHB life cycle including a dominating presence in the slime associated with larval SHB hive destruction. *K. ohmeri* was also isolated from different regions of the gut of adult female and male SHB. These findings support the hypothesis that *K. ohmeri* is an important symbiont of the SHB providing nutritional support for the larval, and possibly, adult stages. Molecular studies revealed the genetic diversity of the Australian isolates of *K. ohmeri* showing there are Australian isolates with identical genetic profiles (via internal transcribed spacer [ITS] sequencing) to the two isolates of *K. ohmeri* previously obtained from adult and larval SHB in Florida and Kenya respectively. Of concern is the similarity of the genetic profile of some Australian SHB derived *K. ohmeri* isolates to that of clinical isolates responsible for fungemia in immuno-compromised patients from Kuwait and Brazil. The results of a mouse model virulence study with two SHB derived *K. ohmeri* isolates were negative. However a more comprehensive study is needed to gain a clear understanding of the potential for human infection from the SHB vectored isolates of *K. ohmeri*.

Studies with the volatiles from hive products, yeast and yeast modified hive products established a hierarchy of attractiveness to adult SHB. Chemical analyses of the volatiles identified compounds common to both pure yeast cultures and the slime produced in a larval SHB mediated hive collapse. However differences in the volatile components were also noted. Traps with an attractant mix consisting of hive products, slime and yeast were successfully deployed in the larval field trial to trap emerging adult SHB, providing support for the concept of an out of hive attractant trap for use in apiary sites.

Implications for relevant stakeholders for

These findings provide a better understanding of the SHB associated yeast *K. ohmeri* and provide data to support further research into the development of a fungal control for SHB larvae and a synthetic SHB attractant. The molecular investigations into *K. ohmeri* provide important information that highlight potential health problems associated with slimed up hives.

Recommendations

The results obtained in this study support further research into a *Metarhizium* control for larval SHB and further studies on the relationship between SHB and *K. ohmeri* in regard to the development of a synthetic SHB attractant and the potential for human infection from *K.ohmeri* in hive slime.

Specifically:

- Further evaluation and development of a *Metarhizium* based commercial product for controlling SHB larvae in the soil under hives and an investigation into the Australian Pesticides and Veterinary Medicines Authority registration status of such a product to help assess the economics of the development of such a product
- Further research into the use of *Beauveria* in traps inside hives is not recommended at this point because of the success of the Apithor® trap currently on the market
- Further studies into the attractiveness of the components of volatiles arising from *K.ohmeri* slimed hive products to SHB including a detailed analysis of SHB behaviour to provide data to

underpin the development of an out of hive attractant trap with synthetic attractant. In addition ecological research should be carried out to provide information to optimise trap design, placement and optimal time of year to deploy for use

- Further investigations into the genetic variation of SHB vectored *K. ohmeri* and potential for virulence towards humans. This should involve more extensive field sampling from SHB, with molecular and growth characterisation of *K. ohmeri* followed by a simple mass screening with the *Galleria* larval model, if this model works with *K. ohmeri*. Further screening of selected isolates with the mouse virulence model using both a different method of exposure, preferably through aspiration, and immuno-suppressed or immuno-compromised mice
- Health warnings in regard to the potential of *K. ohmeri* infection from slimed up hives together with instructions on how to safely clean up slimed up hives should be disseminated to bee keepers. The warning ought to i) Advise beekeepers that simple precautions will minimise exposure to *K. ohmeri* in the slime associated with SHB mediated hive collapse and ii) recommend that a face shield and disposable gloves be worn when handling slimed frames and hive boxes which should be treated with a solution of household bleach (10% dilution) before hosing the slime.
- An investigation into the extent of *K. ohmeri* yeast contamination in honey sent to commercial packers. If cells are in the honey such a study should also aim to establish what level of yeast cells is acceptable and measureable, viability of the yeast in honey and treatments to inactivate yeast cells without affecting the honey.

Introduction

The small hive beetle (SHB) *Aethina tumida* Murray (Coleoptera: Nitulidae) is a scavenger beetle of honey bees, *Apis mellifera* L. first described by Murray (1867). The beetle is native to sub-Saharan Africa where it is a minor pest of little economic importance restricted to infesting weak, stressed or diseased bee colonies (Ellis & Hepburn, 2006; Neumann & Elzen, 2004; Lundie, 1940). These beetles were first detected in Florida (US) in 1998 (Elzen *et al.*, 1999) however the introduction of the SHB was likely earlier as beetles collected from honey bee colonies in South Carolina in 1996 were later identified as SHB (Hood, 2000). The SHB rapidly spread to more than 30 other states, mostly along the eastern coast of the United States (Neumann & Elzen, 2004). SHB soon reached major pest status in the USA when an estimated loss of US\$3 million was attributed to SHB destruction in 1998. SHB has now established in Australia (Gillespie *et al.*, 2003) and been detected in Egypt (Hassan & Neumann, 2008; Mostafa & Willimas, 2002), Portugal (Ritter, 2004) and Canada (Clay, 2006).

Aethina tumida was first confirmed in beehives around Richmond in New South Wales October 2002, followed by reports from SE Queensland, although their potential existence had been flagged during the previous twelve to eighteen months (Australian Honey Bee Industry Council, 2008). By August 2005 SHB were reported in Victoria and the Goulburn Valley (Knoxfield and Ararat, 2005; Fletcher and Cook, 2005; Hood, 2004). Fletcher and Cook (2005) voiced concern about the greater potential for damage to hives in North Queensland if the beetles spread to tropical regions as it is believed that SHB will thrive under warm moist conditions. Since then SHB has proliferated in SE Queensland and is causing major hive damage after a post drought return to moist weather. Surveys conducted in Queensland over the years 2009, 2010, 2011 estimated the losses attributed to SHB destruction of hives to be in excess of \$8 million (Leemon, unpublished).

SHB damage honey bee colonies by eating unprotected bee brood, eggs, honey and pollen (Swart *et al.*, 2001; Ellis & Delaplane, 2008). Larval SHB also cause extensive damage to honey frames, stored combs, pollen and brood when they feed and leave wastes behind. The resulting fermented honey is rejected by honey bees and cannot be marketed by the beekeeper. Heavy infestations may also result in hive death, queens ceasing to lay eggs or bees absconding from their hives (Hood, 2004; MAAREC Publication 4.6, 2000; Hepburn & Radloff, 1998). SHB invasion has also negatively affected the queen and package bee production business and there are concerns for other commodities such as fruits and possible threats to Bumble bee and other non-*Apis* species (Hoffman *et al.*, 2008; Spiewok & Neumann, 2006; Hood, 2004).

Adult SHB are strong fliers and are capable of flying several kilometres. Torto *et al.* (2005) showed that SHB are attracted to a range of hive odours, particularly the odour of adult worker bees. SHB are sexually mature at about one week following emergence from the soil. Adult females will oviposit directly on pollen or brood comb if unhindered by worker bees. It has been estimated that female beetles may potentially lay between 1000 and 2000 eggs in their lifetime (Schmolke, 1974; Somerville, 2003). The beetles will oviposit in cracks and crevices around the periphery of the inside a highly populated bee colony, but they will also oviposit in the brood area if unhindered by adult bees. Most eggs hatch in about three days but the incubation can continue for up to six days (Lundie, 1940). Egg hatching viability is negatively affected by low relative humidity (Somerville, 2003). The larval period lasts an average of 13.3 days inside the bee colony. Mature larvae exit the hive in the late evening and enter the soil to pupate, this process takes about eight days (Schmolke, 1974). Female beetles pupate slightly faster than males with pupation success affected by soil moisture (Ellis, 2004). Drier soils hinder pupation, but pupation success can range from 92-98% if the soil is moist. This implies that beetle pest problems can be expected where soil moisture remains high during the year (Hood, 2004). This may explain why the beetle numbers have increased so dramatically in south east Queensland following good rain periods in the last two years. Another factor contributing to the massive build up in SHB populations is the warmer temperatures during summer when the rains have fallen. Egg incubation is accelerated by high temperature and the exposure of larvae to 34°C also

accelerates their development. Guzman and Frake (2007) reported that at 34°C the SHB life cycle was approximately 23 days, 9 days shorter than the 32 day life cycle reported by Schmolke (1974) at 30°C. Guzman and Frake (2007) also observed an extension of development time to more than 39 days when SHB were exposed to lower temperatures between 24-28°C.

Various methods for controlling all life stages of *A. tumida* have been trialled. Cultural control methods include maintaining strong, clean colonies (Waite and Brown, 2003), encouraging hygienic behaviour in bee colonies (Ellis *et al.*, 2003) and modifying hive entrances to impede beetle access (Ellis *et al.*, 2002). Mechanical control methods include in-hive traps (Hood and Miller, 2003) and light traps (Neumann and Elzen, 2004). Chemical methods of control include coumaphos and fluvalinate in-hive treatments and the treatment of soil surrounding hives using permethrin (Levot and Haque, 2006; Hood 2000). Somerville (2003) conducted a comprehensive study of small hive beetle and its control in the USA. This review noted ways Australia can learn from the USA experience and better manage the beetle problem.

Eradication of *A. tumida* from Australia is not regarded as possible and thus research is focussing on management strategies to minimise damage to honey production and pollinating bees. The use of chemicals such as CheckMite + Strips (coumaphos) and Apistan (fluvalinate) within the hive for SHB control is limited by their toxicity to bees and mammals; increasing issues with resistance and risk of contamination and residues in honey and wax (Sugden *et al.*, 1995). Gardstar (permethrin), a soil drench targeting the soil dwelling stage of the beetle is also highly toxic to bees so extreme caution must be taken to avoid contact with any bees, hive equipment and other surfaces to which bees may come into contact (Hood, 2007; RIRDC 2005).

Various modified hive designs have been developed to aid SHB management. However, inconsistent beetle control has been reported with the use of an upper hive entrance opposed to a lower hive entrance. Decreased production of adult bees and brood, impaired thermoregulation, excessive floor debris and poor drainage have also been associated with an upper hive entrance (Hood, 2004). Traps containing oil such as the “West Beetle Trap” require hives to be completely level to prevent oil leakage and subsequent bee mortality; and supers to be removed in order to be an effective beetle control (Hood, 2004). Some beekeepers have reported success with using diatomaceous earth (DE) in traps instead of oil (Leemon, personal observations). Bucholz *et al.* (2009) also found DE in a bottom board trap was effective at killing adult SHB. The use of traps containing early beetle attractants in apiaries for beetle control has been reported though they proved to be ineffective, most likely due to competing hive odours emanating from nearby honey bee colonies (Hood, 2004; Sugden *et al.*, 1995). More recently reports suggest the efficacy of traps can be increased by the use of attractants from the yeast *Kodamaea ohmeri* (Benda *et al.*, 2008; Nolan & Hood, 2008; Torto *et al.*, 2007a,b).

In addition to the range of hive modifications, traps and some chemical treatments available it is perceived that there is still potential to explore more non- chemical means of managing SHB. One strategy could involve the use of a fungal biopesticides based on spores of *Metarhizium anisopilae* or *Beauveria bassiana*.

Metarhizium anisopilae and *Beauveria bassiana* occur worldwide in the soil and in insects, they are entomopathogenic fungi which have evolved to infect and kill insects. The spores of these fungi adhere to an insect surface, germinate and penetrate the insect killing it as the fungus proliferates throughout the body of the insect (Roberts,1981). The first use of entomopathogenic fungi for insect pest bio-control occurred in the late nineteenth century. The principles of biopesticide control rely upon the application of large numbers of formulated spores to a target insect to ensure a rapid death. In 2000 there were 19 fungal bio-control products based on *Metarhizium* and *Beauveria* registered around the world for insect control (Butt *et al.*, 2001). Since this time more products have been developed and registered. In Australia there are four *Metarhizium* products produced and marketed by Becker Underwood (Australia and New Zealand), for the control of pecan borer; locusts and grasshoppers; sugar cane beetle larvae and southern cockchafer larvae.

Studies by Muerrle *et al.*, (2006) indicated the potential for entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* to be used to control *A. tumida*. They found an increased mortality in *A. tumida* treated with *B. bassiana* (74%) and *M. anisopliae* (28%) compared to control insects. Leemon and McMahon (2008; RIRDC PRJ 000037) identified a number of isolates of *M. anisopliae* and *B. bassiana* endemic to Queensland that showed good efficacy against adult and larval small hive beetles. The *B. bassiana* isolates were more effective against the adult beetles, while the *M. anisopliae* isolates were more effective against the larval SHB. They also found that although the *M. anisopliae* isolates did not kill high numbers of adult beetles the fecundity of the surviving beetles was lowered. This research suggested the potential of *B. bassiana* isolates for the control of adult beetles by needs further investigation. With the recent advances in SHB specific attractants (Benda *et al.*, 2008; Nolan & Hood, 2008; Torto *et al.*, 2007a,b;) it might be possible to combine the spores of virulent isolates of *B. bassiana* with beetle yeast attractants to increase the uptake of fungal spores by adult SHB in devices inside and outside of hives.

Objectives

This research aims to provide information on non-chemical control options for larval and adult SHB as well as a better understanding of the yeast *Kodamaea ohmeri* and its relationship to the SHB. In particular this research focuses on:

1. Characterising and screening a range of *Beauveria bassiana* isolates from Queensland to determine their virulence towards adult SHB, then using a practical method that could translate to in hive testing to expose beetles to spores of the most virulent isolates
2. Determining the effect on SHB fecundity of sub lethal doses of spores of the most virulent isolates of *B. bassiana*
3. Investigating the effect of diatomaceous earth on adult SHB
4. Developing a formulation and application method for applying *Metarhizium anisopliae* spores to the soil around a hive to inhibit the pupation of larval SHB
5. Conducting an investigation into the volatiles associated with *K. ohmeri* and SHB affected hive products, including a chemical analysis of the primary chemical components and the attractiveness of these materials to SHB
6. Exploring the practicality of using *K.ohmeri* associated attractants for trapping adult SHB
7. Confirming the presence of the beetle vectored yeast *Kodamaea ohmeri* in adult SHB collected from at least 10 different locations throughout Eastern Australia
8. Determining if the yeast *K. ohmeri* is present in all stages of the SHB life cycle, including where it might be carried inside adult SHB and any role(s) it might have in the life cycle of the SHB
9. Using molecular techniques to identify and compare isolates of *K. ohmeri* taken from Australian collected SHB to published records *K. ohmeri* including isolates taken from immunocompromised human patients
10. Conducting a preliminary investigation into the virulence of SHB derived isolates of *K. ohmeri* to mammals.

Methodology

Fungal isolates

All *Metarhizium* and *Beauveria* isolates used in these studies were from the Queensland DEEDI entomopathogenic fungal culture collection housed at the Ecosciences Precinct (ESP), Dutton Park. These isolates were obtained from either soil samples or dead insects, including small hive beetle adults and larvae, collected in Queensland. Cultures are stored at 4°C and -22°C on agar slants of Malt Extract agar (*Beauveria* isolates) and Sabouraud's Dextrose Agar (SDA) (*Metarhizium* isolates).

Spores for assays and field trials were produced via a biphasic process. A liquid culture was first grown to inoculate solid media. The liquid culture consisted of 150 ml of sterile yeast peptone broth in 250 ml Erlenmeyer flasks inoculated with spores scraped from Oatmeal agar (Difco™) plates. Cultures were grown for 5 days at 28°C on an orbital shaker. Mushroom spawn culture bags containing 500 g steam sterilised rice or 300 g steam sterilised oat flakes were chemically sterilised with 60 ml 1.5% sodium metabisulphite for 24 hours, then neutralised with 12 ml saturated sodium bicarbonate. Each bag was inoculated with 75 ml of the liquid culture. Rice was used for *Metarhizium* production and oats were used for *Beauveria* production. Extra sterile water was added to the bags to bring the total moisture to 40%. Inoculated bags were incubated for seven days at 28°C on wire racks; the solid cultures were then broken up and left for further 10 days of growth. Bags were opened and left to air dry for 3-4 days at 19°C in a de-humidified room. Spores were harvested from the dried grain through a series of sieves (1 mm, 300 µm and 150 µm) on an Endicott sieve shaker. Spore powder was stored at 4°C. Unharvested dried rice with *Metarhizium* spores was used for some larval control investigations.

Thermal growth characteristics of isolates were determined by measuring radial growth on SDA plates over 14 days at a range of temperatures from 25°C to 35°C.

Small hive beetle colony

General Rearing

The small hive beetles used for this study were from a laboratory colony set up by sourcing adult and larval SHB from various locations around Queensland and New South Wales. The colony was kept in the insectary at The Animal Research Institute Yeerongpilly, Qld (ARI), then at the Ecosciences Precinct (ESP) Dutton Park, Qld. Adult beetles were kept under 12:12 hr light:dark while the larvae and pupae were kept in continuous darkness. The temperature of the insectaries was kept at 28°C and relative humidity of 65%.

Adult beetles were maintained in boxes 22 cm x 21 cm with ventilated lids, and fed on a diet of granular white sugar (sucrose) provided in a 9 cm Petri dish lid. Dampened sponges (6 × 4 cm) and pieces of damp crumpled paper towel provided both moisture and harbourages for the beetles (respectively).

Larvae were reared in a separate container (22 cm x 21 cm) filled with sand (with 10% moisture) to a depth of 12 cm, and a purpose made black plastic bag (19 cm x 14.5 cm) containing broodcomb, pollen and honeycomb was placed on top of the sand. Thirty beetles (15 male and 15 female) were placed inside the bag and the bag was sealed shut. Every 3-4 days the container was checked and moisture added if needed. After 12-15 days the larvae matured to the wandering stage and at day 17-20 the bag was emptied and removed and any larvae which had not entered the sand to pupate were placed on the sand. Pupation lasted 2 weeks, and once beetles started to emerge, 1-2 pieces of wetted,

crumpled up paper towel were placed on the sand to act as a harbourage and prevent beetles which have emerged from dehydrating. Adult beetles were then removed to separate containers as described above.

If adult SHB of a known sex were required the thorax of the beetles was gently squeezed to expose the ovipositor (female), if present.



Figure 1. Materials used for rearing SHB in the laboratory. Purpose made black plastic bag with pieces of honeycomb, pollen and brood comb used for SHB larval production (top left); same larval production bag approximately 10 days later (top right); adult SHB emerging from soil after pupation (bottom left); container used for maintenance of adult SHB (bottom right)

Rearing for yeast investigations

Sterile beetle production

Thirty wandering stage larvae were surface sterilised by washing first in sterile water followed by 70% alcohol and again in sterile water. Larvae were then added to steam sterilised soil (~20 % moisture) in a clear plastic container (9 cm diameter × 16 cm height) for pupation. Larval activity and moisture content were checked every 2-3 days. The stage of development was clearly visible through the clear sides of the containers. This method provided both sterile pupae and beetles for yeast isolations. The effectiveness of the surface sterilisation protocol was checked by allowing samples of larvae to crawl across the surface of SDA plates. Plates were then incubated at 27°C for 3 days.

Virgin beetle production

To obtain truly virgin adults, beetles were pupated as described above and when adults appeared ready to emerge they were physically removed from the sand and stored in separate containers to guarantee these beetles had no contact with other individuals.

Mated beetle production

An even number of sexually mature adult male and female beetles (20 each) were placed into a rectangular plastic container with a small amount of protein cake (C B Palmer Pollen Enriched Bee Feed) (3 cm x 3 cm). After 7 days the substitute was checked for larvae to establish that mating had occurred. The beetles were removed for yeast isolation.

Beetle egg and mucilage harvesting

Two glass microscope slides were joined at each end by 2 small rolls of protein cake (C B Palmer Pollen Enriched Bee Feed), allowing a 2-2.5 mm gap between the slides. The slides were covered loosely with black plastic and placed in a round plastic container (9 cm diameter x 13 cm height) with 30 adult SHB and incubated for 12-24 hours at 28°C and 65% humidity. Beetle eggs were harvested from the gap between the slides using sterile forceps under a stereo microscope. The mucilage around the eggs was carefully removed with sterile fine point forceps immediately after harvesting the eggs to prevent desiccation of the mucilage.

Larval SHB control

Laboratory assays

One new isolate of *Metarhizium*, M91 from soil in the UWS apiary site was screened against SHB larvae under lab conditions. Dried fungal spores on rice (7.5 g) were gently mixed through 200 g moistened sand in a round plastic container (9 cm diameter, 500 ml capacity), then 20 wandering stage larvae were added. The containers were sealed with ventilated lids (gauze inserts) and incubated for 7 days at 27°C and 65% RH. Controls were the same except for the fungal treatment. Three replicates were used for the treatment and control. A destructive assessment was conducted on day 7 by sieving the sand and assessing the number of live and dead larvae and pupae. The assay was repeated twice.

Preliminary field trials

Three preliminary field trials were undertaken testing *Metarhizium* isolates (M16, M81, and M91) in two formulations as soil treatments for controlling wandering-stage SHB larvae seeking subterranean pupation sites. Trials were undertaken onsite at the Animal Research Institute in a shaded location receiving dappled light. One isolate was tested per trial. In each trial nine plastic containers (ea 35 x 25 x 12 cm) with perforated bottoms for drainage were partially submerged in the ground and filled with coarse sand 10cm deep to simulate ambient soil conditions (Figure 2a). Three containers each were treated with 1 L of an oil-based isolate formulation (3 g spores in 150 ml codacide oil, in 3 L of tap water) mixed into the sand; another three containers each were treated with 167 g of dried spores on rice (unharvested spores cultured on rice) mixed into the sand. The remaining three containers received no treatment and functioned as controls. After treatment, 100 laboratory-reared wandering-stage larvae were added to each container, and the containers were sealed with ventilated lids and gauze, which allowed air flow and prevented escape of emerged beetles (Figure 2b). In addition, wandering larvae were reared to emergence in the laboratory (in white sand at 27°C) to indicate when the trial beetles might emerge in the field. Once emergence began, each week counts of emerged beetles were made and pieces of crumpled paper were added to the containers to act as harbourages for the beetles.

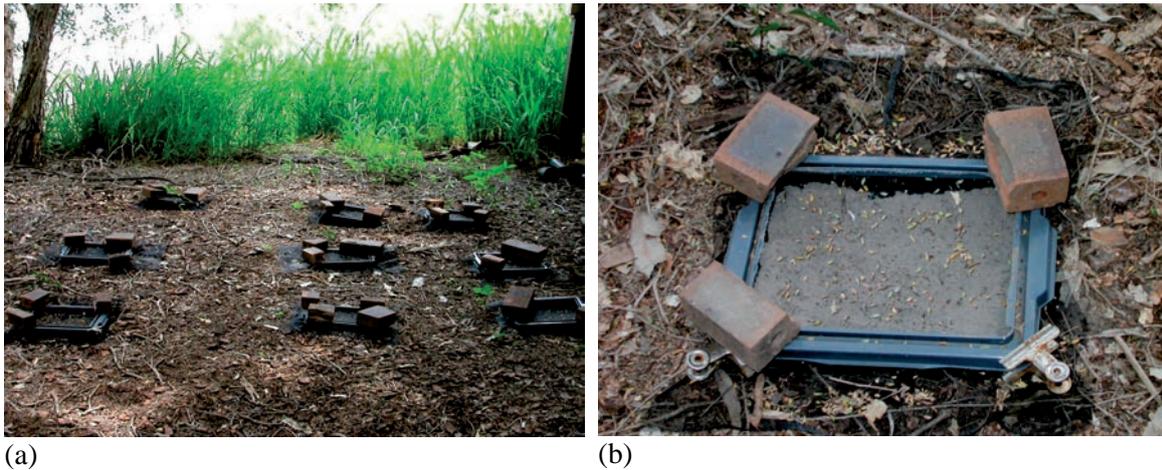


Figure 2. a) Preliminary field trial enclosures buried in the ground; b) A buried enclosure with lid and gauze, held closed with bricks

Field trials

Four field trials were undertaken to test the efficacy of *Metarhizium* as a soil treatment for controlling wandering SHB larvae seeking subterranean sites for pupation. All four trials were undertaken at the Animal Research Institute. Trials 2-4 were conducted on the same site (Figure 4d), while Trial 1 was conducted on a different site (Figure 3d). Each trial used a similar methodology: Large plastic containers with drainage holes were partially submerged in the ground and filled with substrate to simulate ambient soil conditions. The containers were enclosed using cages made of gauze and pipe to prevent egress and ingress of small hive beetles. In half of the containers a *Metarhizium* treatment was applied to the substrate; in the others the substrate was left untreated (controls). The design of the containers and enclosures as well as the source of wandering (late instar) SHB larvae varied in the trials. A simulated beehive containing SHB larvae was placed on the substrate in each container and wandering larvae were allowed to exit and pupate in the substrate for a fixed period, after which the hive was removed. When adult beetles began emerging, pieces of crumpled paper towelling for beetle harbourage were placed on the substrate and traps to attract emerging beetles were suspended from the cage ceiling. Numbers of emerged beetles from each enclosure were removed and counted three times per week when paper and traps were replenished.

Attractant traps were added later in Trial 1, then in each of the following trials (2-4) with the design of the trap and attractant mix being modified to maximise adult capture in each subsequent trial. Traps consisted of cylindrical clear plastic jars (250 ml) containing attractant. Each jar had a screw on plastic lid with three 5 mm holes to allow beetle entry and a mesh funnel to prevent beetle escape (Figure 5). The attractant consisted of honeycomb, yeast culture (*Kodamaea ohmeri*) grown on agar and fermented slime in which SHB larvae were breeding. In the final trial traps had a square (15 x 15 cm) of crumpled paper placed on top of the attractant mix to act as a harbourage and black plastic was inserted around the inside of the trap to provide a dark hiding place for adult SHB. Due to the frequent rain events traps were also fitted with lids suspended above them to prevent rain from filling up the traps.

Trial 1

Trial 1 was conducted in a field with full sun until late afternoon. Six plastic containers (ea 40 x 60 x 13 cm) were used. Each was filled with coarse sand to a depth of 10 cm and enclosed by an 80 cm high dome-shaped cage built from crossed pipe covered with tulle. Bulldog clips and elastic secured

the tulle to the container (Figure 3c). This trial evaluated one treatment against a control replicated three times. The sand in each of three of the plastic containers was treated with a mixture of spore powder (4 g) and dried spores on rice (200 g) of a 50:50 mixture of the *Metarhizium* isolates M16 and M81. This mixture was lightly mixed through the top layer of sand. A “slime out” was generated as a source of larvae by adding 30 adult SHB to a nucleus hive containing two full frames, one of honeycomb and one of brood. The nucleus hives were placed on the sand in each enclosure. Mature larvae began to wander out of the nucleus hives and into the sand after 14 days. The larvae were allowed to exit the nucleus hives for one week before the hives were removed from the enclosures.



Figure 3. Materials used in Field Trial 1. a) Late instar SHB larvae on a frame of brood and honeycomb on top of the nucleus hives used for breeding the larvae. b) Slime generated from the destruction of honeycomb and brood by SHB seeping out of the bottom of a nucleus hive into the surrounding substrate. c) Enclosed nucleus hive used for the production of SHB larvae sitting on top of sand in a plastic container that has been sunk into the ground. d) Six enclosures of Trial 1 in the grounds of the Animal Research Institute

Trials 2-4

Trials 2-4 were conducted in a different field to that used in Trial 1, it had full sun from early mid-morning until late afternoon. Eight plastic containers (ea 56 × 46 × 25 cm) were used per trial. Each was filled with substrate to a depth of 20 cm (30 L). In Trial 2, white sand was the substrate, but in Trials 3 and 4, a 50:50 mix of sterile soil with organic matter (Ki-carma™ garden soil) and white sand was used. Cages (ea 34 cm wide × 40 cm high) were made of plastic tubing covered in mesh hoods and were embedded in the substrate for stability (Figure 4a, d). Elastic string was used to secure the mesh to the container (Figure 4d). Alternate enclosures (No’s 1, 3, 5 and 7) received a *Metarhizium* treatment while the control enclosures (No’s 2, 4, 6, and 8) did not receive any treatment. Treatment entailed 100 grams of fungal spores on dried rice from each of three fungal isolates (M16, M81 and

M91). This equated to approximately 10.49 g of spores in the 300 g of rice which was evenly distributed over the surface of the substrate and mixed into the top 5-7 centimetres.

A simulated “slimed-out” beehive consisting of a Styrofoam cooler (pseudo-hive) containing infested slimed up spoiled brood and honeycomb with wandering larvae was placed on the substrate in each enclosure (Figure 4b). Larvae exited through a hole in the side of each pseudo-hive (Figure 4c) to pupate in the substrate for 1 week, after which the pseudo-hives were removed. Larvae were prepared for Trial 2 by adding 40 adult mixed sex SHB to full frames of brood, honey and pollen in a large moistened plastic bag then incubating the bag for two weeks at 27°C with 70% relative humidity. After two weeks the larval-honeycomb-brood slime was homogenized and 700 gram samples were added to each of the simulated hives. Larvae were reared for Trials 3 and 4 by adding 30 mixed sex adult SHB along with 42 g brood, 90 g honeycomb, 75 g pollen comb and 5 g pollen powder to moistened breeding pouches (29 x 17.5 cm) fashioned from heavy black plastic. The pouches were incubated at 27°C and 66% humidity for two weeks before being added to the pseudo-hives. An extra breeding pouch was maintained in the laboratory as per the general rearing protocol to observe adult emergence. This helped estimate when the field adult beetles should commence emergence so that traps and crumpled paper harbourages could be added at the correct time. For Trials 2-4, 50 g samples of the larval-slime mixtures were taken and the number of larvae in the samples recorded so that the number of larvae exiting the pseudo-hives could be estimated. In Trial 2 there were approximately 2,884 larvae per pseudo-hive and approximately 960 larvae per pseudo-hive in Trials 3 and 4.

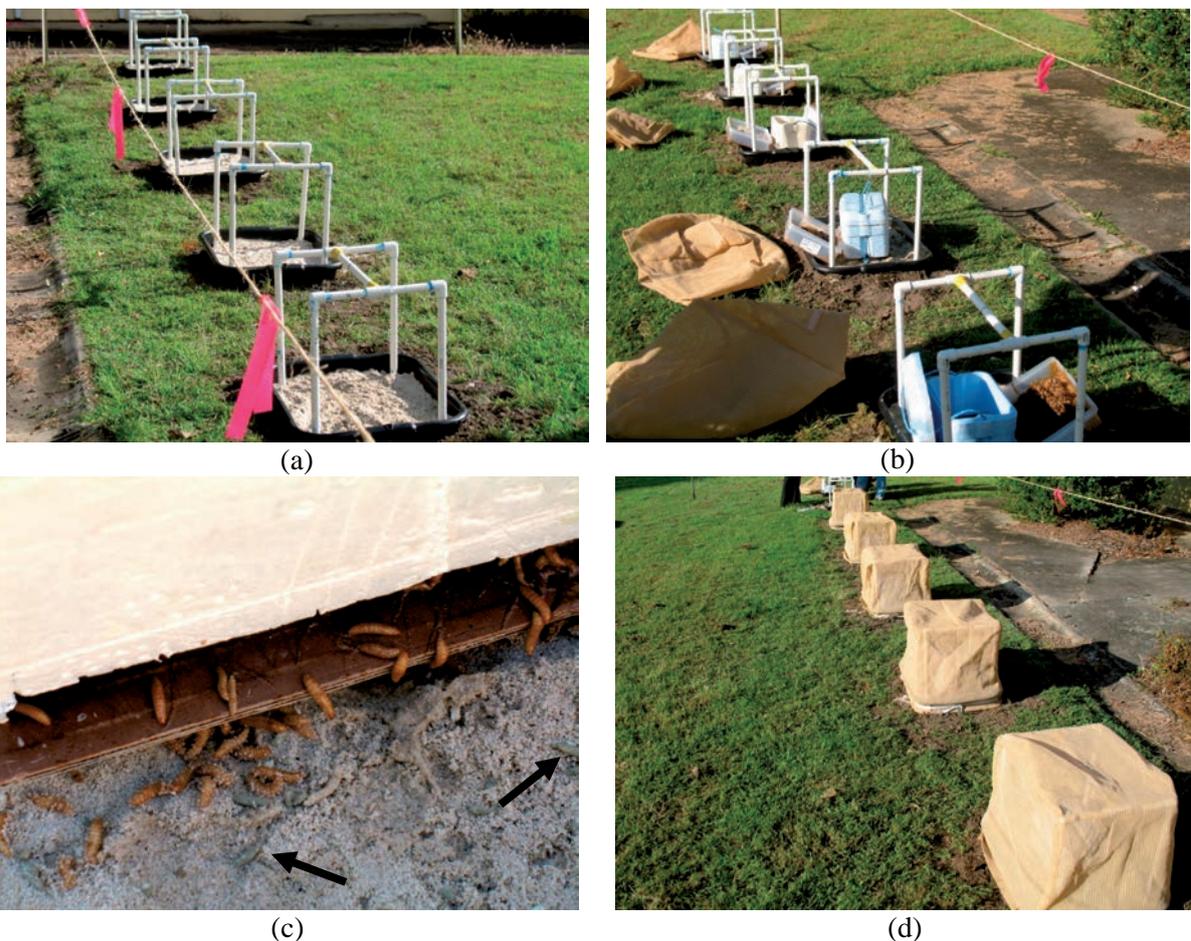


Figure 4. Design of the set up used in Trials 2–4. a) Plastic containers of sand with plastic tubing supports. b) Polystyrene pseudo-hives with containers of SHB larvae and slime ready to add to the hives c) Wandering stage larvae exiting from the pseudo-hives into treated sand with grains of *Metarhizium* covered rice (arrowed). d) Covered enclosures tied at the base with elastic string used in Trials 2–4



Figure 5. Attractant trap used to trap emerging adult SHB in field trials. Trap with honey and yeast mixture before it was added to an enclosure (left); trap with added crumpled paper and adult SHB after removal from enclosure (right)

Adult SHB control

Fungal screening

Seventeen *Beauveria* isolates were screened against adult SHB from which the best six were selected for further screening. Initially adult beetles (20) were dipped in the dry spore powder (0.2 g) of each of the *Beauveria* isolates, then placed into 1 litre rectangular plastic containers and supplied with crumpled paper harbourages, moistened sponge (7 cm x 5 cm Wettex™) and a carbohydrate source (granulated sucrose) and were incubated at 27°C and 65% RH for 14 days. The containers had gauze inserts in the lid for air exchange and a second layer of gauze under the lid to prevent beetle escape. Dead beetles were recorded and removed at day 7 and day 14. Each treatment was replicated three times and each assay was conducted at least twice. Isolations were performed on dead beetles to confirm *Beauveria* infection.

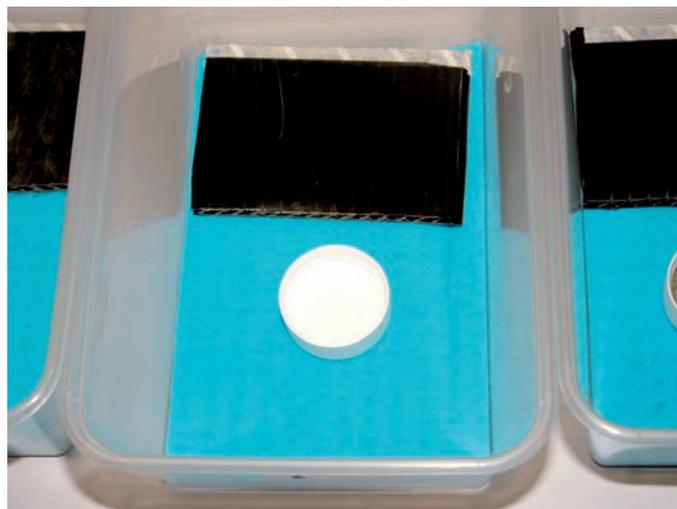


Figure 6. Plastic assay container with corflute refuge used for screening the six best *Beauveria* isolates against adult SHB

The six most effective *Beauveria* isolates were further screened to assess how well adult SHB took up lethal doses of spores from inside corflute refuges. Similar containers were used as before except 0.2 g of spores of each isolate was added to 8 cm x 5 cm corflute refuges. Controls had refuges without spores. An additional treatment in which beetles were dipped in spore powder similarly to the initial

screening assays was included. The crumpled paper harbourages were omitted, and incubation was as for the previous assays as were the mortality assessments. All treatments were replicated three times and the assay was repeated three times.

Fecundity studies

The effect of a sub lethal dose of *Beauveria* on SHB fecundity was investigated using the two most virulent isolates. Forty adult SHB (20 male, 20 female) were fed sucrose (3 g) mixed with spores (0.025 g) of isolates B43 and B46 for 7 days. Controls had plain sucrose (3 g). After day 7, the dead SHB were recorded and removed along with the contaminated sucrose, and then brood comb and pollen (40 g) were added to stimulate breeding. After 7 days the resulting larvae were transferred to breeding containers (12 cm x 17 cm x 19 cm) containing moistened sand. Protein cake (15 g) (C B Palmer & Co. Pollen Enriched Bee Feed) was added as food for the developing larvae. These containers were incubated at 27°C and 65% RH until all larvae had pupated and adult SHB began to emerge. Once adults began to emerge they were removed to separate containers and the numbers recorded. There were four replicates per treatment in each assay and the assay was repeated twice.

Diatomaceous Earth (DE)

The diatomaceous earth (DE) used in these studies was Absorbacide® (Mount Sylvia Diatomite Pty Ltd). This product was chosen because it is readily available to Apiarists and registered by the Biological Farmers of Australia for use on organic farms.

DE in AJ's Beetle Eater ® traps

The survival and behaviour of small hive beetles in AJ's Beetle Eater ® traps with and without DE was assessed in the laboratory. Forty beetles were put in each trap (three traps per treatment and per control – Table 1) and each trap was placed in a separate container (20 x 40 x 8 cm with gauze lid). In each container substrates for oviposition (protein cake and yeast) were placed directly beside the trap to capture eggs so reproduction rates of escaped beetles could be measured. The substrates were a petri-dish containing six filter papers moistened with 12 ml of liquid culture of *Kodamaea ohmeri* (SHB associated yeast) and a 1 cm cube of bee protein cake (C B Palmer & Co. Pollen Enriched Bee Feed). A 2 cm bottle cap of granulated sugar for food, water-moistened sponge for drink, and crumpled paper towel for harbourage were also placed in the containers for the escaped beetles. Containers were incubated at 27°C and 65% RH for 7 days, after which beetle survival, escape, and reproduction were assessed.

Table 1. Bioassays used to assess behaviour and survival of *Aethina tumida* in relation to AJ's Beetle Eater ® traps

Bioassay	Conditions:
1	Treatment: trap half filled with diatomaceous earth Control: trap empty
2 (repeated)	Treatment 1: trap half filled with diatomaceous earth Treatment 2: trap half filled with cornflour Control: trap empty

Assessment of beetle cuticle with scanning electron microscopy (SEM)

Scanning electron microscopy was used to assess damage or scarification caused to the cuticle by exposure to DE. It was also used to examine distribution of DE over the beetle. Ten doused beetles that died after DE exposure were mounted on individual 12 mm aluminium stubs, using adhesive carbon-impregnated tabs, and sputter-coated to a thickness of ~10nm platinum. Beetles were observed using an electron beam of 8-10 kV in a JEOL 6300FE or 6460LV SEM. For comparison, beetles not treated with DE and beetles dusted with cornflour were assessed visually using the same observational protocol.

Yeast investigations

Initial characterisation of yeasts from hive derived slime

Initial investigations were carried out into the presence of yeasts, particularly *Kodamaea ohmeri* in slimed out hives and adult beetles. Samples of slime stained with lactophenol blue were examined under the microscope. Diluted samples of slime were streaked across plates of Sabouraud's dextrose agar (SDA) and incubated at 27°C. Adult SHB (10) were surface sterilised by immersing in 70% ethanol for 2 minutes, washed twice in sterile distilled water before being ground to a homogenate in a sterile mortar and pestle. The homogenate was streaked across plates of SDA and incubated at 27°C for 3 days.

Yeasts with two different morphologies (smooth and wrinkled) were consistently isolated from slime and adult SHB. The optimal growth range of representatives of these two morphologies was examined by inoculating the centre of plates of SDA with sterile 8mm filter paper discs dipped in yeast suspensions. Groups of 4 replicate plates were incubated for 14 days at 20°C; 25°C; 30°C; 35°C; 40°C; and 45°C before the colony diameters were measured along orthogonal axes. Cultures of the two different morphologies were sent to Dr Roger Shivas, Agri-Science Qld for identification. A preliminary investigation of the ability of the wrinkled morphology yeast to grow at different honey concentrations was conducted. Flasks of sterile honey solutions (100%; 75%, 50% and 25%) were inoculated with a 10 µl loop of the wrinkled yeast growing on a plate of SDA. After 7 days incubation at 30°C a 10 µl loop of inoculum from each flask was streaked across a plate of SDA and incubated for 5 days.

The appearance of yeast in the slime generated in a pollen-honey mix as SHB larvae developed was investigated. Ten adult SHB (5 male + 5 female) were added to 2.5 g protein cake (C B Palmer & Co. Pollen Enriched Bee Feed) in a Petri dish (3 replicates) and incubated for 9 days at 30°C to allow mating, oviposition then larval development on the protein cake substrate. The microbial flora growing on the pollen patty as the SHB larvae developed was checked daily. A sterile cotton tip was swabbed across the pollen patty mix then agitated into 0.5 ml of sterile water. The resulting suspension was diluted 1:500 before 10 µl was streaked across a plate of SDA and incubated for 7 days at 27°C.

Volatiles

Analysis of volatiles from yeasts and hive products

Nine samples of hive and hive related products were collected for analysis of their volatile compounds. Honey, wax and slime associated with the SHB induced collapse of a hive (hive slime) were collected from hives. The slime produced when SHB larvae are mass reared on brood and honeycomb in the laboratory was sampled at two different times (early and late slime). Samples of yeasts isolated from the hive slime and grown in liquid culture were also collected (smooth yeast, wrinkled yeast and combination of smooth and wrinkled grown together).

The early and late slimes were sampled from a mixture of brood comb and honey comb used for the mass rearing of SHB larvae and adults. Three frames of honeycomb and brood were taken from a healthy hive naturally infested with SHB were added to a large plastic clinical waste bag (76 x 92 cm) that was placed inside a plastic box (54 x 40 x 32 cm high). The plastic box had a perforated lid covered by gauze to allow for air flow and a 10 cm layer of moist sand on the bottom for larval pupation. Thirty-five adult SHB were introduced to the plastic bag. These were in addition to SHB that were already on the frames when taken from the hive. The early slime was sampled 10 days after larvae began to develop, while the late slime was sampled 6 weeks after the mass rearing container was set up. The hive slime was sampled from a beehive in the field which had succumbed to SHB larvae about 10-14 days earlier. Two different yeasts (smooth morphology = *Candida* sp. and wrinkled morphology = *Kodamaea ohmeri*.) which had been isolated from the hive slime were grown for 4 days in yeast peptone broth amended with honey on an orbital shaker at 27°C (yeast peptone broth: 2% honey; 1% peptone; 0.1% yeast extract). Honey and wax samples came from a hive in the same apiary as the hive from which the slime was taken.

Headspace solid phase microextraction (HS-SPME)

For analyses of the above mentioned samples, 5 g of each were introduced into a 20 ml vial. 5 ml of saturated brine solution was added and mini stir fleas were inserted into each vial to ensure the contents were mixed thoroughly. The vials containing the samples were sealed immediately and frozen until needed. This was done in order to ensure no reaction took place. Analysis was done in triplicate for each sample. In addition to the samples mentioned above, there were also triplicates of the blank and a control. The former contained nothing and the latter contained 5 ml each of Milli-Q water, saturated brine and a stir flea.

Head space mode was chosen in order to prolong the lifetime of the carboxen/Polydimethyl siloxane (CAR/PDMS) fibre used. The incubation temperature was 50°C, incubation time was 10 minutes and the extraction time was set as 45 minutes.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was carried out on a Gerstel Agilent 5890N gas chromatograph equipped with a capillary column (DB-WAX) of 30 m length x 253 µm i.d., 0.25 µm coating thickness, J&W with helium (BOC gasses, ultra high purity), being used as the carrier gas. 1 µl of the Sample was injected into the Gerstel programmable temperature vaporise (PTV) injector, which then was analysed in splitless mode at an injector temperature of 200°C for 5 minutes. The average linear velocity was 56 cm/sec, and the constant flow rate was 2.4 ml/min. The pressure was 124 KPa and the total flow was 118.1 ml/min. The initial temperature of the oven was 40°C, at which it was held for 2 minutes and then increased to 220°C. The temperature increase was gradual and was elevated by 4 degrees and held constant for 5 minutes. The total run time was 52.00 minutes and the data was collected using the MSD ChemStation D.02.00.275 (Agilent Technologies, 1989-2005, USA) software.

The peaks obtained in the spectra were compared to the National Institute of Standards and Technology (NIST) spectral library (2005) data to tentatively identify the compounds.

SHB responses to different volatile sources

A series of preliminary investigations into the attractiveness of a range of yeast and hive associated odours and volatiles to adult SHB were carried out by three groups of students from the University of Queensland (3rd Animal Behaviour, BIOL3207) using Y-tube olfactometers. The investigations were conducted under controlled conditions of constant temperature (24°C) in a darkened room lit by a red lamp to minimise phototactic interference. Each Y-tube olfactometer (Figure 7) was able to compare the attractiveness of two different odours to adult SHB, with one adult beetle examined independently against a choice of two odours in each test.

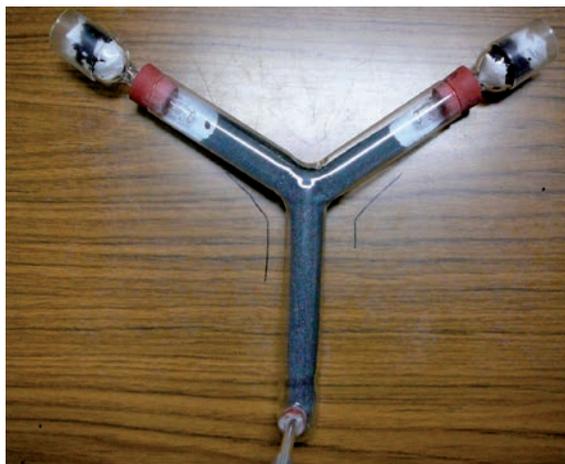


Figure 7. Y-tube olfactometer used to compare the attractiveness of different odours to SHB. One beetle at a time was introduced to the bottom of the “Y” which was then connected to a flow meter to pull air through the charcoal filters and past odours placed at the top of each arm of the “Y”

Each arm of the y-tube olfactometer was connected to a glass cylinder and odour sources were placed inside on a 300 mm² strip of filter paper. Air was drawn through the y-tube olfactometer using a vacuum with a constant airflow of 0.36 L/minute. The air was filtered through activated charcoal+glass fibre to purify the air streaming into each arm. The charcoal was changed at regular intervals to ensure no saturation of scents occurred. A new beetle was placed downwind of the airflow in each olfactometer for each test. Each trial ran for a maximum of 10 minutes or until the beetle had made a ‘choice’. The first instinctive move within an arm that lasted at least 30 seconds was determined as the beetle’s choice of scent. Each y-tube olfactometer was regularly rotated after tests to ensure there was no directional bias. Each trial was conducted using 20 independent beetles in the late afternoon when SHB were noted to be most active (personal observations of laboratory colonies of SHB).

One group of students investigated the attractiveness of materials that were also chemically analysed (see previous section). These included pure cultures of smooth and wrinkled yeasts, slime collected from collapsed hives, slime from mass rearing of larvae in the laboratory, isopentyl acetate (IPA - the bee alarm pheromone) and samples of wax and honey. A second group of students investigated another set of natural beehive attractants including honey, brood, IPA, pollen dough and early and late slime sampled from larval rearing. The third group of students investigated a range of synthetic chemical combinations of volatiles identified as common to yeasts and slime through chemical analyses (ethanol; phenyl ethyl alcohol; 1-butanol 3-methyl; 1-propanol 2-methyl, ethyl acetate and IPA).

Molecular identification

Yeast isolation from adult SHB from different locations

Adult small hive beetles of mixed sex were sampled from laboratory colonies and beehives in New South Wales (NSW) and Queensland (QLD). Ten beetles from each sample were surface sterilised by immersing in 70% ethanol and agitating for 2 min. After decanting the ethanol the beetles were rinsed in sterile water twice. The surface sterilised beetles were next ground to a homogenate with a sterile mortar and pestle. A 1 µl aliquot of each homogenate was spread across a plate of Sabouraud’s Dextrose Agar (SDA) and incubated at 27°C for 2-3 days. A single representative yeast colony was chosen for sub culturing and streaked across a fresh plate of SDA to obtain a pure colony. On some occasions the yeast had to be streaked more than once to obtain a clean pure culture. Pure cultures were maintained on SDA slopes at -4°C and also stored either at -80°C or as freeze dried cultures. For

some samples from nine locations in Qld and NSW a 1µl aliquot of the homogenate was plated onto four separate plates of SDA. A representative colony was then sub cultured from each of these four plates.

Yeast isolations from different parts of SHB lifecycle

Groups of ten individuals were sampled from each stage of the SHB lifecycle; eggs, larvae, pupae and newly emerged adults. Larvae were pre-washed in sterile water to remove as much of the yeast infested slime as possible. Pupae and newly emerged adults were produced through sterile culture as outlined previously in this report. Isolation of yeasts was then carried out as described above by surface sterilization and plating a homogenate on SDA plates. Mucilage from around eggs was removed as previously described and mixed with sterile water on a sterile microscope slide before a small amount was streaked across a plate of SDA with a sterile plastic inoculating loop (1 µl). Isolations were replicated 4 times and all plates were incubated at 27°C for three days.

Yeast isolations from adult SHB internal organs

The gut and other internal organs, including the reproductive organs, were removed from adult SHB for yeast isolation. Both mated and virgin adult female and male SHB raised as previously described in this report were dissected on a wax plate under a NIKON SMZ800 stereo microscope. Beetles anaesthetised with CO₂ were placed on a wax plate cooled by sitting on a cold pack (Medi-Pak™) to prevent the beetles reviving. Beetles were pinned at the head and lower abdomen and the elytra, wings and legs were removed before an incision was made from the oesophagus to the rectum. The interior was flushed with sterile water to remove the fat body so that the alimentary canal could easily be seen. The alimentary canal was removed and rinsed with 70% ethanol followed by sterile water before being cut to separate the fore, mid and hind gut sections. These sections were transferred to a sterile glass microscope slide and cut open to expose the internal gut wall. Liquid from this surface was transferred to a plate of SDA with a sterile plastic inoculating loop (1 µl) for incubation at 27°C for 3 days. A white kidney shaped organ, and either the testes or ovaries were then removed, squashed on a sterile slide and plated as above. All samples were replicated four times for each internal part.

The initial identification of yeasts with two different morphologies was conducted by Dr Roger Shivas. Later only yeasts with a specific wrinkled morphology were selected and subcultured to obtain pure colonies. These pure colonies were sent to the molecular mycology laboratory of A/Prof Wieland Meyer (University of Sydney) at Westmead hospital for DNA isolation and identification through micro-satellite fingerprinting and ITS sequencing.

DNA isolation

Isolates were sub-cultured onto SDA and incubated at 27°C for 72 h prior to DNA extraction. High molecular weight DNA was extracted according to Ferrer *et al.* (2001) with minor modifications. Half an inoculation loop of the culture was transferred to a microcentrifuge tube and kept at -20°C overnight. Thereafter, the fungal material was incubated at 65°C for 1 h with 500 µl of lysis buffer (17.3 mM SDS, 0.25 M NaCl, 25 mM EDTA, 0.2 M Tris-HCl) and 5 µl of 2-mercaptoethanol. After incubation, 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1), vol/vol/vol) was added to the tube and the mixture centrifuged at 14,000 rpm for 15 min. The upper phase was removed and mixed with an equal volume of isopropanol then left at -20°C overnight for DNA precipitation. After washing in 70% ethanol, the DNA pellet was resuspended in sterile deionized water. The DNA concentration for each isolate was determined by reading the UV absorbance at 260 nm (BioPhotometer, Eppendorf) and diluted to 10 ng/µl.

Species identification by ITS sequencing

Twelve *Kodamaea ohmeri* isolates, representative of each of the major clades obtained in the genotype analysis, were selected for ITS sequencing. Two yeasts with smooth morphology

representing non-*K. ohmeri* yeasts were also selected for ITS sequencing. The ITS1, 5.8S and ITS2 regions of the rDNA gene cluster were amplified with the primers SR6R (5' GTARAAGTCGTAACAAGG 3') and LR1 (5' GGTTGGTTTCTTTTCCT 3') [Vilgalis et al. 1990]. Amplifications were carried out in a 50 µl reaction volume, containing 100 ng of genomic DNA, 5 µl of PCR Buffer (100 mM Tris-HCL, pH 8.3, 500 mM KCL, 15 mM MgCl₂, 0.01% w/v gelatine), 5 µl of 2 mM dNTPS (2mM dATP, dCTP, dGTG, dTTP) (Invitrogen, Carlsbad, USA), 3 µl of 50 mM MgCl₂, 5 µl of primer SR6R, 5 µl of primer LR1, and 0.5 µl of 5 U/µl BIOTAQ™ DNA polymerase (Bioline Pty Ltd, Australia). The PCR was performed in a Perkin Elmer DNA Thermal Cyclor 480 (Norwalk, USA) as follows: 1 cycle of initial denaturation at 97°C for 3 min, followed by 20 cycles of 35 seconds denaturation at 97°C, 55 seconds annealing at 50°C, and 45 seconds of primer extension at 72°C, and 10 cycles of 45 seconds denaturation at 97°C, 55 seconds annealing at 50°C, and 2 min of primer extension at 72°C, followed by a final extension cycle for 6 minutes at 72°C. Purified PCR products were sent to MACROGEN (Seoul, Korea) for commercial sequencing using SR6R or LR1 as forward and reverse primers. Sequences were edited using Sequencer version 4.7 (Gene Codes, Ann Arbor, MI). ITS sequences obtained from the 12 isolates in this study (see above) were blasted against Genbank for species identification. To determine the relationship of the Australian *K. ohmeri* isolates with other global isolates the obtained ITS sequences were aligned with the ITS sequences of various other global isolates that have been deposited in genbank (Table 2). Phylogenetic analyses were performed using the program PAUP* version 4.06.10 [Swofford 2003].

Table 2. Reference numbers of ITS sequences from different isolates of *Kodamea ohmeri* deposited in Genbank, along with the source and location from which these isolates originated

GenBank Reference Number	Source type	Specific source	Location	Reference
EU569326	SHB	Larva	Kenya	[gb3]
AY911385	SHB	Adult	Florida, USA	[gb3]
HQ696999	Insect	<i>Trichogramma chilonis</i>	India	NRRL Y27634
FR734183	clinical	human blood cultures	Shuwaikh, Kuwait	[gb7]
FR734182	clinical	human blood cultures	Shuwaikh, Kuwait	[gb7]
FJ215865	clinical	Human blood and central venous catheter	Natal, Brazil	[gb4]
FM178297	Bird	Guano	Chile	Meyer <i>et al.</i> (unpubl)
EF192218	Environment	Seawater	China, south sea	[gb14]
EF196810	Environment	Sediments	China, east sea	[gb14]
EF198010	Environment	Mud of salterns	Indian ocean	[gb14]
EF190226	Environment	Marine algae	China, coast of Qinlao	[gb14]
AB469381	Environment	Coastal seawater	Yilan, Taiwan	TL0803 [gb10]
AY168786	Environment	Water samples	Taiwan	ST5-3 [gb11]
GU246263	Environment	Cucumber brine	USA	[gb1] CBS 5367
FJ810135	Environment	unknown	China	Dd08014 Jiang <i>et al.</i> (unpubl)
DQ666194	Fish	Gut (<i>Hexagrammes otakii</i>)	China	NRRL Y27634 [gb13]
DQ681362	Fish	Marine	Pacific ocean	[gb14]

PCR-fingerprinting based genotyping

Yeast genotypes were obtained via PCR-fingerprinting with the microsatellite specific primer M13 (5' GAGGGTGGCGGTTCT 3') and the minisatellite specific primers (GACA)₄ and (GTG)₅ (Meyer *et al.* 1993). Amplification reactions were performed in 50 µl reaction volume containing 25ng genomic DNA, 5 µl of PCR Buffer (100 mM Tris-HCL, pH 8.3, 500 mM KCL, 15 mM MgCl₂, 0.01% w/v gelatine), 5 µl of 2 mM dNTP_s (2mM dATP, dCTP, dGTG, dTTP) (Invitrogen, Carlsbad, USA), 3.5 µl of 50 mM MgCl₂, 3 µl of primer and 0.5 µl of 5 U/µl BIOTAQTM DNA polymerase (Biolone Pty Ltd, Australia) The PCR was performed for 35 cycles in a Perkin Elmer DNA Thermal Cycler 480 (Norwalk, USA) as follows: 35 seconds denaturation at 94°C, 60 seconds annealing at 50°C (Primer M13 and (GTG)₅) or 43°C (primer (GACA)₄), and 20 seconds of primer extension at 72°C, followed by a final extension cycle for 9 minutes at 72°C.

Blank control tubes containing all reagents except template DNA were included for each run; each sample was analyzed at least twice. PCR products were separated on 1.4% agarose gels at 60 V for 14 cm and photographed under UV light after ethidium bromide staining. PCR-fingerprinting patterns were analysed using the gel analysis module in the software package BioloMICS version 8.7.7.8 (BioAware®, Belgium). Images were normalized for lane to-lane differences in mobility by the alignment of patterns obtained on multiple loadings of the 1kb DNA size marker (GIBCO-BRL, USA) included at regular intervals in each gel. The unweighted-pair group method using arithmetic averages (UPGMA) and NeiLi was used to generate dendrograms based on the coefficient of similarity between the isolates. In addition to the agglomerative-clustering method, principal coordinate analysis (PcoA, BioloMICS version 8.7.7.8) was conducted to give an overall representation of the observed strain variation.

Mouse virulence study

Three *Kodamaea ohmeri* strains, two obtained from mucilage around SHB eggs and one isolated from mixed sex adult SHB sampled from a beehive in Kurrajong NSW, were investigated for their virulence in an intravenous murine model of fungal infection. Yeast cells were grown on SDA plates for 48 h at 27°C. Groups of three six to eight weeks old Balb/C female mice were inoculated via tail vein injection with 100µl of either 5x10⁴, 1x10⁵, 1x10⁶, 1x10⁷, 1x10⁸ and 1x10⁹ cells/ml as well as a saline control without yeast cells in accordance with the approved animal ethics protocol #5071 (Western Sydney Area Health Animal Ethics Committee). The mice were placed in standard mice filter top cages with access to water and food ad libitum. The mice were observed for signs of infection (e.g. difficult breathing, neurological symptoms, ruffled fur, lethargy, poor eating) daily. As no signs of infection were visible after 1 month the mice were euthanised with 5% CO₂ and the lung, brain and spleen were harvested, homogenised in 85% saline and 100µl of the 10⁻² and 10⁻⁴ dilutions of the organ suspensions were plated on SDA plates and incubated for 48h at 27°C.

Results

Fungal isolates

Fungal isolates used in this research were isolated from a range of sources over a period of 8 years and are maintained in the DEEDI animal science entomopathogenic fungal collection held at the Ecosciences Precinct (Table 3). Most of the *Beauveria bassiana* strains were isolated from insects, with three strains originating in SHB. Two of the *Metarhizium anisopliae* strains were isolated from soil and the third was isolated from an adult SHB.

Table 3. Temperature characterisation and source of fungal isolates (*Beauveria bassiana* or *Metarhizium anisopliae*) investigated for adult and larval SHB control

Isolate number	Species	Source of isolate	Isolated	Av growth rate (mm/day) 25°C	Av growth rate (mm/day) 30°C	Av growth rate (mm/day) 35°C
B4	<i>B. bassiana</i>	<i>Lucilia cuprina</i>	Aug, 01	3.06	2.56	0.18
B6	<i>B. bassiana</i>	<i>Musca domestica</i>	Oct, 01	2.84	2.38	0
B14	<i>B. bassiana</i>	<i>Musca domestica</i>	Oct, 01	3.02	2.04	0
B23	<i>B. bassiana</i>	<i>Haemotobia irritans</i>	Feb, 04	2.00	2.14	0.29
B25	<i>B. bassiana</i>	<i>Bovicola ovis</i>	Sept, 04	2.16	1.14	0
B26	<i>B. bassiana</i>	<i>Bovicola ovis</i>	Sept, 04	2.84	1.34	0
B27	<i>B. bassiana</i>	<i>Bovicola ovis</i>	Sept, 04	1.79	0.85	0
B28	<i>B. bassiana</i>	<i>Bovicola ovis</i>	Oct, 04	1.68	0.76	0
B30	<i>B. bassiana</i>	<i>Bovicola ovis</i>	Oct, 04	1.71	1.27	0
B34	<i>B. bassiana</i>	<i>Bovicola ovis</i>	Oct, 04	NA	NA	NA
B39	<i>B. bassiana</i>	Laboratory mutant	Sept, 06	NA	NA	0
B42	<i>B. bassiana</i>	<i>Aethina tumida</i>	Feb, 07	1.49	1.45	0
B43	<i>B. bassiana</i>	<i>Musca domestica</i>	Feb, 07	2.52	2.14	0
B44	<i>B. bassiana</i>	<i>Musca domestica</i>	Mar, 08	1.52	1.41	0
B45	<i>B. bassiana</i>	<i>Aethina tumida</i>	Jun, 09	2.52	2.25	0
B46	<i>B. bassiana</i>	Laboratory mutant	Oct, 09	2.43	2.07	0
B47	<i>B. bassiana</i>	<i>Aethina tumida</i>	Oct, 09	NA	NA	NA
M16	<i>M. anisopliae</i>	Soil, Aratula, Qld	May, 99	5.11	4.8	0.7
M81	<i>M. anisopliae</i>	<i>Aethina tumida</i>	Jan, 07	NA	4.0	1.0
M91	<i>M. anisopliae</i>	Soil, UWS apiary site NSW	Oct, 09	NA	NA	NA

NA = not available

All *B. bassiana* strains were noted to grow much slower than the *M. anisopliae* strains at both 25°C and 30°C. Only two strains of *Beauveria* showed any growth at 35°C, whereas the 2 strains of *Metarhizium* tested grew at 35°C. The *B. bassiana* strains had lower thermal growth optima than the *M. anisopliae* isolates. This is consistent with previous observations for *B. bassiana* and *M.*

anisopliae. Because of their higher growth optima a number of *M. anisopliae* strains were previously screened in a prior study (RIRDC Honeybee PRJ-000037).

Larval SHB Control

Laboratory Assays

Of the isolates selected for further investigations with larval SHB, M16 and M81 had been screened in a previous RIRDC project (PRJ-000037). When the new isolate M91 was screened against larval SHB no adult beetles emerged from either of the assays.

Preliminary field trials

Of the four trials set up two were destroyed by very intense rain events. In the other two trials the *Metarhizium* soil treatments were able to disrupt the emergence of adult small hive beetles. The number of adults emerging from soil treated with both M81 formulations was significantly lower ($P < 0.001$) than the number emerging from the non treated (control) soil. There was no significant difference between either of the M81 formulations (Figure 8) with 4% emergence from soil treated with the M81 oil formulation and 6% emergence from the soil treated with the M81 granules. In the last preliminary field trial only the granulated formulation of *Metarhizium* isolate M91 had a significant effect ($P < 0.001$) on SHB pupation with merely 1% of adults emerging (Figure 9). Although only 16% of adults emerged from soil treated with the M91 oil formulation this was not significantly lower than the emergence from the control soil (29%) ($P < 0.05$). In the trial testing isolate M81 adult emergence took from 32 to 39 days while in the trial with isolate M91 adult emergence took from 28 to 38 days. This reflected the slightly higher ambient temperatures when the later trial was conducted.

The level of pupation success for wandering larvae introduced to the untreated soils was quite low compared with that achieved in laboratory assays (up to 100%, PRJ-000037) with only 42% in one trial and 29% in the other. This may reflect the level of exposure pupating larvae face from both weather and predation. Slugs and ants were observed in the area of the enclosures.

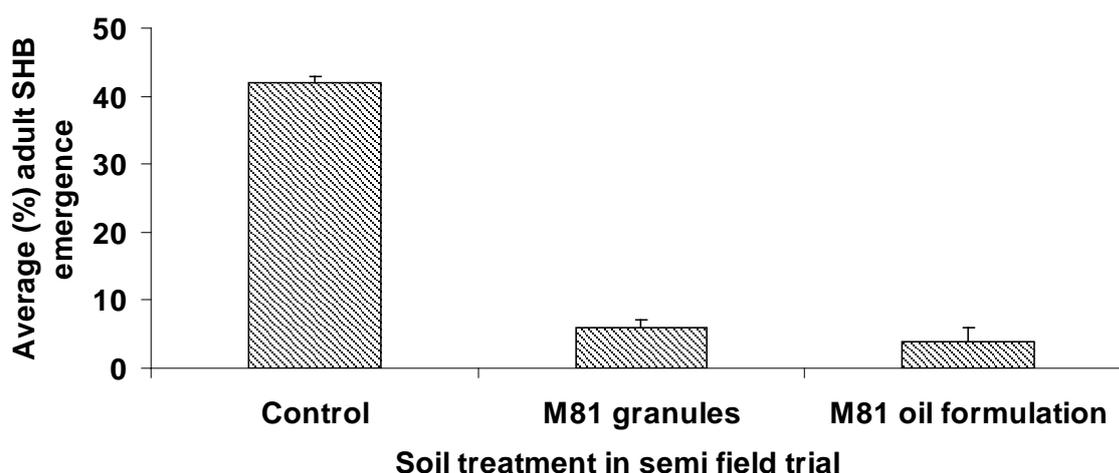


Figure 8. Average percentage of adult SHB emerging from untreated soil and soil treated with formulations of the *Metarhizium* isolate M81 after larvae were introduced for pupation

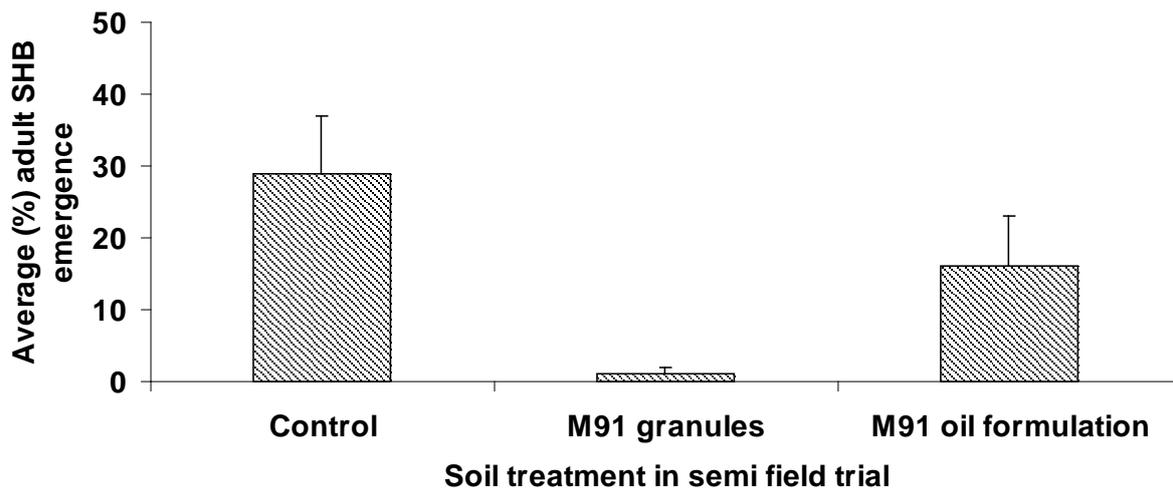


Figure 9. Average percentage of adult SHB emerging from untreated soil and soil treated with formulations of the *Metarhizium* isolate M91 after larvae were introduced for pupation

Field trials

The first field trial was carried out in autumn 2010 with lower ambient temperatures than the other three trials which were carried out over the summer of 2010-2011. This difference in ambient temperature influenced the time taken for pupation. The trials conducted over summer were also subject to heavy rain events. In all trials a range of arthropods and even slugs were observed in the enclosures with ants in some observed carrying off SHB larva.

Trial 1

Adult SHB were collected from the six enclosures over 52 days with a significantly ($P < 0.05$) lower number (974) collected from treated enclosures compared to untreated enclosures (1759). This trial was set up in late March, hence the night temperatures decreased over the course of the trial slowing down the emergence of the adults. The mean numbers of adult SHB collected in the control and treated enclosures were 586 (± 47) and 325 (± 29) respectively (Figure 10). However the netting used for the enclosures developed small holes over the course of the trial. Thus a number of adult SHB may have escaped through these holes.

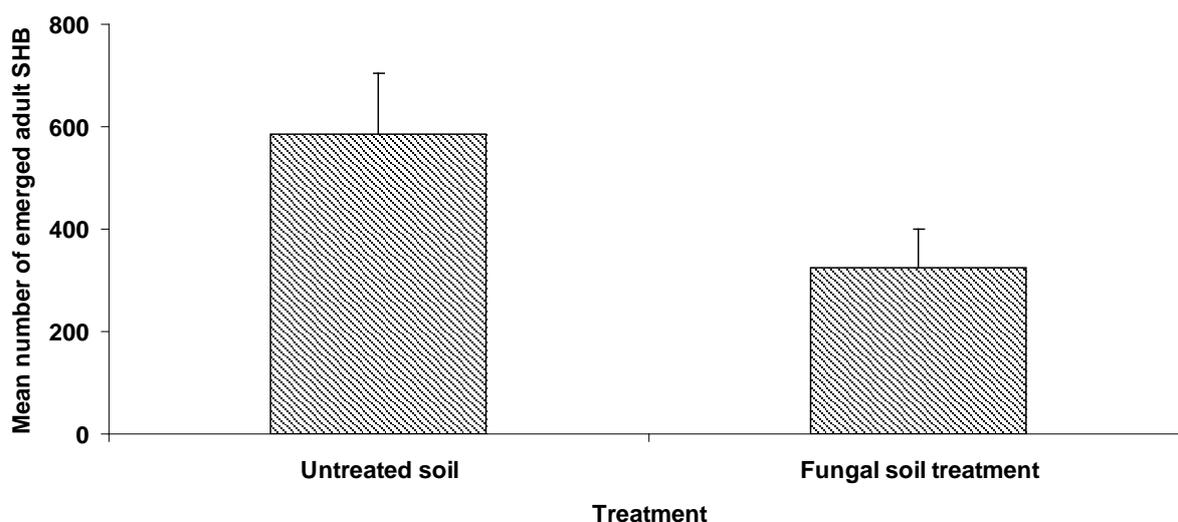


Figure 10. Mean number of adult SHB emerging from fungal treated and untreated soil under nucleus hives containing SHB larvae breeding in a ‘slime-out’ in Trial 1

Trial 2

This trial was conducted during early summer when ambient temperatures were quite high (29°C-33°C). Adult SHB were collected from the enclosures over a period of 12 days. 3014 beetles were collected from the control enclosures (average of 754 per enclosure) compared to 3737 beetles from treatment enclosures (average of 935 per enclosure). The difference in emergence between control and treatment enclosures was not significant ($p=0.53$). An abnormally heavy rain event during the trial appeared to affect the trial; it is possible that the fine grained pure white sand provided for pupation may not have ‘held’ the fungal spores allowing them to be washed through the sand. Both black and green ants were seen in all enclosures, some were noted to be removing both live and dead larvae. Six dead adult SHB collected from the fungal treated enclosures were noted to be covered in *Metarhizium* growth.

Trial 3

Because of concerns of the effect of the pure white sand substrate on the efficacy of the fungal treatment in Trial 2 the substrate in Trial 3 was modified by adding 50% potting mix with a high level of organic matter. During this trial there was an extremely high level of rainfall as well as flooding in Brisbane which both affected the trial and prevented access to the trial site at the time when adult SHB emergence was expected. This prevented the addition of the traps until a week later than originally planned. Overall fewer adult SHB were collected in this trial compared to the other trials. However the SHB emergence from the treated enclosures was significantly lower than from the untreated enclosures ($p=0.03$). A total of 420 adult SHB were collected from the untreated enclosures (average 10/ enclosure) compared to 34 SHB from treated enclosures (average 8.5/enclosure) (Figure 11). Four adult SHB collected from the treated enclosures were covered in *Metarhizium* growth. A 335 g sample of soil was taken from each treatment and incubated in the laboratory to observe the effect of the high rainfall on pupation just before access to the trial was cut off due to flooding. In these samples 100-150 adult SHB emerged from the untreated soil while only 20-40 adult SHB emerged from the fungal treated soil. In addition there was evidence of fungal infection on larvae in the soil sampled from the treated soil.

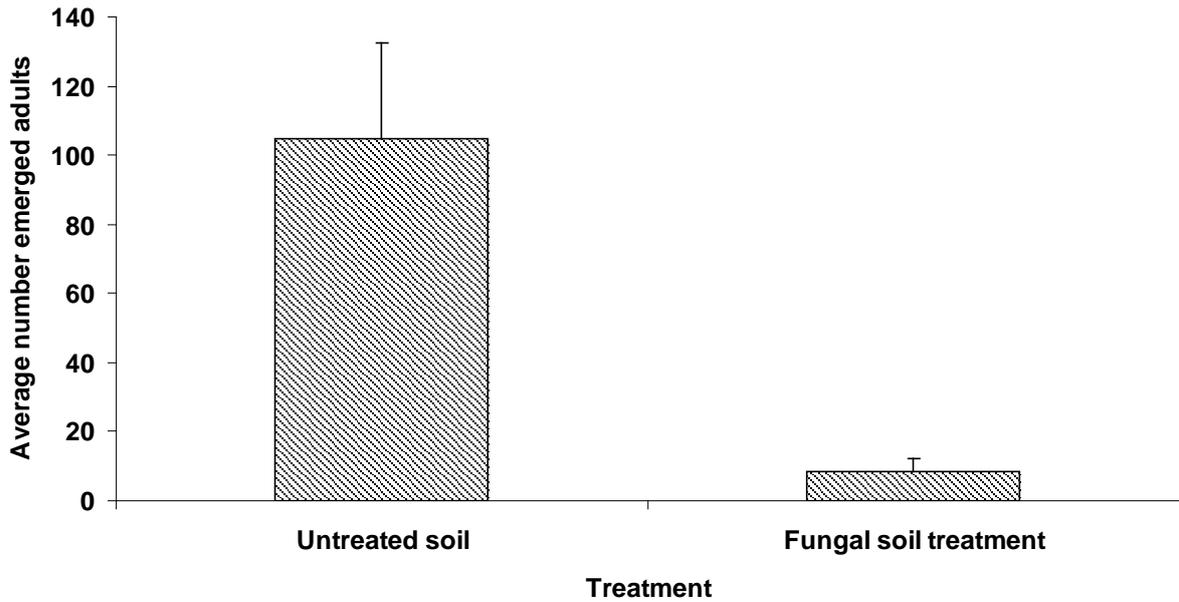


Figure 11. Mean number of adult SHB emerging from treated and untreated soil under pseudo-hives with SHB larvae emerging from a measured amount of 'slime-out' in Trial 3

Trial 4

This trial was conducted during late summer without the heavy rainfall experienced during the previous trial, the ambient temperatures were also quite high (29°C-33°C). Adult SHB were collected from the enclosures over 15 days. 1704 beetles were collected from the untreated enclosures (average 426±171) compared to 865 beetles from treated enclosures (average 216±61) (Figure 12). Although the fungal treatment resulted in fewer adult SHB emerging the decrease was not significant (P=0.29).

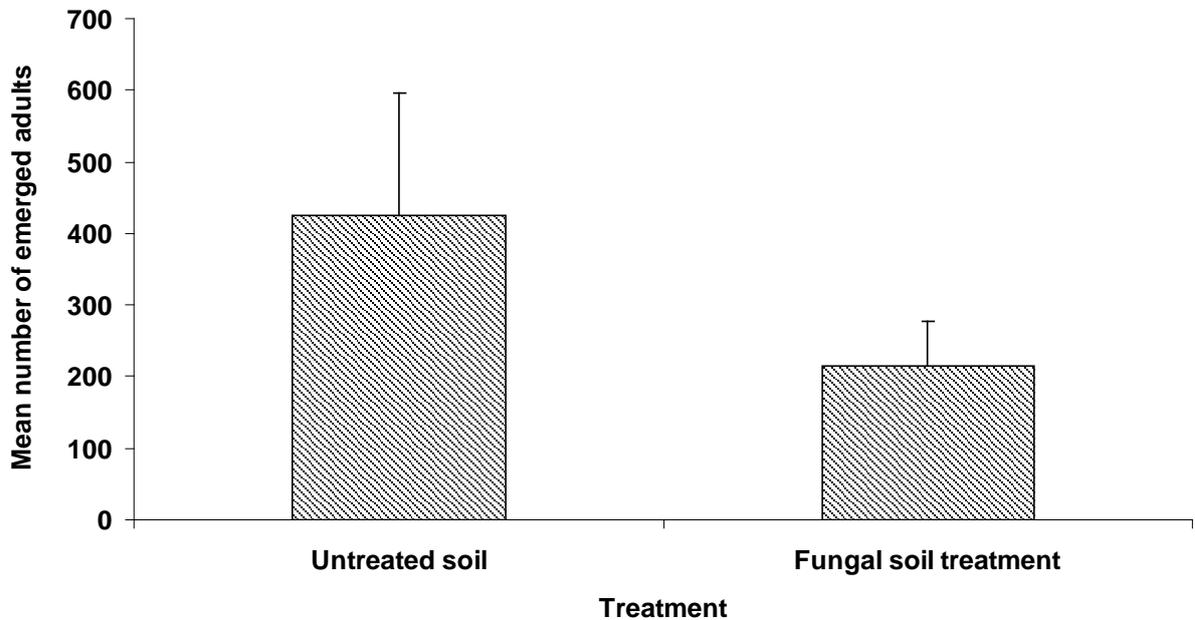


Figure 12. Mean number of adult SHB emerging from treated and untreated soil under pseudo-hives with SHB larvae emerging from a measured amount of 'slime-out' in Trial 4

Attractant traps in field trials

The number of emerging small hive beetles caught in the attractant traps was significantly higher ($P < 0.001$) than the number of beetles found in the crumpled paper harbourages. Furthermore the proportion trapped in each trial increased as the trap and the attractant mix were modified (Figure 13). By the fourth and last trial all beetles were attracted into the traps in the enclosures.

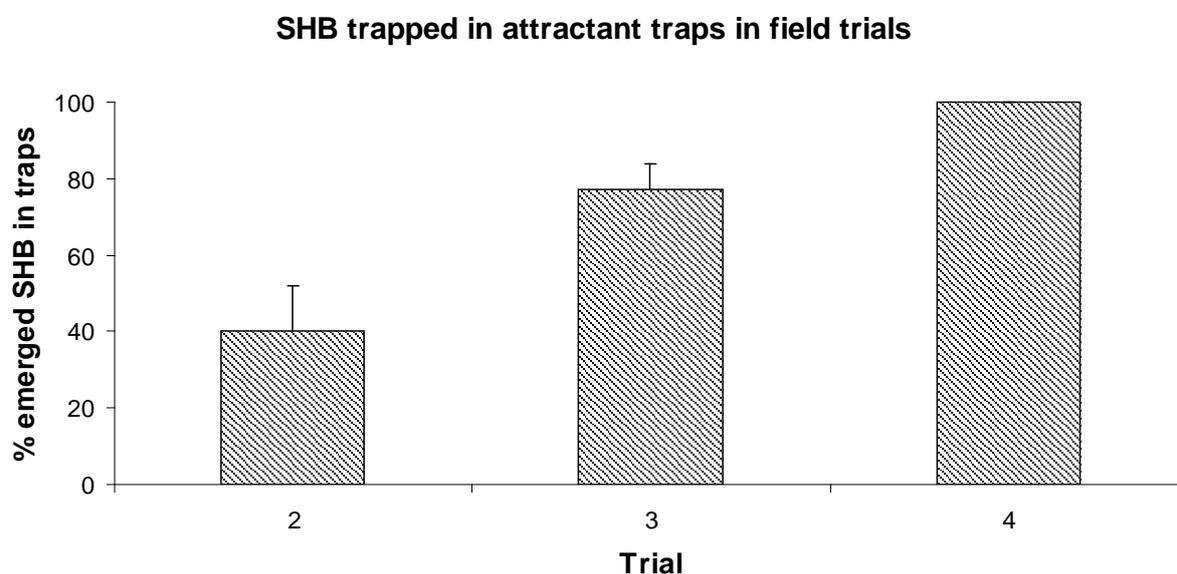


Figure 13. Average percentage of emerging small hive beetles caught in attractant traps during the larval field Trials 2-4

Adult SHB control

Fungal screening

In the initial screening of the 17 *Beauveria* isolates listed in Table 3, the mean mortality of adult SHB at day 7 ranged from 2 (± 2) % to 100 %. By day 14 the mean mortality of adult SHB ranged from 23 (± 5) % to 100 %. Of the six isolates chosen for further investigation three caused 100 % mortality in adult SHB after 14 days while the other three caused 99 (± 1) %; 98 (± 1) %; and 97 (± 2)% mortality in the same period (Table 4).

Adult SHB exposed to spores of the six most effective *Beauveria* isolates in corflute refuges all had much lower rates of mortality than when directly dipped in the spores (Table 4). The isolate which caused the highest SHB mortality through exposure in a refuge (B26) at days 7 and 14 caused the lowest mortality in SHB dipped in its spores. There was no significant difference ($P < 0.05$) in SHB mortality when the beetles were exposed to the spores of the different *Beauveria* isolates in corflute refuges. However when SHB were dipped in the *Beauveria* spores three of the six isolates caused a significantly higher mortality after 7 days (Table 4). *Beauveria* grew from all dead SHB incubated with moisture (Figure 14).

None of the six best isolates screened against adult SHB were able to grow at 35°C (Table 3). The three isolates growing best at 30°C were B45, B43 & B46 (2.25, 2.14, 2.07 mm/day respectively) while the other three isolates (B42, B26 & B30) showed slower growth rates (1.45, 1.34, 1.27 mm/day respectively). The temperature maintained in a bee hive around the brood ranges from 32 - 35°C (av. 34°C).

Table 4. Mean (%) corrected mortality (\pm SE) of adult SHB exposed to spores of the 6 most effective *Beauveria* isolates of through either direct dipping in spores or self contamination with spores inside corflute refuges. Isolates are in decreasing order of efficacy at day 14

Adults dipped in spore powder			Adults exposed to spore powder in corflute refuge		
Isolate	Mean % corrected mortality day 7 (\pm SE)	Mean % corrected mortality day 14 (\pm SE)	Isolate	Mean % corrected mortality day 7 (\pm SE)	Mean % corrected mortality day 14 (\pm SE)
B43	97 _a	100 _a	B26	18 _c	65 _c
B46	90 _a	100 _a	B42	11 _c	61 _c
B45	88 _a	100 _a	B46	15 _c	60 _{cd}
B42	59 _b	99 _a	B43	11 _c	50 _{cde}
B30	61 _b	95 _{ab}	B45	7 _c	46 _{de}
B26	53 _b	80 _b	B30	15 _c	38 _e

Means with the same subscript in the day 7 columns or day 14 columns are not significantly different at the P = 0.05 level



Figure 14. *Beauveria* growing from adult SHB beetle which died after exposure to *Beauveria* spores in a refuge

Fecundity

The fungal treatments in both assays caused a significant reduction in the fecundity of beetles which survived even though the effect of exposure to *Beauveria* spores on adult mortality varied between the two assays (Table 5). In the first assay more than half of the beetles were dead after 7 days of exposure to the two different fungal isolates compared to only 11% death in the control beetles. In the second assay exposure to the fungal spores resulted in much lower mortalities for all treatments. In the two assays the mixed sex control beetles produced the equivalent of 4.04 and 4.55 offspring per adult. While the highest number of offspring per adult beetle produced by beetles exposed to *Beauveria* spores was 1.97 and the lowest number was 0. Thus the reduction in fecundity of the beetles which survived the *Beauveria* treatments ranged from 100% to 57%. There was no significant difference between the mean weights and sizes of the offspring from the either the controls of *Beauveria* treated survivors.

Table 5. Effect of exposure to spores of two *Beauveria* isolates (B43, B46) on adult SHB mortality and subsequently, the fecundity of the surviving adults

Treatment	Mean % adult mortality after 7 days (\pm SE)	Mean number of offspring produced (\pm SE)	Mean number of offspring per adult	Mean % reduction in fecundity
ASSAY 1				
Control	11 (\pm 1) _a	144 (\pm 18) _a	4.04 _a	NA
B43	57 (\pm 6) _b	13 (\pm 5) _b	0.87 _b	78
B46	59 (\pm 2) _b	0 _b	0 _b	100
ASSAY 2				
Control	4 (\pm 1) _c	175 (\pm 20) _c	4.55 _c	NA
B43	18 (\pm 4) _d	44 (\pm 16) _d	1.33 _d	71
B46	5 _c	75 (\pm 10) _d	1.97 _d	57

Means with the same subscript are not significantly different at the P = 0.05 level

Diatomaceous Earth

Behaviour and survival of beetles in relation to traps used in-hive

Figure 15a shows two AJ's Beetle Eater® traps and Figure 15b shows small hive beetles removed from beetle traps after exposure to DE. In Bioassay 1 only one beetle escaped from the traps in which DE was used (< 1%; n = 120). The rest died (mortality >99%). In contrast, beetles in the control, with no DE present, moved indiscriminately into and out of the traps. Three were counted as dead in the trap by day 7 (mortality 2.5%; n = 120). They appeared to be using the traps as a harborage of sorts.

In all controls, breeding had commenced on the protein cake by 7 d, however no breeding occurred on the protein cake in the treated replicates. For both replications of Bioassay 2, no beetles escaped from traps with DE (all died) while beetles moved indiscriminately into and out of the traps with cornflour. Although a small number died in the cornflour traps (7 beetles: mortality 5.8%; n = 120) in the first of the replications. Breeding commenced within the controls (cornflour) by day 7 with larvae visible on the protein cake.

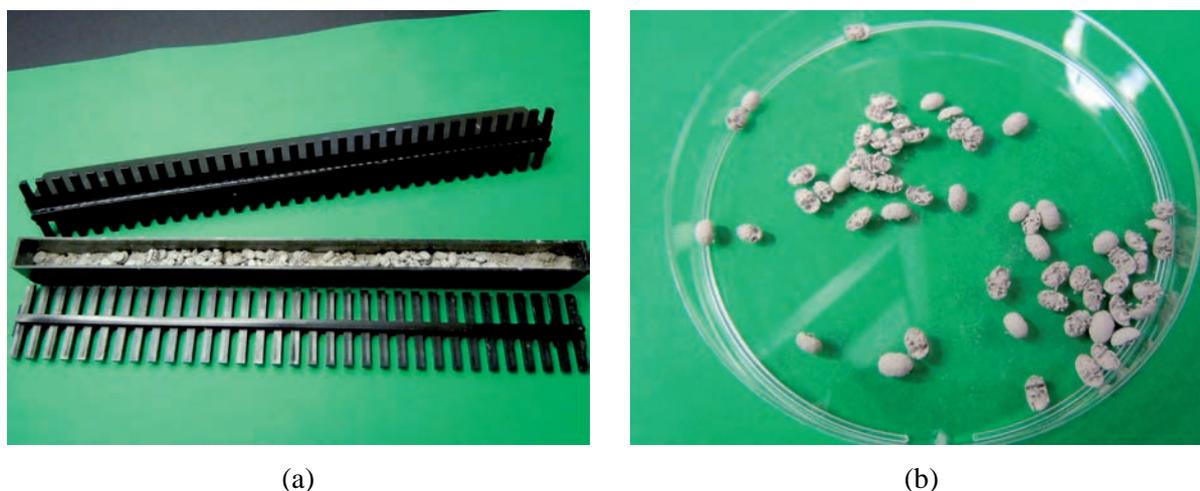


Figure 15. a) Dead SHB caught in the DE in an AJ's Beetle Eater® trap b) Dead SHB coated in DE recovered from an AJ's Beetle Eater® trap

Assessment of beetle cuticle with Scanning Electron Microscopy (SEM)

No scarification of adult beetle cuticle was observed after exposure to DE; however DE particles were found clustered onto sensilla, and in particular were seen to heavily coat the antennal sensilla (Figure 16 a-d).

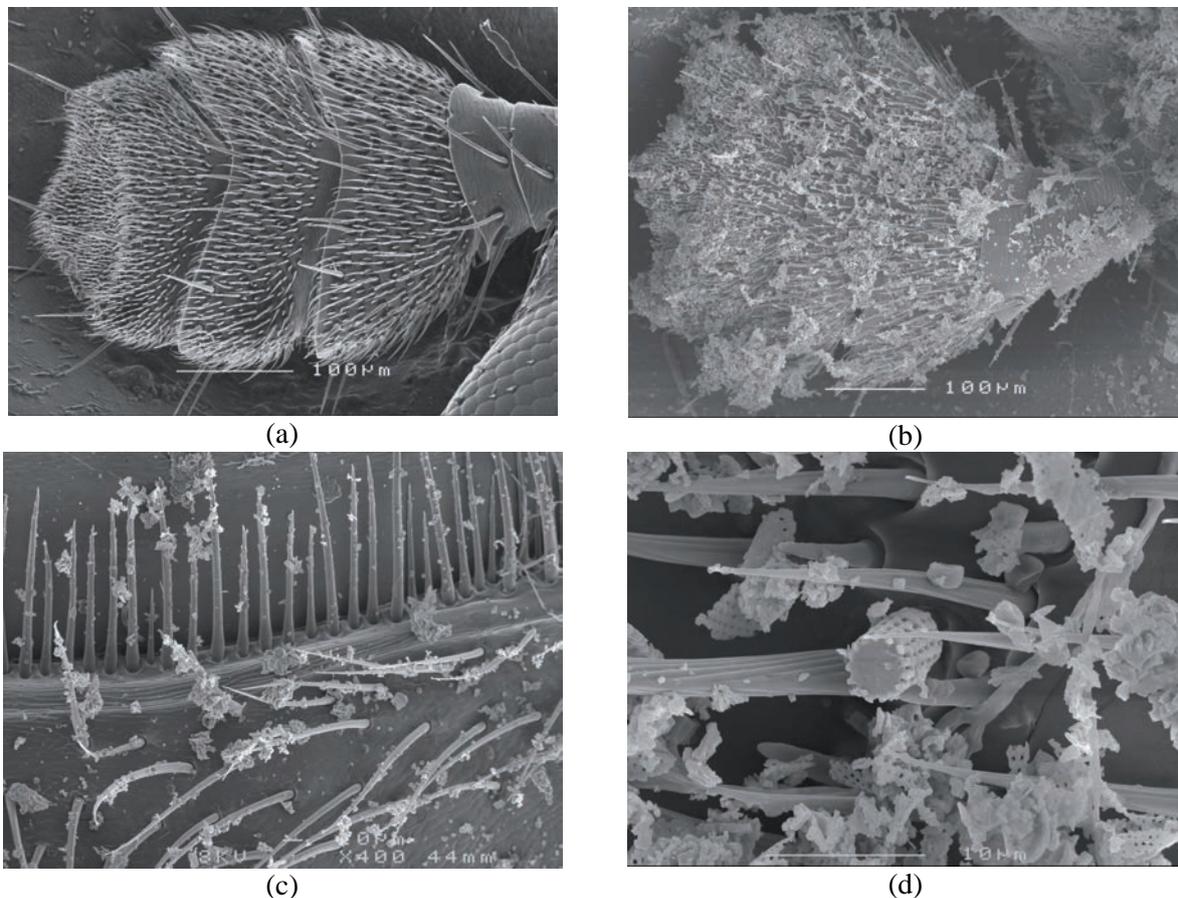


Figure 16. a) Scanning electron micrographs showing antenna of untreated adult SHB. b) Antenna of treated adult SHB treated with diatomaceous earth. c) Sensilla around the neck of an adult SHB coated with DE. d) Higher magnification of DE coating sensilla. Scale bars are present on individual photographs

Yeast Investigations

Initial characterisation of yeasts from hive derived slime and adult SHB

An examination of SHB larval infested slime sampled from a collapsed hive revealed that the slime is largely composed of yeast cells along with the expected grains of pollen, honey and wax (Figure 17).

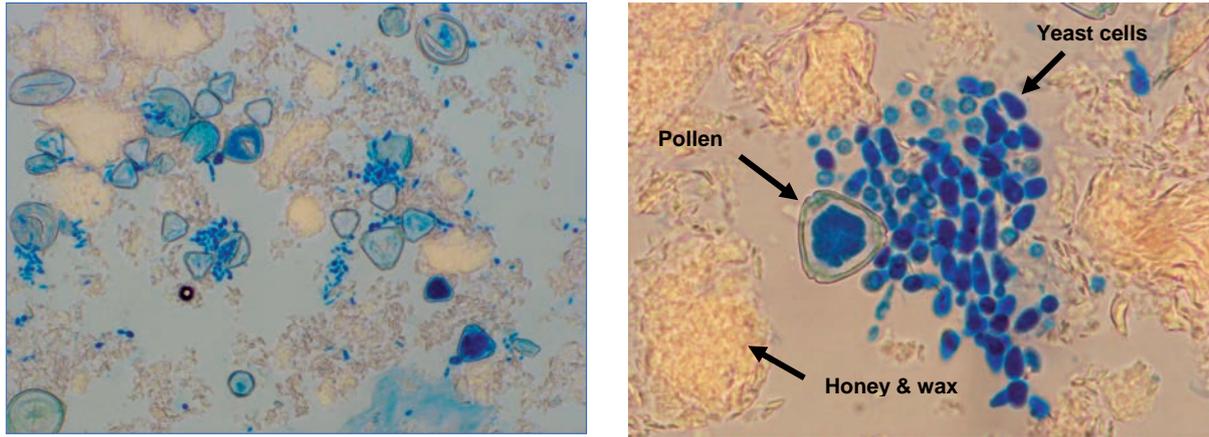


Figure 17. Micrograph of hive slime showing the yeast cells as well as pollen grains, honey and wax (× 200 mag left, × 400 mag right)

Initial isolations from the slime produced yeasts of two different morphologies, one wrinkled the other smooth (Figure 18). Further isolations from adult SHB produced the same types of yeast colonies. Benda *et al.* (2008) described the yeast, *Kodamaea ohmeri*, which they isolated from adult SHB as having 2 different morphologies of smooth and wrinkled. They also noted that the smooth morphology usually became wrinkled after five days. However the smooth morphology yeasts isolated from the hive slime and adult SHB were never observed to later develop a wrinkled morphology. Similarly the wrinkled morphology colonies appeared wrinkled from the beginning. The variation between colonies with the smooth morphology led to speculation that this morphology represented a number of different yeasts. Subsequent molecular identification revealed the wrinkled morphology yeast to be *Kodamaea ohmeri* and the smooth morphology yeasts to be different *Candida* species.

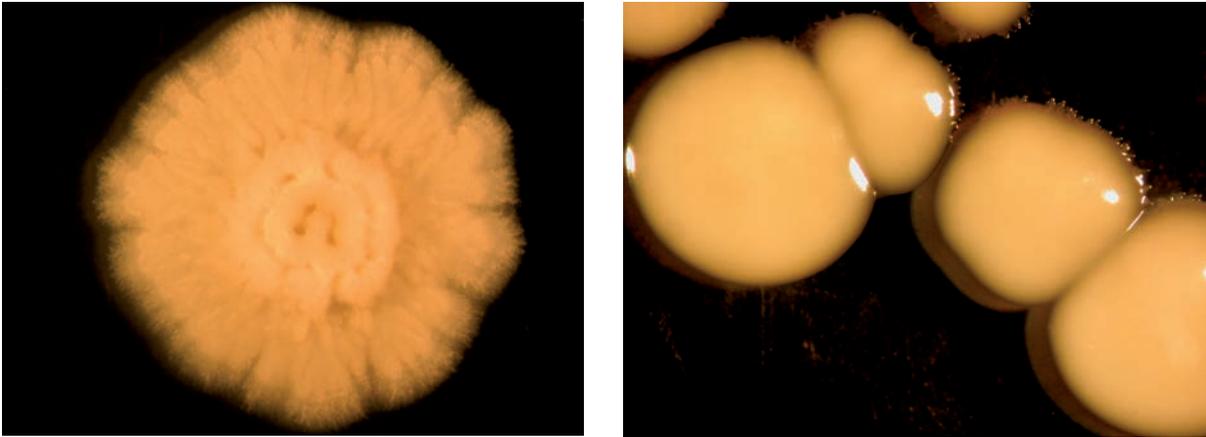


Figure 18. Colony of wrinkled morphology yeast (left) and colonies of smooth morphology yeast (right)

A comparison of the growth of colonies of the two different morphologies showed a clear difference with the wrinkled exhibiting a faster growth rate (Figure 19). The wrinkled morphology yeast also grew at higher temperatures than the smooth morphology yeast.

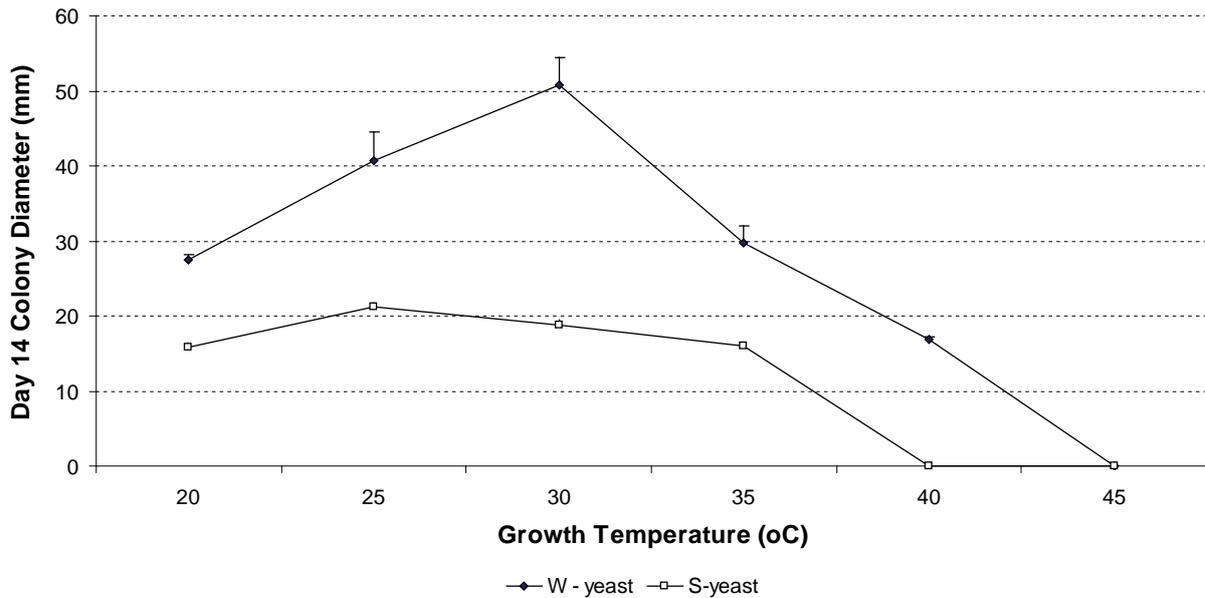


Figure 19. Comparison of growth response to temperature of two different yeast morphologies (W = wrinkled; S = smooth)

K. ohmeri isolates from SHB showed high osmo-tolerance when inoculated into a sterile mixture of honey and water. *K. ohmeri* cells were able to multiply in 25% and 50% concentrations of honey (Figure 20). At 75% honey concentration *K. ohmeri* cells did not appear to multiply but were able to survive for at least 5 days. *K. ohmeri* cells inoculated into 100% honey did not survive.



Figure 20. *Kodamaea ohmeri* colonies growing from 10µl samples of honey: water mixtures which had been inoculated with this yeast 5 days before

The presence of yeast, in particular *K. ohmeri* in the medium on which SHB larvae fed and developed was investigated. It had been observed that when adult SHB oviposit on firm protein cakes containing a mixture of honey and pollen the cakes will become slimy progressing to semi-liquid consistency with a strong “yeasty” odour as the developing larvae grow to maturity. Once the larvae have completed development and wandered off to pupate they leave behind only a dry “fluffy” matter. Under the stereo-microscope this dry fluffy matter appeared to be strands of dry insect frass, which was found to contain some yeast cells when examined under the compound microscope. Daily swabs were taken from the protein cakes to investigate what fungi are present on the surface of the protein cakes as SHB larvae develop. Colonies of different fungi grew on SDA plates inoculated with the swabs.

Typically a range of common airborne fungal species, including *Aspergillus*, *Penicillium*, and *Cladosporium*, grew on SDA plates inoculated with swabs taken one day after adult SHB had oviposited on the protein cakes (Figure 21). However by 4 days post oviposition these common fungal species had disappeared and yeast colonies were becoming apparent. Between 7 and 9 days post oviposition the swabs taken from the now slimy semi-liquid protein cakes resulted in an almost exclusive presence of wrinkled morphology yeast colonies (Figure 21). *K. ohmeri* which was not originally present on the protein cakes came to dominate the colonies growing on the SDA plates indicating a parallel development of this yeast and SHB larvae on protein cakes. Moreover the dry frass remaining after the mature larvae had wandered off to pupate indicated that the larvae had consumed the yeast as part of their development.

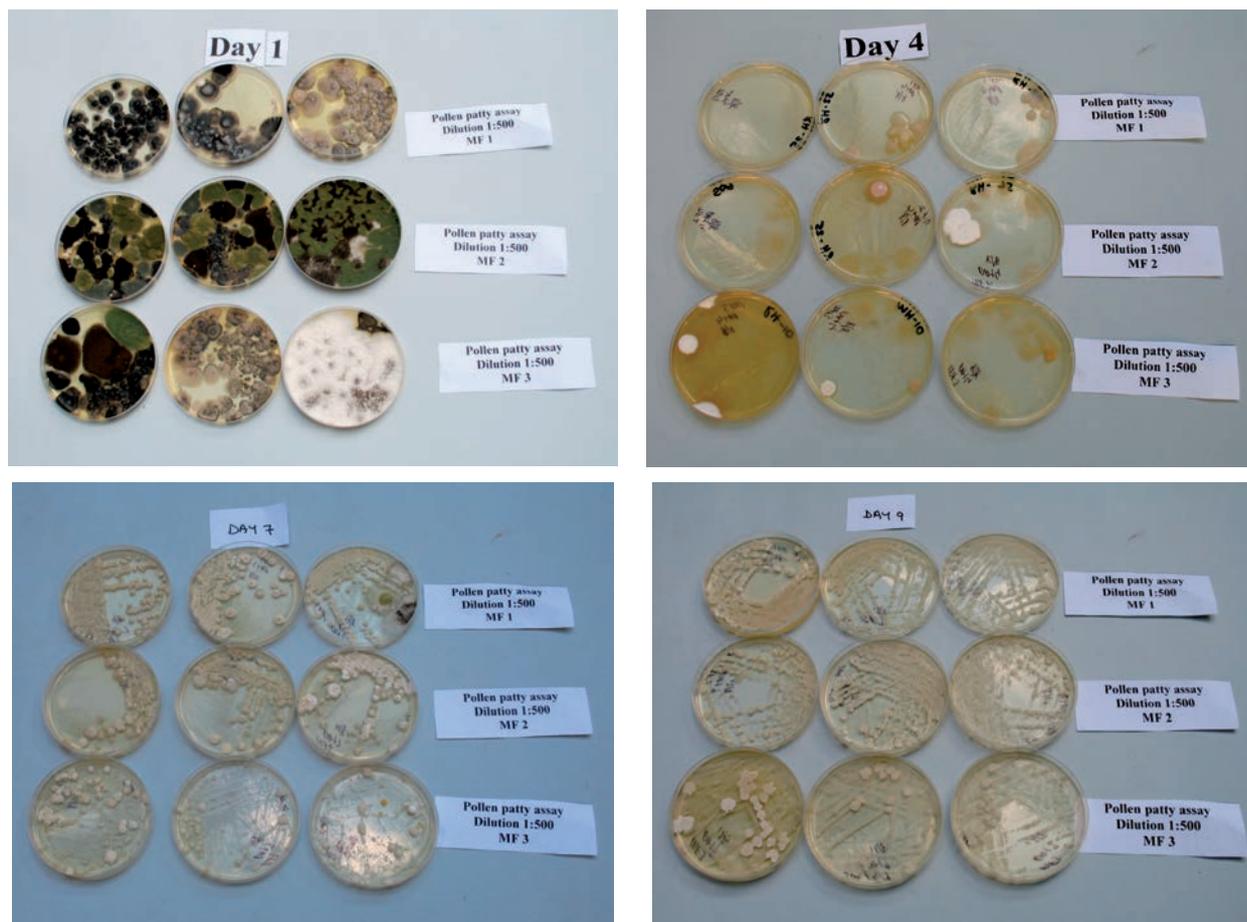


Figure 21. Fungal colonies (7days) growing on plates of SDA streaked with swabs taken from protein cakes on which SHB larvae developed on days 1, 4, 7 and 9 post oviposition by adult SHB

Volatiles

Volatile analyses

The GC-MS traces (Figure 22) show that volatiles associated with yeast cultures (smooth and wrinkled morphology) and slime associated with the breakdown of hive products by SHB share a number of similar peaks. The peaks represent chemical components of the volatiles. The GCMS traces also show that the wax and honey samples have few components in common with each other or with any of the slime or yeast samples or each other. The volatiles produced by early slime have few compounds in contrast to volatiles from late slime which contain many different compounds.

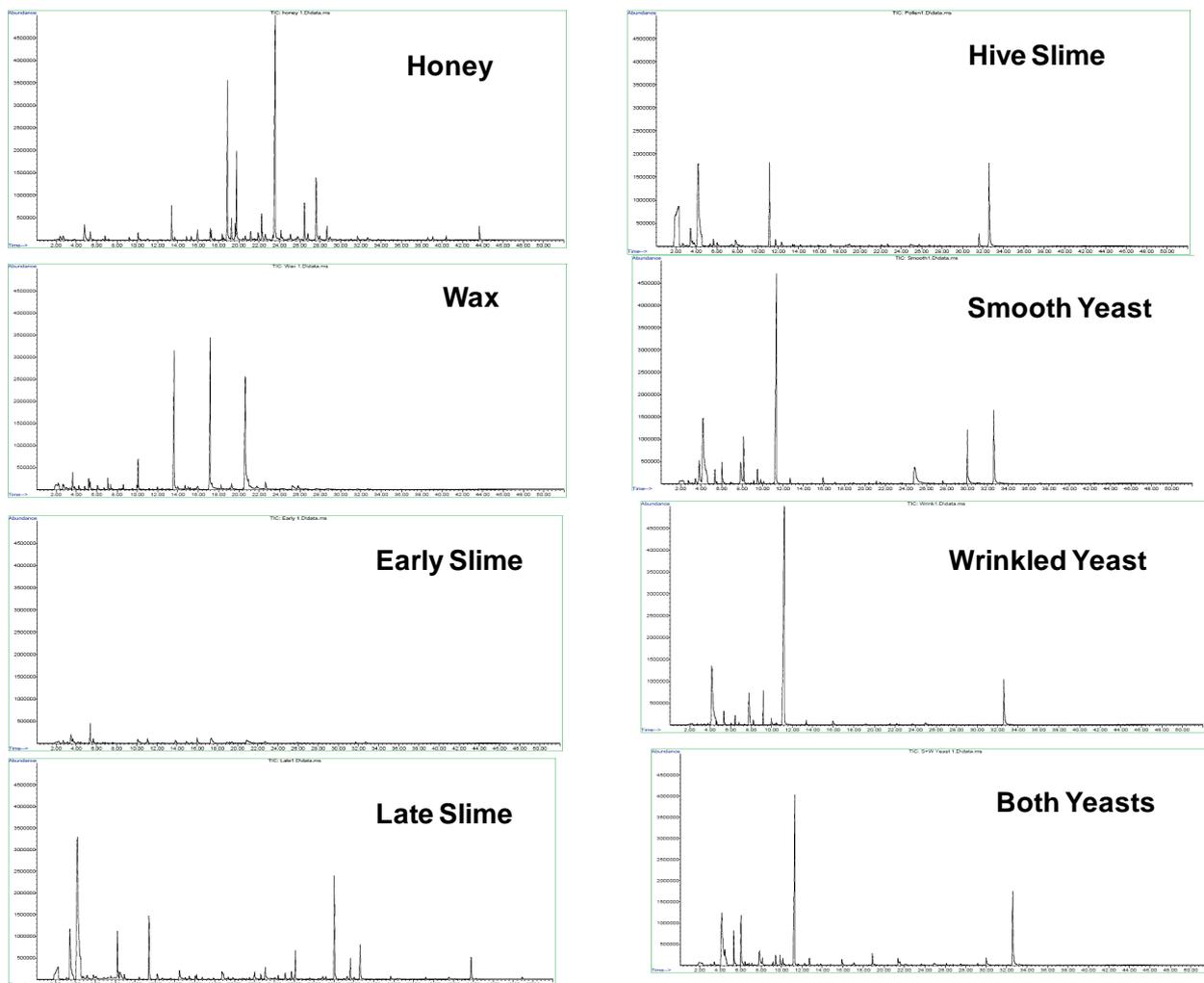


Figure 22. Comparison of GC-MS traces of volatiles emanating from hive products, SHB fermented hive products and cultures of pure yeasts isolated from SHB induced hive fermentation products

Seven compounds were found to be common to both slime and yeast volatiles (Table 6). Of these compounds, ethyl alcohol was present in very high amounts. Substantial amounts of ethyl acetate were also present although this compound was not present in the volatiles produced by the wrinkled yeast.

A number of different butanoic and propanoic compounds were present in the volatiles produced by the yeast cultures yet were not present in any of the slime volatiles. One of these compounds was 1-butanol 2- methyl acetate also known as isopentyl acetate. It is interesting that this compound was found in the volatiles emanating from all three of the yeast samples but it was not detected in any of the volatiles associated with the slime samples.

Compounds present in volatiles from honey and wax as well as slime and yeast volatiles were octanal [wax, early & hive slime, S + W yeast]; nonanal (wax, honey, early & hive slime, S + W yeast); decanal [wax, early & hive slime] and benzyl alcohol [honey, late & hive slime].

Table 6. Compounds common to volatiles emanating from slime samples and yeast cultures

Compounds in volatiles	Volatile source					
	Early slime	Late slime	Hive slime	S yeast	W yeast	S + W yeast
Ethyl acetate	✓	✓	✓	✓		✓
Ethyl alcohol	✓	✓	✓	✓	✓	✓
Butanoic acid ethyl ester			✓	✓	✓	✓
1 Butanol 3 Methyl			✓	✓	✓	✓
Hexanoic acid, ethyl ester		✓	✓			✓
Benzene acetaldehyde			✓	✓	✓	✓
Phenyl ethyl alcohol	✓	✓	✓	✓	✓	✓

Attractant studies

SHB responses to different volatile sources

The attractiveness to adult SHB of the slime and yeast volatiles analysed through GC-MS were evaluated against a standardised “hive odour”. The hive odour consisted of a mixture of pollen powder, honey and wax. The SHB showed no significant attraction for any of the yeast volatiles or the early slime over the hive odour. However volatiles from both the late and hive slimes were highly attractive to adult SHB, with the hive collected slime proving to be the most attractive. Isopentyl acetate, also known as the bee alarm pheromone and found to be present in the yeast volatiles analysed was not found to be a strong attractant to adult SHB. This finding was repeated in two subsequent student investigations. Isopentyl acetate at the range of dilutions tested across three separate investigations with the Y-tube olfactometer was never found to be attractive to adult SHB.

In a second investigation the attractiveness of a range of hive products, including slime, to adult SHB was tested. Pollen dough consisting of a honey, wax and pollen freshly extracted from a hive proved to be the most attractive. However in this experiment the sources of slime were different to those previously investigated through GC-MS and SHB behavioural studies. The slime used in this investigation included a very early slime and a very late slightly dried slime.

In the third investigation the attractiveness of five of the compounds (ethanol; ethyl acetate; phenyl ethyl alcohol; 1 butanol 3 methyl; and 1 propanol 2 methyl) found to be present in yeasts and slime (Table 6) was tested. A reverse stepwise process was used to evaluate the attractiveness of these compounds to SHB. Based on the findings in the literature isopentyl acetate was also included. The compounds were mixed together in ratios which reflected the amounts found in yeast and slime volatiles through GC-MS analyses with each compound omitted in turn from the mix for each adult SHB challenge. Ethanol and Ethyl acetate were found to be highly attractive to adult SHB while the isopentyl acetate may have had a repellent effect on adult SHB. A combination of honeycomb, yeast and slime was found to be far more attractive than the synthetic mix which included the isopentyl acetate.

Molecular identification

Molecular identification was carried out on 75 yeasts isolated from SHB, this included 70 yeasts of wrinkled morphology and 5 yeasts with a smooth morphology. Representative isolates with the wrinkled morphology were positively identified as *Kodamea ohmeri* through ITS sequencing (Tables 7, 8, Figure 26). No yeasts with the smooth morphology were identified as *K. ohmeri*. However three

of the smooth yeasts were identified as 3 different *Candida* species: *C. apicola*; *C. nivariensis*; *C. bituminiphilia*. The other two smooth morphology yeasts were different *Pichia* species: *P. guilliermondii*, *P. caribbica*.

Yeasts were isolated from all life stages of the Small hive beetle including the mucilage surrounding eggs and the slime associated with larval development whether on protein cake or a mixture of honey comb and brood. Yeasts were also isolated from the fore, mid and hind gut of adult beetles. The sterile isolations revealed two different morphologies of cream yeast, smooth and wrinkled, (Figure 18), although the wrinkled morphology usually dominated.

Because preliminary identifications showed the wrinkled morphology to be *Kodamaea ohmeri*, only the wrinkled morphology colonies were selected for sub-culturing to produce pure cultures for identification and genotyping. In all isolations from the different SHB life stages the wrinkled morphology dominated with fewer yeast colonies of smooth morphology, this was especially pronounced in adult male and female SHB, whether virgin or mated. Smooth morphology yeast colonies were evident in isolations from the slime sampled from either larval infested hives or produced in association with larval rearing in the laboratory, although the wrinkled morphology still dominated.

Table 7. *Kodamaea ohmeri* and 2 *Candida* yeasts isolated from different stages of the small hive beetle life cycle. The identity of the yeasts were confirmed through ITS sequencing and MALDI-TOF MS. The MLMT genotype of the *K.ohmeri* isolates was generated via three different PCR sequencing patterns (M13, [GACA]₄ and [GTG]₅)

Westmead deposit number	Date isolated	Stage of SHB life cycle	Molecular species identification confirmation				
			ITS Sequence	M13 pattern	(GACA) ₄ pattern	(GTG) ₅ pattern	MLMT*
WM 10.191	03.11.10	Adult virgin males & females	ND	18	17	16	20
WM 10.202	03.11.10	Adult virgin females	ND	21	7	13	33
WM 10.201	03.11.10	Adult virgin female, mid-gut	ND	15	15	18	29
WM 10.205	03.11.10	Adult virgin males	ND	18	18	17	22
WM 10.206	03.11.10	Adult virgin male, mid-gut	ND	18	17	16	20
WM 10.228	03.11.10	Adult virgin male, hind-gut	ND	18	20	15	23
WM 10.203	03.11.10	Adult mated females	ND	15	14	19	28
WM 10.194	03.11.10	Adult mixed female	ND	18	19	16	21
WM 10.196	03.11.10	Adult female mated, mid-gut	ND	18	17	16	20
WM 10.197	03.11.10	Adult female mated, hind-gut	ND	22	25	12	32
WM 10.204	03.11.10	Adult mated males	ND	15	14	19	28
WM 10.193	03.11.10	Adult mixed males	ND	18	17	16	20
WM 10.207	03.11.10	Adult mated males, fore-gut	ND	18	17	16	20
WM 10.198	03.11.10	Eggs	ND	15	14	19	28
WM 09.208	02.11.09	Egg mucilage	□	7	5	4	8
WM 10.11	23.11.09	Egg mucilage	ND	7	5	4	8
WM 10.199	03.11.10	Larvae	ND	15	14	19	28
WM 10.195	03.11.10	Larvae	ND	14	27	11	31
WM 10.4	06.05.09	Hive slime	ND	17	26	14	30
WM 09.206	02.11.09	Laboratory slime	□	1	1	1	1
WM 10.12	23.11.09	Pupae	ND	6	4	2	7
WM 10.200	03.11.10	Pupae	□	15	14	19	28
WM 09.209	02.11.09	Newly emerged adults	□	1	2	1	2
WM 10.192	03.11.10	Newly emerged adults	□	18	17	16	20

* MLMT = Multilocus microsatellite type (derived from the 3 patterns generated from different primers); ND = not done

K. ohmeri was found to be present in all SHB life stages including both male and female adult beetles, eggs, larvae, pupae and the slime associated with larvae (Table 7, Figure 23). Isolations from the gut of adult SHB showed *K. ohmeri* to be present in the foregut, midgut and hindgut of both virgin and mated males and females (Table 7, Figure 24). No yeasts were isolated from either the ovaries or testes of female or males adult SHB. In addition, no specialised structures that might serve as repositories for fungal cells, such as the mycetomes found in some insects, were seen.

The 24 isolates of *K. ohmeri* obtained from different stages of the SHB life cycle represented 14 different MLMT genotypes. (Table 7, Figure 23). MLMT genotypes 20 and 28 were isolated from a number of different life stages of the SHB including adult male and females, newly emerged adults, eggs, larvae and pupae (Table 7, Figure 23). Furthermore MLMT genotype 20 was isolated from the midgut of adult mated females and virgin males as well as the foregut of a mated adult male SHB. The range and frequency of MLMT genotypes of *K.ohmeri* isolated from different life stages of the small hive beetle are clearly illustrated in Figure 23, emphasising the presence of the same genotypes across different life stages.

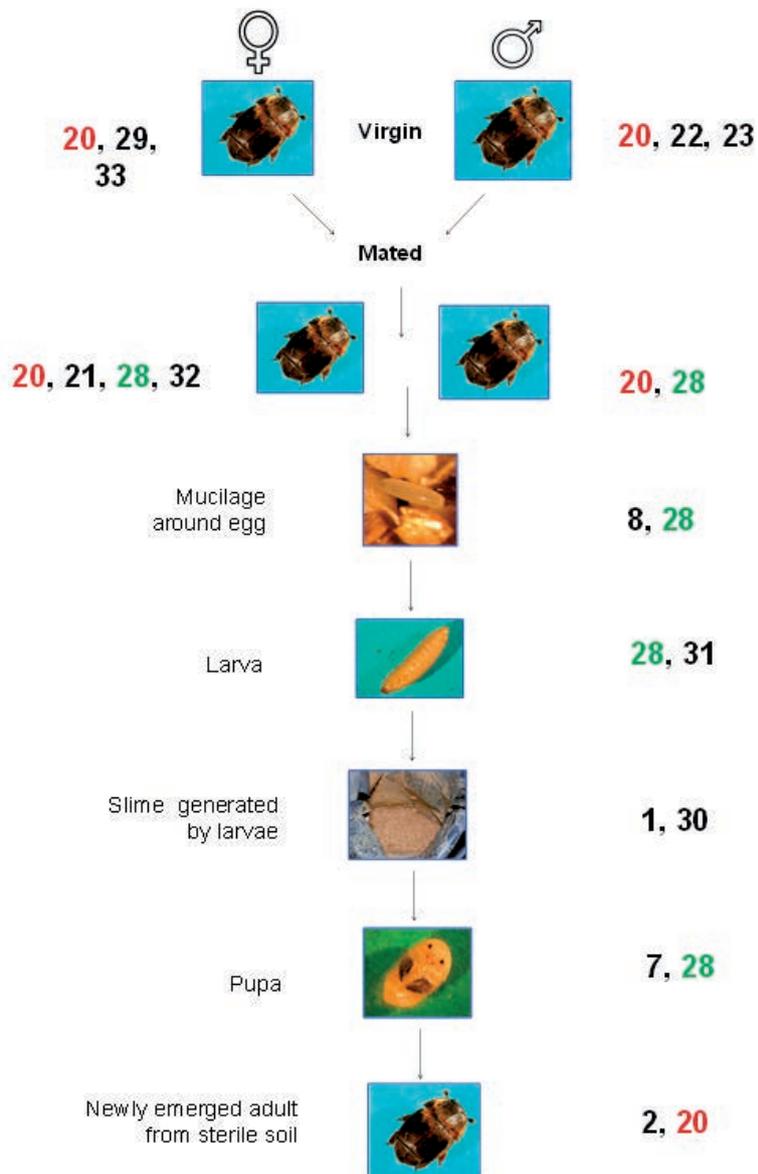


Figure 23. Range of *K. ohmeri* MLMT genotypes isolated from different stages of the small hive beetle life cycle. Genotypes 20 (red) and 28 (green) were isolated from several stages of the life cycle

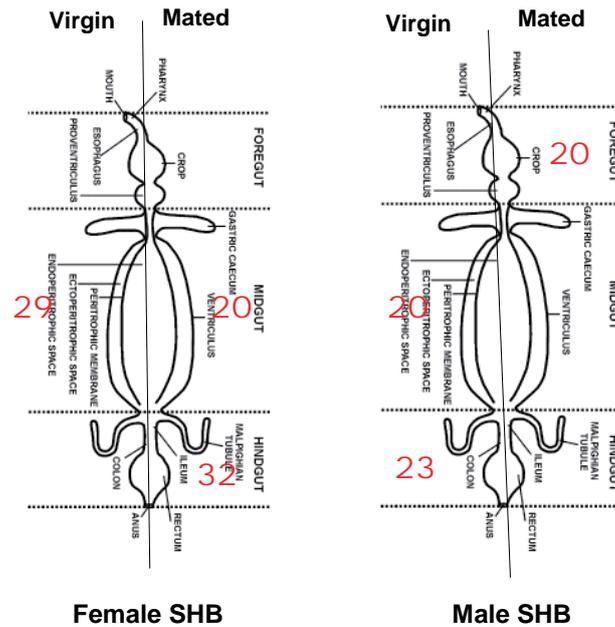


Figure 24. MLMT genotypes of *K. ohmeri* isolated from different regions of the alimentary tract of male and female SHB (virgin and mated)

The forty-six isolates of *K. ohmeri* obtained from adult SHB sampled from different sites in Qld and NSW showed a similar level of genetic variation to that in the isolates obtained from different stages of the SHB lifecycle. The 26 *K. ohmeri* isolates from Qld collected SHB represented 13 different MLMT genotypes, while the 20 isolates from NSW collected SHB represented 9 different genotypes (Table 8). This compares to the 24 *K. ohmeri* isolates from different stages of the SHB life cycle which are represented by 14 different genotypes. These results suggest extensive genetic variation in *K. ohmeri* isolates associated with SHB in Australian apiaries. However some genotypes were found in widely separated locations. MLMT genotype 6 was isolated from SHB collected in both NSW (Bathurst, St Ives, Kurrajong) and Qld (Wondai) (Table 8, Figure 25). While MLMT genotype 2 was isolated from both adult SHB collected in the field (Murgon, Qld) and an adult SHB emerging from sterile soil in a laboratory colony (Table 7, 8). Similarly MLMT genotype 8 was isolated twice from the mucilage around eggs produced in the laboratory colony and nine times from adult SHB collected from three different field sites in NSW (Richmond, UWS Richmond, Kurrajong) (Tables 7, 8).

The level of variation in *K. ohmeri* genotypes found in adult SHB collected from the limited sampling across 9 locations in Qld and NSW is very interesting. From each location 10 randomly selected adult SHB were pooled for yeast isolation. Four representative *K. ohmeri* colonies were then randomly selected for purification and DNA extraction. In two locations (Sherwood, Richmond) all four *K. ohmeri* isolates were identical for that location, while in four locations (Bellbird Park, Eastern Heights, St Ives, Kurrajong) there were three different *K. ohmeri* genotypes per location. The remaining three locations (Blacksoil, Bellbowrie, UWS Richmond) each yielded two different *K. ohmeri* isolates (Table 8, Figure 25).

The *Kodamaea ohmeri* ITS sequences deposited in Genbank illustrate both the cosmopolitan nature of this yeast and the diverse range of substrates that it appears capable of colonising (Table 2). This yeast has previously been isolated from environmental sources (including seawater, cucumber brine, mud and sediments, marine algae); fish; birds; insects (including SHB) but more disturbingly, blood from immuno-compromised humans. A phylogenetic tree constructed with the ITS sequences from selected Australian SHB *K. ohmeri* isolates and those deposited in Genbank (Figure 26) shows the relationship between these isolates and suggests 2 large groups that could be further discriminated into 6 clades. SHB derived isolates of *K. ohmeri* occur in all but 1 of those clades and entirely make up the bottom

4 clades. It can also be seen there are Australian isolates of SHB derived *K. ohmeri* identical to the two isolates previously derived from SHB in Florida and Kenya.

The ITS sequence of the *K. ohmeri* isolate (AY911385) from an adult SHB collected in a honey bee colony in Florida is identical to three isolates derived from SHB in Australia (WM09.206, WM09.207, WM09.209). The MLMT genotypes of these three isolates are 1, 2, 2 (respectively). The ITS sequence of the *K. ohmeri* isolate (EU569326) from a SHB larva collected in Kenya is identical to the Australian isolate WM10.192, which has the MLMT genotype of 20.

Figure 26 shows there are SHB derived isolates of *K. ohmeri* more closely related to the clinical isolates from Kuwait than these isolates are to the other clinical isolate from Brazil. The difference between the Kuwait and Brazil clinical isolates is 6 changes. Whereas the difference between the two Kuwait isolates and the Australian isolates WM10.192 (MLMT 20) and WM10.200 (MLMT 28) is represented by one and two changes (respectively). Additionally, Australian isolates WM10.192 (MLMT 20) and WM10.221 (MLMT 16) are both only five changes different to the clinical isolate from Brazil.

Table 8. MLMT genotypes of *K. ohmeri* isolates obtained from adult SHB sampled throughout Queensland and New South Wales. The MLMT genotype of the *K.ohmeri* isolates was generated via three different PCR sequencing patterns (M13, [GACA]₄ and [GTG]₅)

Westmead deposit number	State	City where SHB collected	Date isolated	Molecular species identification confirmation				
				ITS Sequence	M13 pattern	(GACA) ₄ pattern	(GTG) ₅ pattern	MLMT
WM 09.207	Qld	Murgon	22.07.09	✓	1	2	1	2
WM 10.1	NSW	Bathurst	08.04.09	ND	4	6	9	17
WM 10.2	NSW	Bathurst	08.04.09	ND	5	1	1	6
WM 10.3	Qld	Bellbowrie	30.04.09	ND	13	16	10	19
WM 10.5	NSW	Moruya	18.07.09	ND	8	8	8	10
WM 10.6	NSW	Camden	22.07.09	ND	16	13	20	27
WM 10.7	Qld	Wondai	27.07.09	ND	5	1	1	6
WM 10.8	Qld	Weyba	07.08.09	ND	1	3	2	3
WM 10.9	Qld	Nambour	07.08.09	ND	8	9	5	11
WM 10.10	Qld	Mullet Creek	07.08.09	ND	8	9	5	11
WM 10.208	Qld	Blacksoil	03.11.10	✓	8	10	5	13
WM 10.209	Qld	Blacksoil	03.11.10	ND	8	10	5	13
WM 10.210	Qld	Blacksoil	03.11.10	ND	9	11	6	14
WM 10.211	Qld	Blacksoil	03.11.10	ND	9	11	6	14
WM 10.212	Qld	BellBird Park	04.11.10	ND	10	12	6	15
WM 10.213	Qld	BellBird Park	04.11.10	ND	19	22	21	24
WM 10.214	Qld	BellBird Park	04.11.10	ND	10	12	6	15
WM 10.215	Qld	BellBird Park	04.11.10	ND	12	21	3	18
WM 10.216	Qld	Eastern Heights	04.11.10	ND	19	24	21	25
WM 10.217	Qld	Eastern Heights	04.11.10	ND	20	23	22	26
WM 10.218	Qld	Eastern Heights	04.11.10	ND	20	23	22	26
WM 10.219	Qld	Eastern Heights	04.11.10	ND	10	12	6	15
WM 10.220	Qld	Bellbowrie	04.11.10	✓	19	24	21	25
WM 10.221	Qld	Bellbowrie	04.11.10	✓	11	12	6	16
WM 10.222	Qld	Bellbowrie	04.11.10	ND	19	24	21	25

Westmead deposit number	State	City where SHB collected	Date isolated	Molecular species identification confirmation				
				ITS Sequence	M13 pattern	(GACA) ₄ pattern	(GTG) ₅ pattern	MLMT
WM 10.223	Qld	Bellbowrie	04.11.10	ND	19	24	21	25
WM 10.224	Qld	Sherwood	04.11.10	✓	9	11	6	14
WM 10.225	Qld	Sherwood	04.11.10	ND	9	11	6	14
WM 10.226	Qld	Sherwood	04.11.10	ND	9	11	6	14
WM 10.227	Qld	Sherwood	04.11.10	ND	9	11	6	14
WM 10.230	NSW	St Ives	18.11.10	✓	8	8	5	12
WM 10.231	NSW	St Ives	18.11.10	ND	8	8	5	12
WM 10.232	NSW	St Ives	18.11.10	ND	2	2	2	4
WM 10.233	NSW	St Ives	18.11.10	ND	5	1	1	6
WM 10.234	NSW	Richmond (UWS)	18.11.10	✓	7	5	4	8
WM 10.235	NSW	Richmond (UWS)	18.11.10	ND	7	5	4	8
WM 10.236	NSW	Richmond (UWS)	18.11.10	ND	7	5	7	9
WM 10.237	NSW	Richmond (UWS)	18.11.10	ND	7	5	4	8
WM 10.238	NSW	Richmond	18.11.10	ND	7	5	4	8
WM 10.239	NSW	Richmond	18.11.10	ND	7	5	4	8
WM 10.240	NSW	Richmond	18.11.10	ND	7	5	4	8
WM 10.241	NSW	Richmond	18.11.10	ND	7	5	4	8
WM 10.242	NSW	Kurrajong	18.11.10	ND	5	1	1	6
WM 10.243	NSW	Kurrajong	18.11.10	ND	3	1	1	5
WM 10.244	NSW	Kurrajong	18.11.10	ND	7	5	4	8
WM 10.245	NSW	Kurrajong	18.11.10	ND	7	5	4	8

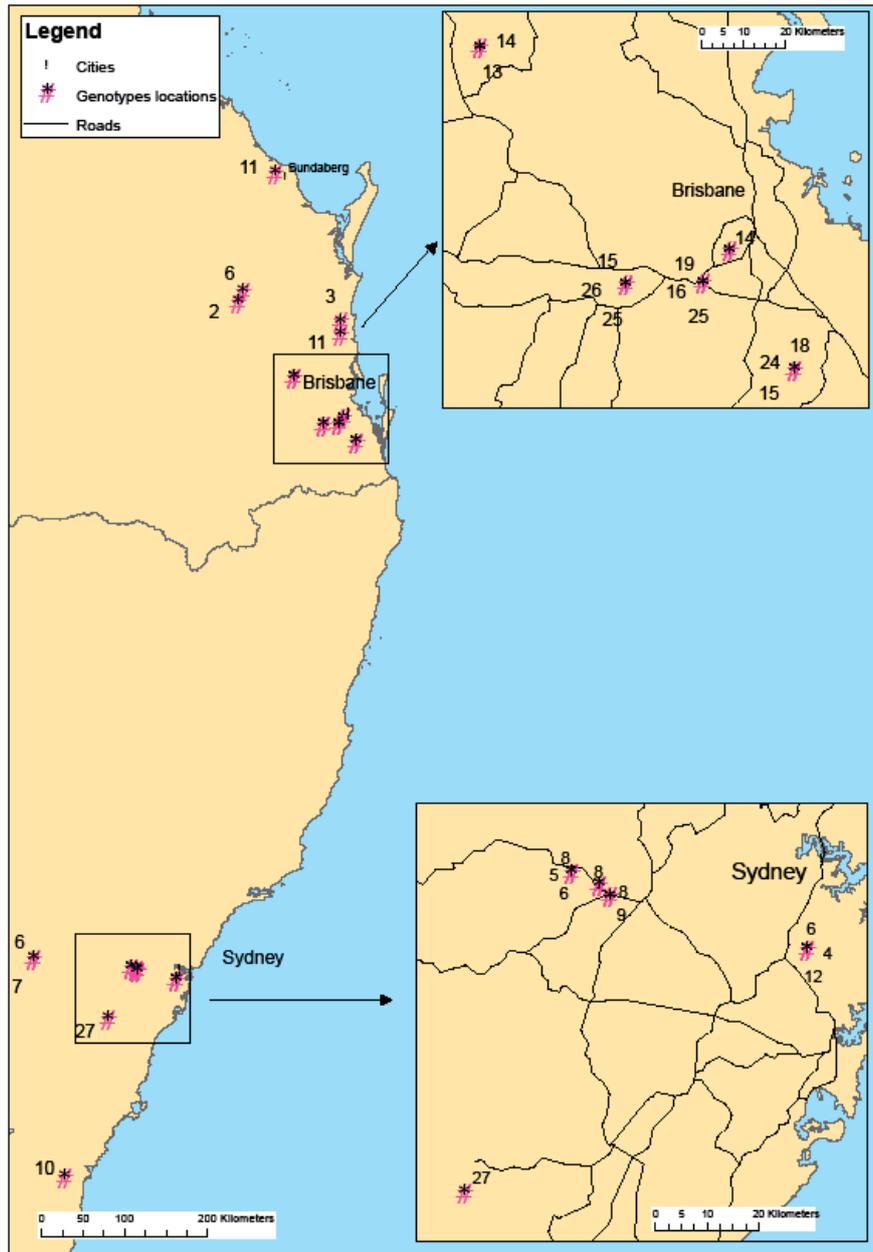


Figure 25. Location of *Kodamaea ohmeri* MLMT genotypes arising from adult SHB collected in the field in Qld and NSW

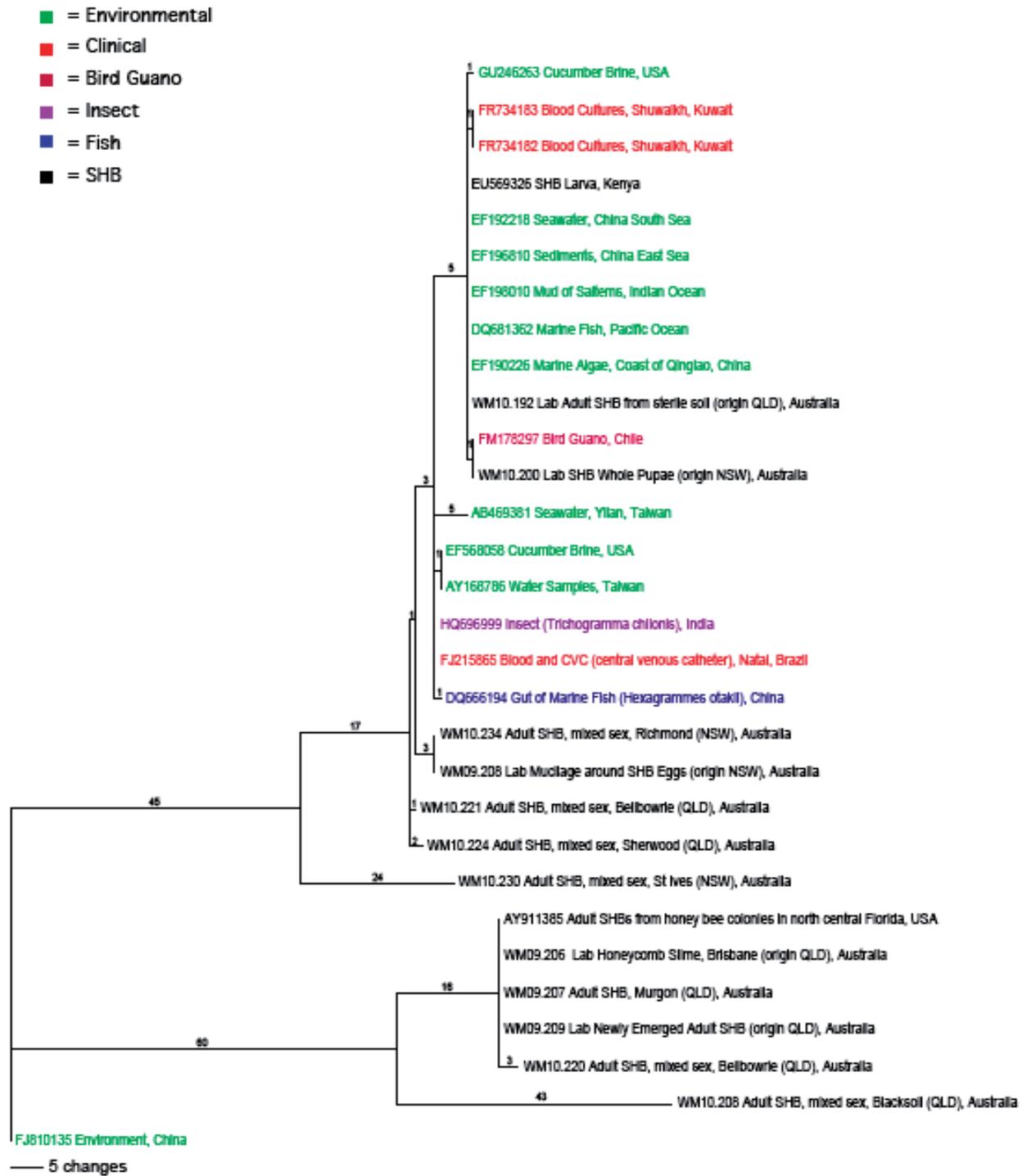


Figure 26. Phylogenetic tree constructed from a comparison of ITS sequences from Australian SHB isolates of *K. ohmeri* to ITS sequences from *K. ohmeri* isolates deposited in Genbank. The *K. ohmeri* isolates represented by the sequences deposited in Genbank originated from a diversity of sources. The sequence sources have been colour coded as labelled in the top left corner

Mouse virulence study

No mice showed any symptoms of infection or ill health after inoculation with even the highest dose (1×10^9 cells/ml) of *K. ohmeri*. In addition no yeast grew when organ suspensions were plated onto SDA after the mice were euthanised. Because there was no evidence of *K. ohmeri* in any of the mice one month after being injected with a range of doses, it appears that the immunocompetent mice successfully eliminated it from their systems.

Discussion

Larval investigations

The results of both the semi-field trial and field trials are encouraging despite the variability in the results. They demonstrate a proof of concept that fungal spores added to the soil can disrupt the SHB life cycle by killing SHB larvae exiting hives to pupate in the treated soil. The variability could have been offset by more trials, however the number of trials conducted was limited by the time required to run each trial. Each semi-field and field trial took a minimum time of 35–45 days during the warmest weather while the trial during late summer and early autumn took up to 81 days for adult emergence to complete. Moreover some of the trials were adversely affected by the weather. The trials also demonstrated that the fungus applied as a granular product was more effective than when applied in an oil based formulation. A granular product would be cheaper to produce and market than an oil based formulation

These investigations showed that a fungal control added to the soil will infect and kill a large proportion of larvae exiting the hive, thus preventing the build up in SHB numbers around a hives. Beekeepers using bottom board traps have reported regularly catching larvae in these traps even when there is no obvious larval damage to combs visible inside the hive (personal communication). This suggests a low level of background breeding of SHB can occur in hives without obvious damage, enabling the gradual increase in SHB numbers from this localised breeding to a level where severe damage can be inflicted. Breaking the SHB life cycle in the soil around hives should be an essential management strategy.

These trials also highlighted the effects of both predation and weather on SHB pupation. In the preliminary field trials only 44% of adult SHB emerged in one trial and 29% in another while there was no pupation in two trials due to extreme summer rain events. It is important to understand how both the environment and weather may limit the emergence of adult SHB. The results of this study also suggest that predation on larvae around hives should be encouraged. It is worth noting that chemical treatments will not only kill larval SHB in the local area around hives but they will also reduce or even kill off the populations of potential predators. This is undesirable, especially if the chemical treatment is then washed from the soil in heavy rain or breaks down, there will be no natural controls for SHB larvae left in the soil. A biological control such as *Metarhizium* is likely to be more selective and thus less damaging to non-target beneficial organisms that can prey on larvae.

Adult investigations

The results obtained for the fungal control of adult SHB are promising. Several isolates of *Beauveria bassiana* caused high rates of mortality in adult SHB (100% for some isolates) when beetles were dipped in the spore powder. Adult SHB were also exposed to the best isolates in refuge traps to test a mechanism for applying the fungus inside a hive. In previous studies (PRJ-000037) it was noted that fungal spores distributed inside a hive for SHB control were quickly cleaned up by worker bees. Therefore fungal spores will need to be separated from bees by applying inside a refuge trap for SHB. The *Beauveria* isolates controlled adult SHB through infecting and killing some adults and reducing the fecundity of surviving adults. The overall effect was to significantly reduce the number of eggs and the resulting destructive larval stage. This project was unable to carry out an in hive assessment of the *Beauveria* controls due to a change to hive access when the research laboratories were moved to a new site which lacks suitable facilities for maintaining hives. Further work is recommended to investigate improving the formulation of the *Beauveria* spores to optimise both the spore viability and the uptake of the spores by adult SHB under hive conditions. In hive testing in different locations should then be carried out.

Richards *et al.* (2005) studied the treatment of the small hive beetle with DE in the presence of fungal spores to assess whether it increased mortality. The possibility of using DE as a carrier for fungal spores served as one motivation for investigating the effects of DE on SHB in the current study. Richards *et al.* (2005) observed surface lacerations between segments of SHB larvae treated with DE. This was consistent with speculation that DE would kill adult SHB through scarification of the cuticle disrupting their waterproofing. However our investigations with the scanning electron microscope (SEM) did not reveal any evidence of scarification on the cuticle of adult SHB after the application of DE. Larvae however, are likely to have softer cuticles than adults and this may explain the difference in the results in this study compared to Richards *et al.* (2005).

Interestingly Richards *et al.* (2005) found that despite the scarification on SHB larvae by DE it did not assist in pupal mortality. However the current study showed that nearly all SHB perished in the DE added to AJ's beetle eater traps. The SEM investigations showed how the small particles of DE adhere to the sensilla of adult SHB leading to adults becoming totally coated when they enter a trap such as the AJ's beetle eater trap. Coated beetles did not escape from such traps and died within 1–2 days. While no actual mechanism of SHB death from DE was proven it could be speculated that death occurred through a combination of effects; including a disruption of waterproofing via DE adsorption to surface waxes and a blocking of sensory input when DE particles coat the sensilla. This study provides support for the practice of using DE in traps instead of oil. The use of DE has advantages over oil when traps are not completely level and hives are moved about as the DE does not easily spill out of the traps.

Buchholz *et al.* (2009) added DE (in this case Fossil Shield ® 90) to bottom boards under hives and found that more than 50% of the beetles in naturally infested hives perished in 2 d. But concern was raised about endangering bees and bee products via DE dust circulating through the hive driven by thermoregulation (bee fanning behaviour). The current study finds the use of DE in the AJ's Beetle Eater® trap is effective for inducing mortality (>99%), it therefore provides a suitable method to both contain and introduce DE to the hive. The trap is placed between frames at the top of the hive rather than the bottom where air is drawn in for ventilation; the DE is contained in a covered trench; and the trap contains the DE in such a way that honeybees will not come in contact because the lid slots are too small to allow bee ingress but of a size that allows beetles to pass through.

Yeast Investigations

The preliminary yeast studies showed that the slime generated when larval SHB destroy hives is largely composed of yeast cells. Moreover the dominant yeast was identified as *Kodamaea ohmeri* although a range of other yeasts was also found. *K. ohmeri* isolated from the slime was found to be osmo-tolerant thus able to grow at honey concentrations inhibitory to many other fungi. *K. ohmeri* was also noted to grow well at temperatures between 30°C and 40°C, while one of the other yeasts isolated from slime did not grow well at the higher temperatures. This suggests *K. ohmeri* is well adapted to growing at the temperatures experienced inside a hive, while the other yeast, possibly one incidentally brought into the hive by bees, would not grow well until the hive had collapsed and its temperature dropped to ambient.

The yeasty slime associated with hive destruction was also generated in the laboratory when small hive beetles were allowed to breed on protein cake. *K. ohmeri* was also present in this slime and came to dominate the slime as larvae developed on the protein cake. However when the larvae had completed development and moved away to pupate the slime disappeared being replaced with a dry insect frass that did contain some yeast cells. This suggests a mutually beneficial relationship, possibly nutritional, between *K. ohmeri* and SHB larvae as the larvae grow and develop.

The volatile studies conducted during this project build on prior research in which various hive related materials characterised with GC-MS were investigated for their attractiveness to SHB (Torto *et al.* 2005, 2007b, Suazo *et al.* , 2003) and the work of Benda *et al.* (2007) in which volatiles generated by

K. ohmeri were similarly investigated. Benda *et al* (2007) investigated volatiles emanating from amended synthetic media (Sabouraud Dextrose Yeast Agar) inoculated with *K. ohmeri*. One of the aims of this study was to identify volatiles common to both SHB associated slime and pure cultures of yeasts isolated from slime. Therefore this study analysed volatiles from hive products, pure yeast cultures and hive products modified by yeast and SHB larvae (slime). The attractiveness of these materials and some of their chemical components to adult SHB was then examined.

The GC-MS profiles of the samples of SHB derived slime and yeast cultures found seven compounds were common to pure yeast cultures and the slime. Both ethyl alcohol and ethyl acetate were produced in large amounts. These compounds are typical yeast products. Bartelt and Hossain (2010) listed them as model materials abundant in yeast fermentation that feature in many of the blends attractive to *Carpophilus* beetles. The only compound common to yeasts and slime in our study that was also reported in the studies by Benda *et al* (2007) and Torto *et al* (2005, 2007) was 1-butanol 3-methyl. It is interesting that their studies also reported the presence of 1-butanol 2-methyl known as isopentyl acetate, a major component of the honey bee alarm pheromone (Torto *et al*, 2007a). In our study this compound was present in volatiles emanating from all three of the pure yeast cultures but not from any of the volatiles associated with the slime collected from hives destroyed by SHB larvae. Furthermore while the studies of Torto *et al* (2007 b) reported isopentyl acetate to be attractive to adult SHB our studies did not find it to be as attractive as the yeast and slime volatiles. When searching for compounds attractive to adult SHB the investigation of the slime emanating from a destroyed hive which has been observed to attract large numbers of adult SHB (Leemon: pers. observation) should provide a more realistic baseline than laboratory produced volatiles from pure yeast cultures.

Our investigations, although of a very preliminary nature, did establish a hierarchy of SHB attractiveness for the various hive materials, yeast modified hive materials and pure yeast cultures with fresh hive collected slime appearing to be most attractive. These studies provide a basis and direction for any future studies aimed at developing a synthetic SHB attractant for use in traps outside of hives.

The data collected during the larval field trials provide an early proof of concept for the use of an attractant trap to capture emerging adult SHB. It is presumed that such traps will also have the potential to be effective in trapping adult SHB flying into apiaries from distant sites if a strong enough attractant is used. These studies suggest that the best attractant will be based on a synthetic mix of volatiles from hive products as well as yeast modified products. The development of a synthetic SHB attractant will require considerable effort to choose the best chemical components from hive products and yeast modified hive materials and optimise the ratios of these chemical ingredients to produce a highly attractant and cost efficient attractant for use in traps to be placed in apiaries.

The results from the molecular investigations clearly demonstrate that not only was *Kodamaea ohmeri* present in all SHB sampled from a range of locations in Qld and NSW but was also present throughout the whole lifecycle of the small hive beetle. Moreover this investigation found *K. ohmeri* had a dominating presence in the slime generated by SHB larvae as they develop on hive materials. Importantly *K. ohmeri* has been isolated from small hive beetles produced via sterile methods and virgin adult beetles. The same genotype (MLMT20, Figures 23, 24) was isolated from both virgin male and female adult SHB as well as mated male and female adults and newly emerged adults that had begun pupation in sterilised soil as surface sterilised larvae. Another very similar genotype (MLMT 28, Figures 23, 24) was isolated from both mated male and female adult SHB, the mucilage around SHB eggs, larvae and pupae that had also begun pupation in sterile soil as surface sterilised larvae. This finding of clones of *K. ohmeri* from different stages of the SHB life cycle suggests an intimate, possibly symbiotic association between *K. ohmeri* and the small hive beetle with this yeast being taken in by larvae and carried internally in pupae and adult beetles. *K. ohmeri* has previously been reported from small hive beetle adults, larvae and infested hives (Benda *et al* 2008). It has been shown that other Coleopteran species which harbour yeasts carry them in their gut (Paine *et al*. 1997;

Rivera et al 2009), contributing to a close relationship or symbiosis between the beetle and yeast. Benda *et al.* (2008) also noted that when a hive is heavily infested with small hive beetle larvae the only species of yeast present appeared to be *K. ohmeri*. This suggests *K. ohmeri* may either block or out-compete other yeast species. The investigation in our study of SHB larval development on protein cake corroborates this observation (Figure 21).

Frank (1996) postulated that within host-symbiont relationships the host benefits via increased fitness when harbouring only one symbiont. A symbiont can be described as an organism whose normal habitat is part of another living organism for part or all of its life (Smith, 1979). Fungal associations and symbioses with arthropods of numerous families are quite well known (Noda & Kodama, 1996; Suh *et al.*, 2001; Vega & Dowd, 2005; Nguyen *et al.*, 2007). Dowd (1992) noted that the degree of intimacy varies in fungal arthropod associations and Suh *et al.* (2005) suggested that the relationship can be crucial to keeping the arthropod host alive. The fungus may provide additional nutrients where the diet is poor (Suh *et al.* 2005), aid digestion (Vega & Dowd 2005), aid metabolism or assist with defensive mechanisms (Frank 1996) and help detoxify or metabolise toxins (Dowd 1992). Many beetle species inhabiting nutrient poor environments harbour yeast symbionts, as evidenced with the drugstore beetle (*Stegobium paniceum*) and the cigarette beetle (*Lasioderma serricorne*). Both these Anobiidae family beetles are pests of stored products and harbour yeast symbionts between the fore and mid gut (Plant and Fraenkel, 1954; Noda and Kodama, 1996). Scolytid bark beetles harbour a fungus in specialised structures on their body. This beetle benefits nutritionally by feeding on the fungus to supplement an otherwise nutrient poor diet (Paine *et al.* 1997). Bark beetles of the genus *Dendroctonus* are known to harbour yeasts which have been speculated to aid with nutrition, detoxification and pheromone production (Rivera *et al.* 2009).

Within the sap beetle or Nitidulidae family, of which the small hive beetle (*Aethina tumida*) is a member, a number of fungal-arthropod associations have been reported. (James 1993; James 1995; Rosa *et al.* 1999; Lachance *et al.* 2001; Starmer & Lachance, 2011). Lachance *et al.* (2001) noted that Nitidulid beetles vector a highly specific yeast community that may serve as food for the larvae of the host insects carrying them. At least three species of *Kodamaea* have previously been reported in association with beetles from the Nitidulid genus *Aethina* (Lachance *et al.* 2001). Specifically, *K. anthophila* was noted to be dominant in *Aethina concolor* on Maui and Kauai in the South Pacific (Lachance *et al.* 2001). Moreover *K. nitidularum* was named in recognition that it is vectored by members of the nitidulid family (Rosa *et al.* 1999). From molecular evidence Rosa *et al.*, 1999 noted that *K. anthophila* and *K. nitidularum* appear to be relatively recent species. They speculated that *K. ohmeri* by virtue of its less specialized ecology and position in their molecular phylogeny may represent the ancestral form to these species which both show greater substrate specificity than the less specialized *K. ohmeri*. However our current studies suggest *K. ohmeri* has a very specialized relationship with a Nitidulid beetle, the small hive beetle.

The presence of *K. ohmeri* in the mucilage around SHB eggs supports the idea that the transmission of *K. ohmeri* in the SHB occurs vertically from parent to offspring. Gut yeasts being maternally transferred from generation to generation by smearing eggs with yeast cells has been reported for other Coleopteran species of Anobiid and Cerambycid beetles (Suh & Blackwell 2004; Douglas 1989; Jones et al 1999). Some beetle species are reported to purge their gut in preparation for pupation (Nijhout 2001; Emlen and Nijhout 1999). Whether the SHB purges its gut is unknown. But if it does this may be the reason that other species of yeast are not as prevalent at the pupa and egg life stages, assuming that *K. ohmeri* is retained in the fore gut region during purging. No specific yeast harbouring structures were detected during dissection of adult SHB, and *K. ohmeri* was isolated from the fore, mid and hind gut regions therefore it is assumed that *K. ohmeri* primarily resides and is carried in the gut of adult SHB.

The primary habitat of adult SHB is inside honey bee hives (Lundie 1940; Schmolke 1974; Hepburn & Radloff 1998) which consists of large amounts of hydrocarbons (wax) and carbohydrate (a complex range of sugars) and a limited amount of proteins (brood, pollen stores). Adult SHB need a protein source such as brood to reproduce (Ellis 2002; Neumann & Elzen 2004). To support the development

of very large numbers of protein hungry larvae a hive represents a nutrient limited environment if the honey and wax cannot be utilised. It is possible that *K. ohmeri* may provide critical nutritional support to larval SHB as a food source and possibly to adult SHB by aiding digestion.

A strain of *K. ohmeri* (BG3) isolated from the gut of the marine fish species *Hexagrammes otakii* was found to produce phytase (Li *et al.* 2008 a & b; Li *et al.* 2007). This enzyme catalyses the release of inorganic orthophosphates from phytates and phytic acid (Mullaney and Ullah 2003). Barrientos *et al.* (1994) showed phytic acid to be present in pollen grains and Kuang *et al.* (2009) showed pollen grains store phosphorous as phytate. Glucose, readily available in hives was demonstrated to be the best carbon source for phytase production by *K. ohmeri* (Li *et al.* 2008a; Chi *et al.* 2009). The presence of yeasts within a species of *Drosophila* fly was shown to assist phosphorus uptake by the fly (King 1954; Lipke and Fraenkel 1965). Thus it could be speculated that *K. ohmeri* may help SHB with phosphorous uptake. Anecdotal observations suggest that hives are more vulnerable to high larval breeding leading to slimeouts when they have large stores of pollen. The yeast may be crucial in releasing nutrients including phosphorus from the pollen for use by the SHB. However further investigation in this area is recommended.

A proposed nutritional role of *K. ohmeri* in the life cycle of the SHB (*Aethina tumida*) is outlined in Figure 27. This speculation is based on observations and results of investigations noted in the current study. Further research to confirm this speculation is warranted. It is proposed that *K. ohmeri* is carried within the gut of adult SHB as a symbiont which may aid digestion. Adults add this yeast to the mucilage they coat their eggs with. However germination and growth of the yeast on hive products, mostly honey and wax, requires some type of stimulation from the developing larvae, possibly larval wastes. *K. ohmeri* is then able to grow on the substrate converting it to very large numbers of yeast cells. These cells can then be consumed by the developing larvae as a rich food source containing protein. Once larvae stop feeding and begin pupation some *K. ohmeri* cells are retained, possibly in the in the gut, and so will be present in the digestive system when they emerge as new adults.

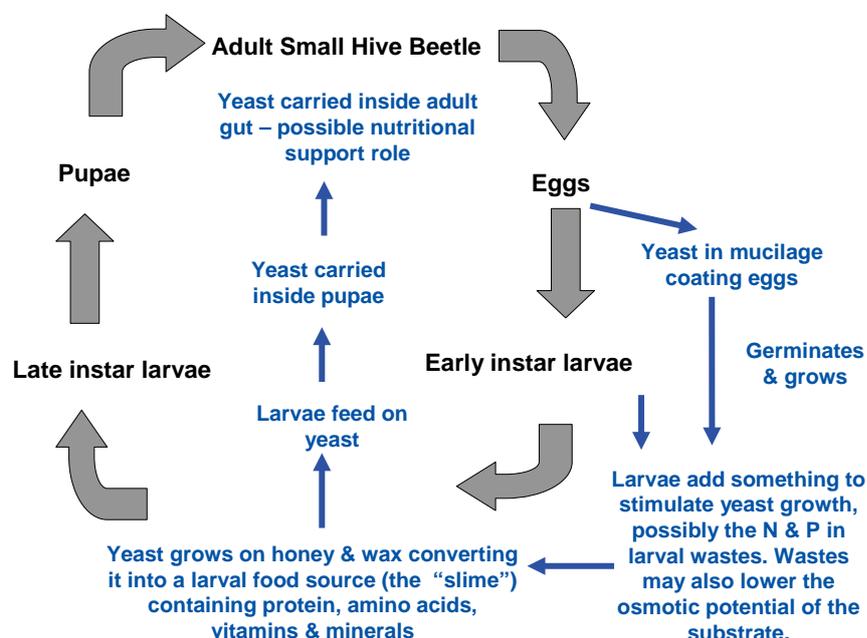


Figure 27. A proposed nutritional role of the yeast *Kodamaea ohmeri* in the life cycle of the small hive beetle *Aethina tumida*

Benda *et al.* (2008) isolated different stains of *K. ohmeri* from SHB sourced from Florida and Kenya and demonstrated, with limited sampling, an association between this yeast, SHB and honeybee hives in two distinct locations. Our study has verified the presence of *K. ohmeri* in SHB in Australia. Benda *et al.* (2008) noted that the mechanism by which *K. ohmeri* enters the hive is not well understood. They speculated that adult beetles originating in heavily damaged hives or from some unknown substrate may inoculate healthy hives with yeast residues. This current study suggests that *K. ohmeri* is a symbiont carried by all stages of SHB, and enters hives with adult beetles. It is therefore likely that SHB brought their own strains of *K. ohmeri* with them when they first entered Australia before 2002.

K. ohmeri appears to be cosmopolitan yeast that has been isolated from a wide range of substrates including cassava roots, fruits films on brine, pickles, cassava roots, soil and sea water (Suh and Blackwell 2005; Ferreira *et al.* 2009; Chiu *et al.* 2010). However it has also been recently identified as the etiological agent of human fungal infections (Yang *et al.* 2009; de Barros *et al.* 2009; Chiu *et al.* 2010; Shang *et al.* 2010).

Yang *et al.* (2009) note that *Kodamaea ohmeri* was formerly considered a contaminant, but is now known to be a significant human pathogen that has been shown to cause fungemia, endocarditis, funguria, and peritonitis in immuno-compromised patients. De Barros *et al.* (2009) summarising the clinical features of *K. ohmeri* infections in the literature noted that *K. ohmeri* infections occur in a broad range of patient categories, including neonates and children and on different continents. They suggest it likely that the number of described cases represents a low estimate of the actual incidence because it seems likely that the species may not be properly diagnosed in many routine hospital labs. Chiu *et al.* (2010) observed that opportunistic fungal infections due to uncommon fungal pathogens have increased over the last decade. They suggest that *K. ohmeri* is an emerging pathogen in immuno-compromised patients citing 21 cases of *K. ohmeri*, some successfully treated with fluconazole or caspofungin but others untreated, caused mortality.

Shang *et al.* (2010) also reviewed the literature on human infection by *K. ohmeri* noting that although *K. ohmeri* has been recognised as an opportunistic pathogen especially in immuno-compromised patients, there have been two reports of infection in immuno competent patients. They concluded that *K. ohmeri* is an emerging opportunistic pathogen of clinical practice that should not be regarded as a contaminant of blood cultures.

When beekeepers are confronted with hive losses through slime outs many will try to retrieve the hive boxes and some frames because of the high cost of replacement. Cleaning such hives can involve hosing out the slime. This will create aerosols which may contain large numbers of *K. ohmeri* cells. These cells could be inhaled by beekeepers. Many amateur beekeepers are retired males of 70+ years and therefore more likely to belong to a demographic confronting health issues such as prostate cancer treatment and hip or knee replacement and hence could have suppressed immunity. Therefore caution is recommended to minimise exposure to the yeast when dealing with a slimed up hive. Treating the slime with a 10% dilution of household yeast should kill the yeast and appropriate protective equipment such as water proof gloves and face shields should also be used.

Although no mice showed any signs of infection or ill health when inoculated with SHB derived strains of *K. ohmeri* the tests were undertaken with healthy immuno-competent mice. This current study revealed genetic variation across the strains isolated from SHB with the 70 isolates falling into 32 different MLMT genotypes. Only 2 SHB isolates were tested against mice. A comparison of the ITS sequences of selected Australian SHB *K. ohmeri* isolates and the 3 clinical isolates suggested a close similarity between there isolates and the clinical isolates. Some of the SHB isolates appeared to be more closely related to the clinical isolates than the clinical isolates were to each other. ITS sequence data is used as a measure of the relatedness between organisms and is now perhaps the most widely sequenced DNA region in fungi (Peay *et al.*, 2008). It has also proven especially useful for elucidating relationships among clinically important yeast species (Chen *et al.*, 2001). However ITS sequence data cannot be used as a predictor of virulence of yeast towards mammals. To better assess the clinical potential of the *K. ohmeri* isolates from SHB more screening should be undertaken,

preferably with immuno-suppressed mice. The fact that a SHB derived isolate of *K. ohmeri* was found to be able to grow well at Human body temperature (Figure 19) should also be of concern. Most fungi do not grow well at temperatures above 33°C and any that do should be handled with caution.

Conclusion

This research showed that a *Metarhizium* based control added to soil can infect and kill a large proportion of larvae entering the soil to pupate, thus preventing the build up in SHB numbers around hives. The negative impact of predation and weather on pupating SHB was also highlighted. Several isolates of *Beauveria bassiana* highly virulent to adult SHB with the potential for in hive testing were identified, furthermore it was shown that sub-lethal doses of these fungi will significantly reduce the fecundity of surviving beetles.

The ability of diatomaceous earth (DE) to kill adult SHB when exposed to it in traps was quantified, while electron microscopy showed that DE particles adhere to and coat the sensilla of adult SHB rather than scratching the surface as has been postulated.

Extensive studies into yeasts and the SHB found that *K. ohmeri* was present in all samples of adult SHB collected throughout NSW and Qld, furthermore this yeast was also found in all stages of the SHB life cycle including a dominating presence in the slime associated with larval SHB hive destruction. *K. ohmeri* was also isolated from different regions of the gut of adult female and male SHB. These findings support the hypothesis that *K. ohmeri* is an important symbiont of the SHB providing nutritional support for the larval, and possibly, adult stages. Molecular studies revealed the genetic diversity of the Australian isolates of *K. ohmeri* showing there are Australian isolates with identical genetic profiles (via ITS sequencing) to the two isolates of *K. ohmeri* previously obtained from adult and larval SHB in Florida and Kenya respectively. Of concern is the similarity of the genetic profile of some Australian SHB derived *K. ohmeri* isolates to that of clinical isolates responsible for fungemia in immuno-compromised patients in Kuwait and Brazil. Although the results of the mouse model virulence study with two SHB derived *K. ohmeri* isolates were negative a more comprehensive study should be undertaken to gain a clear understanding of the potential for human infection from the SHB vectored isolates of *K. ohmeri*.

Studies on the attractiveness of volatiles from hive products, yeast and yeast modified hive products established a hierarchy of attractiveness to adult SHB. Chemical analyses of the volatiles identified compounds common to both pure yeast cultures and the slime produced in a larval SHB mediated hive collapse. However differences in the volatile components were also noted. Traps with an attractant mix consisting of hive products, slime and yeast were successfully deployed in the larval field trial to trap emerging adult SHB, provided support for the concept of an out of hive attractant trap for use in apiary sites.

Recommendations

The results obtained in this study support further research into a *Metarhizium* control for larval SHB and further studies on the relationship between SHB and *K. ohmeri* in regard to the development of a synthetic SHB attractant and the potential for human infection from *K.ohmeri* in hive slime.

Specifically:

- Further evaluation and development of a *Metarhizium* based commercial product for controlling SHB larvae in the soil under hives and an investigation into the APVMA registration status of such a product to help assess the economics of the development of such a product
- Further research into the use of *Beauveria* in traps inside hives is not recommended at this point because of the success of the Apithor® trap currently on the market
- Further studies into the attractiveness of the components of volatiles arising from *K.ohmeri* slimed hive products to SHB including a detailed analysis of SHB behaviour to provide data to underpin the development of an out of hive attractant trap with synthetic attractant. In addition ecological research should be carried out to provide information to optimise trap design, placement and optimal time of year to deploy for use
- Further investigations into the genetic variation of SHB vectored *K. ohmeri* and potential for virulence towards humans. This should involve more extensive field sampling from SHB, with molecular and growth characterisation of *K. ohmeri* followed by a simple mass screening with the *Galleria* larval model, if this model works with *K. ohmeri*. Further screening of selected isolates with the mouse virulence model using both a different method of exposure, preferably through aspiration, and immuno-suppressed or immuno-compromised mice
- Health warnings in regard to the potential of *K. ohmeri* infection from slimed up hives together with instructions on how to safely clean up slimed up hives should be disseminated to bee keepers. The warning ought to i) Advise beekeepers that simple precautions will minimise exposure to *K. ohmeri* in the slime associated with SHB mediated hive collapse and ii) recommend that a face shield and disposable gloves be worn when handling slimed frames and hive boxes which should be treated with a solution of household bleach (10% dilution) before hosing the slime.
- An investigation into the extent of *K. ohmeri* yeast contamination in honey sent to commercial packers. If cells are in the honey such a study should also aim to establish what level of yeast cells is acceptable and measureable, viability of the yeast in honey and treatments to inactivate yeast cells without affecting the honey.

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In-hive Fungal Biocontrol of Small Hive Beetle

By Diana Leemon

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The small hive beetle (SHB) is a native scavenger of bee hives in South Africa where it is regarded as a minor pest. It was discovered in Australia in 2002. Since this time SHB populations have increased in number and range in the eastern states of Australia where in some areas they are causing significant losses.

This report targets the beekeeping industry in Australia, particularly beekeepers in the warmer regions affected by the small hive beetle, and extension staff in advisory roles. It is intended to provide information on non-chemical control options for the small hive beetle as well as important information about the yeasts associated with this pest which aid in the destruction of bee hives and stored comb and potential health risks.

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Cover image: Small hive beetle larvae