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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PH0503

2. Project title:
   Development of a monitoring system for the small hive beetle, Aethina tumida (Murray) (Coleoptera: Nitulidae)

3. Contractor organisation(s):
   National Bee Unit
   Food & Environment Research Agency (Defra)
   Sand Hutton
   York
   YO41 1LZ

4. Total Defra project costs (agreed fixed price): £ 225,772

5. Project: start date ..... 01 May 2006
   end date ..... 31 March 2009

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

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

There is a need to maintain robust bee stocks in the UK, not only to protect the honey industry but, more importantly, to ensure a healthy supply of vital pollinators, whose activities are key to the successful production of many commercially important crops. Globalisation, trade and movement of bees around the world have increased the potential risks to bee health posed by exotic pests. Recent concern has focused on *Aethina tumida* [the Small Hive Beetle (SHB)]. Although still absent from the UK, SHB is considered to be a major threat to the long-term sustainability and economic prosperity of EU and UK apiculture. The primary aim of the current project (PH0503) was to establish a system for early detection and monitoring of SHB, by developing an effective lure and trap system; it also sought to investigate key aspects of behaviour for optimum deployment of monitors and to examine the potential of novel control measures. To this end, a culture of SHB from mixed origins (South Africa and USA) was successfully established at Fera. Under closely controlled laboratory conditions, this has subsequently produced multiple thousands of standard adult and larval beetles, over several generations, for use in the experimental programme:

Development and validation of lure and trap

- In order to identify natural cues that are attractive to the SHB, volatiles were extracted from a range of hive-associated materials, either by aeration onto porous polymer, and/or by solid phase microextraction.
- These trials confirmed that slumgum, a waste product from honey production, contained a particularly rich array (80 compounds) of potentially attractive substances.
- Adult beetles were exposed to these extracts and their responses measured by two complementary methods: electroantennography (EAG), and behavioural bioassay using olfactometry, respectively.
- Further elucidation of electrophysiologically active compounds was achieved by means of coupled solid phase microextraction-gas chromatography–electroantennographic detection (SPME-GC-EAD). A total of 16 chemical components were identified as potential attractants for adult SHB.
- The ten chemicals that elicited the greatest EAG responses were subjected to further bioassays to assess their individual effects on the behaviour of adult SHB.
- After complex multiple comparisons between different candidate chemicals, and thorough statistical analyses, two substances were found to be consistently attractive to SHB, each eliciting highly significant ($P<0.001$) positive behavioural responses from adult beetles compared to all other chemicals tested: when exposed to either of these volatiles, SHB became much more active, displaying significantly increased levels of activity ($P<0.001$); it was also found that a significantly greater proportion of beetles ($P<0.001$) entered assay chambers that contained either of the test chemicals. (Unnamed to protect IP)
- A prototype lure and trap for adult SHB, incorporating a mixture of the proven attractants was developed at Fera, and its efficacy evaluated under controlled conditions.
- Laboratory trials showed that traps baited with the Fera chemical lure consistently caught more than four times as many beetles than similar unbaited traps, and this difference in trap performance was highly significant ($P<0.001$).

- Field validation began in South Africa in January 2009. Initial results are inconclusive. It is possible that the lure will only be effective over short range. However, a lack of flying beetles at the chosen field site has prevented sufficiently critical testing of the lure. Further prototype lures and traps have therefore been dispatched to two alternative test sites in the USA, and data is awaited.

Investigating SHB behaviour to assist in development of novel control practices

- To elucidate which substances SHB may use for intraspecific communication, adult *A. tumida* were extracted in a solvent, and gross EAG was used to examine the ability of male and female beetles to perceive volatiles from these extracts.
- Both male and female SHB gave EAG responses to solvent extracts from either sex. As was observed for the responses to volatiles from hive-associated components, the EAG response of female beetles was greater than that of male beetles for all sources tested.
- The respective behavioural responses of male or female SHB adults to beetle extracts were assessed using a static four-way olfactometer.
- On average, male beetles spent more than twice as much time in quadrants containing extracts derived from female beetles than they did in quadrants containing extracts derived from their own sex, but this apparent attraction was not found to be statistically significant ($P=0.15$).
- The proportion of time spent by female beetles in the quadrant with same-sex extract was significantly less than expected ($P=0.02$), suggesting some repellence.
- Analysis of solvent extracts derived from adult male or female SHB by Gas Chromatography-Mass Spectrometry (GC-MS) did not reveal any consistent differences between their chemical profiles.
- SPME-GC-MS did not show a difference between volatiles released by male and female beetles.
- Preliminary observations of the behaviour of immature ("wandering") life stages of SHB, made at Fera, suggest that a chemical cue may exert a powerful influence on larval wandering behaviour which could be exploited for the purposes of novel control practices.
- Residues were obtained from surfaces with which wandering SHB larvae had been in contact, and SHB larvae at contrasting stages of their (behavioural) development were also extracted.
- All larval-derived solvent extracts samples were analysed using GC-MS, but no consistent differences were found to exist between the profiles of wandering and non-wandering larvae, respectively.
- Many insect-derived peptides have high potency and specificity of action towards invertebrates, and as such are ideal candidate novel insecticides. Although such substances are typically ineffective when administered orally, fusion protein technology enables the oral delivery of insecticidal peptides to their site of action. Project PH0503 sought to examine the effect of one such fusion protein, in which a toxic peptide was linked to the carrier protein GNA, on larval SHB (no details provided to protect IP).
- Methods were developed to deliver the fusion protein to immature SHB, through appropriately modified diets. Larval growth, and survival were subsequently monitored.
- Although no significant increase in mortality was observed in larvae that had consumed fusion protein diet, as demonstrated for other insect orders, ingestion did result in a significant retardation of the development (weight) of SHB larvae ($P<0.05$).
- This suggests that fusion protein technology may have potential in the development of novel control methods for SHB.
- Intact fusion protein was found in larvae exposed to modified diet, but there was also some evidence of proteolytic cleavage – i.e. in at least some cases, the fusion protein may have been broken down prior to delivery to target tissues within SHB larvae. However, it is not yet known at which point this proteolytic cleavage may have occurred.
- The potential of insecticidal fusion proteins as a novel control method for both larval and adult SHB has been further explored in a separate Defra-funded project, PH0505.

Overall conclusions and opportunities for further research

- This project has clearly identified two volatile chemicals that elicit highly significant positive behavioural responses in adult SHB. These have been incorporated into a lure and trap, which proved to be effective at trapping adult beetles when deployed under controlled laboratory conditions. This system is currently being field tested in both South Africa and the USA. There is a need to undertake further laboratory and field trials to refine the trap and lure design.
- This project also identified certain volatiles that have significant negative effects on adult SHB. These have the potential to be exploited as repellents, and require further investigation.
- For the purposes of this project PH0503, bioassays were conducted under a very limited range of controlled environmental conditions, chosen to ensure that adult SHB were behaviourally active. There is a pressing need to ascertain the biology (development and behaviour) of *A. tumida* under a range of environmental conditions that are more representative of the UK (i.e. likely survival, activity and fecundity of adult SHB at cooler temperatures, and at compromised nutritional status). Such information is not only important in elucidating the efficacy of the lure components under UK conditions, but is also crucial to our understanding of the true threat (likely establishment and spread)

posed by SHB in the event of its introduction to the UK.

- The observation that in their final stages of larval development, immature "wandering" SHB cause other younger larvae to follow them away from their shared food source (bee brood) requires further investigation. While the current study was unable to determine any specific chemical characteristic(s) underlying this phenomenon, such a marked behavioural trait must have some physiological basis that, if properly understood, has clear potential to be exploited as control method.
- Experiments to ascertain the effects of an insecticidal fusion protein on the growth and survival of larval SHB were inconclusive. Although the potential of this technology in SHB control has been investigated more thoroughly elsewhere (Defra project PH0505), there is still a need to improve the stability of fusion proteins within appropriately modified beetle diet.

Project Report to Defra

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

Introduction

Honey bees (*Apis mellifera*) make significant contributions to the national economy, not only as honey producers[1] but, more importantly, as the primary pollinators of a wide range of valuable commercial crops. Recent estimates for agricultural/horticultural crops grown commercially in the UK that benefit from bee pollination are in the region of £200m p.a.[2] , while the value of honey production in the UK fluctuates between £10m and £35m p.a. Their foraging activities also help to sustain the biodiversity of myriad natural and semi-natural (e.g. garden) ecosystems. For these reasons, it is vital to maintain robust, healthy honey bee stocks. At their worst, outbreaks of pests and disease cause significant colony losses[3].

There are an estimated 274,000 colonies of honey bees in the UK (England and Wales). Of these, approximately 40,000 colonies are managed on a professional basis, by ~200 commercial beekeepers. Small-scale producers and hobbyists keep the remainder. The National Bee Unit (NBU), based at the Food and

Environment Research Agency (Fera), implements the national bee health programme in England and Wales, underpinned by a programme of research and development to provide up to date technical support to beekeepers. The work includes disease/pest diagnosis, development of contingency plans for emerging threats, import risk analysis, related extension work and consultancy services to both government and industry. Increased globalisation, trade and movement of bees around the world has amplified the risks facing to bee health. Potential exists for major pest threats of the honey bee to reach Europe and the UK. Recent concern has focused on *Aethina tumida* [the small hive beetle (SHB)] and the *Tropilaelaps* mites. In 2003, the European Commission stepped up measures to protect EU apiculture against these pests by making both notifiable throughout the Community and establishing additional import controls to reduce the risk of their introduction from third countries[4].

The small hive beetle (hereafter referred to as SHB) is a major threat to the long-term sustainability and economic prosperity of EU and UK apiculture and to agriculture/environment through disruption to pollination. It has the potential to become a problem for apiculture on a global scale[5]. The beetle belongs to a family of scavenger beetles indigenous to Southern Africa. However, although primarily a sub tropical insect, SHB has recently escaped its native range and established populations in North America and Australia, including more temperate regions of the USA due to the capacity to successfully over-winter in honeybee clusters. From its first detection in Florida, it has spread to over 30 US States[6]. The economic damage it has caused is significant. In Florida in 1998 alone, estimates of colony losses and economic damage from beetle infestations and honey contamination cost the industry $3 million, with over 30,000 colonies lost[7]. The SHB would cause significant colony losses and economic damage if it were to become established in the UK and Europe. SHB was intercepted in October 2004 in an unauthorised consignment of queen bees imported into Portugal from Texas, USA[8]. The rapid response from the authorities and destruction of the affected apiaries eradicated the pest. However, this incident shows that the beetle arriving in the EU is a very real prospect. To date, all medicament control methods employed against the SHB have been inadequate, with highly variable success rates. Additionally experiments to develop traps or exclusion devices for the beetles have not been successful. The Portugal incident highlights the urgent need to face this new threat and find appropriate and environmentally safe methods for both detection and control before arrival of the SHB in the UK, to protect the beekeeping industry and pollination services provided to the economy.

Traps with attractant lures have proven to be an effective means of monitoring for the presence and population levels of insects in a wide variety of situations, for example, moth species in orchard ecosystems[9]. Attractant lures may be based on pheromones produced naturally by the insect, derived from food sources or in the case of parasitoids and predators, on volatile cues from the host/prey of the insect. Little knowledge exists about the behaviour of the SHB in relation to mate location and location of hives. The greater our knowledge of the behaviour the greater the opportunity to develop effective control measures. It is important that any control measures developed do not affect bee behaviour or health. Therefore, measures that are specific for the SHB would be advantageous. At present there is no early detection method or trapping system available for inspection services or beekeepers to use. This current project aimed to establish a system for early detection and monitoring of the SHB by developing an effective lure and trap system and to investigate key aspects of behaviour for optimum deployment of monitors and to examine the potential of novel control measures.

Objectives

1. Plant Health Division will establish a project steering group to discuss project progress and results obtained. The group will meet annually. Scientists within the project will meet on a regular basis to discuss results and next steps.
2. Establish and maintain a laboratory culture of the SHB in Fera's quarantine licensed facilities.
3. Identify sources of natural cues to attract the SHB, establish the chemical identity of the attractants and the feasibility of their use in attractant lures.
4. Establish whether pheromone communication is used by the SHB and if so to establish the identity and function of the pheromones.
5. Develop a trap incorporating an attractant lure and establish a protocol for its use.
6. Validate a monitoring system for SHB in field trials (e.g. in South Africa and/or in the USA, where SHB occurs).
7. Examine behaviour of non-wandering and wandering larval stages and identify the possibility of a chemical cue to influence behaviour.
8. Investigate novel control methods for the SHB that could be used in conjunction with the attractants/monitoring systems, or larval chemical cues if found.
9. Disseminate information to stakeholders, beekeepers and inspectors.

Methods

Method 1. Establishing project steering group and project team meetings

A project steering group was established, comprising Defra, NBU, Regional Bee Inspectors (RBIs) and Fera representatives, to allow discussion of project research, and reporting of results to the interested parties. Scientists within the project met on a regular basis to discuss results and next steps.

Method 2. Establishing and maintaining SHB culture

The first stage of the project sought to establish a culture of SHB in the UK at Fera's quarantine facilities, using standard published protocols[10] (and other USDA material). Specimens of *A. tumida* larvae were imported and used under the conditions laid down in Plant Health Licence No. PHL 251B/5328(02/2006) Amended (04/2006), from Dr Jeff Pettis, USDA, Beltsville and Dr Mike Allsopp, Plant Protection Research Institute, South Africa. SHB were imported from Florida and Maryland (USA) and South Africa (two separate importations) in June 2006 and March 2007, respectively. During transport, larvae were maintained within three layers of containment. Packages were not opened until placed within the secure quarantine facilities. The larvae received were at the 'wandering phase' and therefore ready to go to 'ground' for pupating. They were introduced into 2 litre plastic bottles three quarters filled with damp sand (approx 10-15% v/v). The larvae immediately burrowed down into the sand. Bottles were maintained within a controlled environment room according to the methods described by Cuthbertson *et al*. (2008)[11]: temperature 30°C, 65% r.h. and a Light:Dark regime of L:D 16:8h with an artificial dawn and dusk period. Adult beetles began to emerge within 13 days. These were collected off the sand from which they emerged, into plastic containers baited with honey. Upon collection, adult beetles were reared in plastic boxes (15 x 6 x 10 cm) (50 beetles/box) similar to those used by Haque and Levot (2005)[12], and fed a mixture of honey, pollen and breakfast cereal bran. Within 3-4 days these adults began to lay eggs in-between specially prepared glass slides (Jeff Pettis, USDA, *unpublished methodology*). Slides were removed at intervals and placed within new culturing boxes in order for the eggs to hatch and the larvae to begin the lifecycle again. Cultures were subsequently reared at 20°C, 65% r.h.

Method 3. Identifying natural cues to attract SHB

In order to collect substances that may serve as natural attractants for the SHB, volatiles were extracted from a range of hive-associated materials, either by aeration on to porous polymer, and/or by solid phase microextraction. It was found that slumgum, (a waste product from processing of honey, wax and old combs, that includes dead bees, cocoons, honey, beeswax and propolis) contained more compounds that the other materials (bees, honey, pollen) screened (Table 1). Adult beetles were exposed to these extracts and their responses measured by two complementary methods: electroantennography (EAG), and behavioural bioassay using olfactometry, respectively. Methodology for EAG is described in detail in Section 3.1, below. Static four-way olfactometry was conducted as follows:

Table 1. Number of compounds found from four hive associated volatile sources using two different collection techniques

Source of volatiles	Aeration on to porous polymer	Solid phase microextraction
Bees	24	65
Honey	17	42
Pollen	18	59
Slumgum	31	80

The volatiles, in a solvent carrier, were applied to a filter paper disk that was located in one of the four quadrants below a fine weave nylon mesh, which served as a walking platform for the beetle. A single beetle was introduced into the arena above the quadrants and it's movements recorded, via a video camera, for a period of five minutes. The video camera was linked to a computer running Ethovision behavioural analysis software. This software enabled the time spent in each quadrant, velocity, time spent stationary and other parameters to be easily assessed. The response of male and female beetles to the volatiles collected by aeration from bees, pollen, honey and slumgum was recorded. A typical trace of a beetle responding to an attractive odour source is compared to a trace when the carrier solvent only was present (Figure 1A,B).

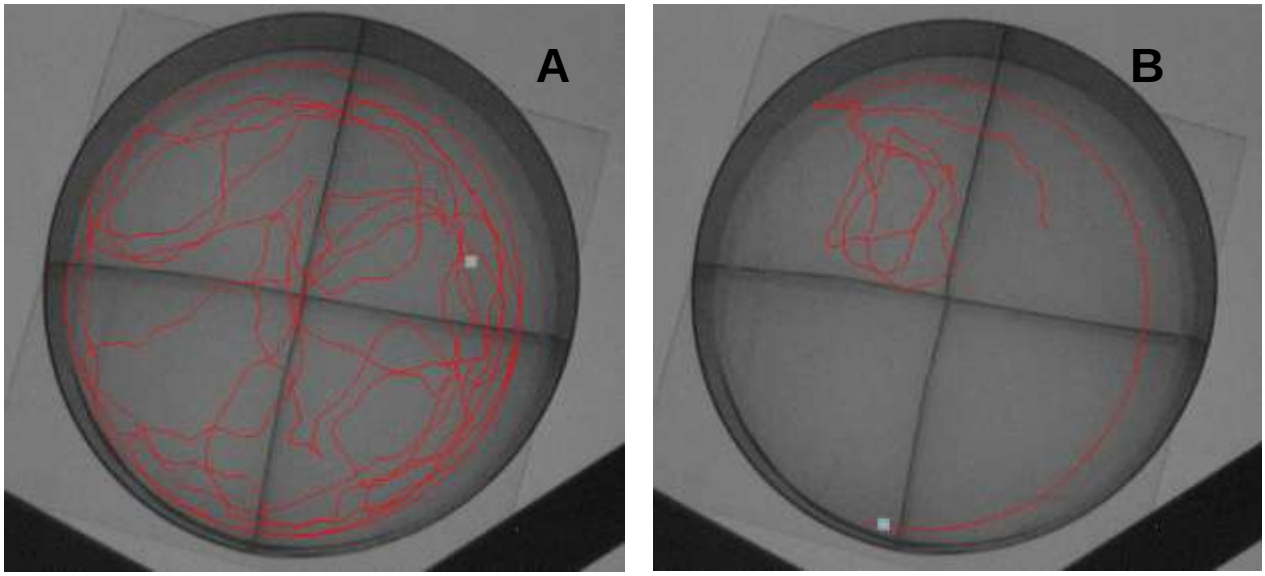


Figure 1. Traces showing the typical movement of *A. tumida* over a 5 minute period in response to solvent (A); or an attractive odour source (B). Odour source was present in the upper left quadrant.

Having established that volatiles extracted from the hive product slumgum elicited positive behavioural responses in adult SHB, the project subsequently focussed on two required outcomes: 1) Firstly, the identification of electrophysiologically active compounds contained within these volatiles; 2) Secondly, the effect of individual, potentially attractive, chemicals on the behaviour of adult SHB.

3.1) The identification of electrophysiologically active compounds

This was achieved by means of coupled solid phase microextraction-gas chromatography–electroantennographic detection (SPME-GC-EAD). Solid phase microextraction was used to collect the volatile compounds from the headspace above slumgum and desorbed into the GC port. This was done both as SPME-GC-EAD, in order to determine the responses of the insects to each compound, and as SPME-GC-MS, so that those compounds to which the insects responded could be identified. Responses were confirmed by EAD tests with standards of the electrophysiologically active compounds. Details of methodology are as follows:

- Collection of volatiles using solid phase microextraction

Test material: volatile compounds were collected from the headspace above 100g of slumgum in a 1l glass beaker. A piece of aluminium foil was stretched across the top of the beaker and the volatiles left to equilibrate in the headspace for 1h. The volatiles were sampled for 1h by pushing the needle of a portable SPME sampler (Carboxen/Polydimethylsiloxane 75µm film thickness) through the foil. The SPME fibre was first conditioned by heating at 300°C in the injection port of a GC for 1h. Volatiles were collected at 20°C.

- Electrophysiological responses to volatiles (Coupled SPME-GC-EAD)

Gas Chromatography: The SPME fibre was desorbed for 1min in the injection port of the GC (Hewlett Packard 5890 Series II gas chromatograph) at 250°C in splitless mode (purge on after 1min) onto a Chrompack CPSil-5CB column (100% polydimethylsiloxane, 50m x 0.32mm internal diameter, 1.2µm film thickness). The carrier gas was helium and the GC was operated in constant pressure mode (15 psi, 31.9cm/s at 40°C GC oven temperature, split vent flow 50.0ml/min) with flame ionisation detector (FID) at 280°C. GC temperature programme: the oven temperature was initially at 40°C for 1min, rising at 6°C/min to 180°C and then at 10°C/min to 280°C and held at 280°C for 10min.

Electroantennography: Recordings were made by placing the head of a living beetle into an electrical circuit, passing an olfactory stimulus over the antenna and recording the response. Each electrode consisted of a piece of chloridised silver wire (0.5mm dia.) inserted into a glass capillary tube. The capillary for the indifferent electrode was filled with Ephrussi-Beadle saline[13], and the capillary for the recording electrode contained Ephrussi-Beadle saline with the addition of 0.02% v/v of a surfactant (Triton X-100 supplied by Alfa Aesar, Heysham, Lancashire, UK) to give a good contact. The head of the insect was removed using a sharp scalpel blade and the indifferent electrode placed in the back of the head. The recording electrode was cut such that the surface area of the cut end was equal in size to the area of the underside of the antennal club. The electrode was pushed against the underside of the antennal club. Signals from the recording electrode were interfaced to a PC via an AC/DC amplifier in DC mode and an Autospike Interface Box (Syntech, Hilversum, The Netherlands). The odour delivery system was as described by White and Birch[14]. The GC column effluent was split 1:1 between two identical lengths of 0.32 mm fused silica capillary column using a universal quick seal splitter. One length led to the FID detector while the other led, through a copper tube surrounded by a heating tape maintained at 230°C, to a glass stimulus delivery tube. The effluent (0.9 ml/min) was diluted with humidified air to give a total airflow of 1000 ml/min, regulated by a Stimulus Controller (Syntech, Hilversum, The Netherlands) and passed over the antenna of the test insect. The gender of each insect was determined after the head has been removed. Ten male and ten

female insects were tested. The data from both the FID and the EAD were analysed simultaneously using GcEadPro v4.1 software (Syntech, Hilversum, The Netherlands).

- GC-MS analysis of volatiles

The samples were analysed on a Hewlett Packard 5890 series II gas chromatograph coupled to a VG Trio-1 mass spectrometer (VG Masslab Ltd., Altrincham, UK). The SPME sampling method, GC temperature programme and column were identical to that used for SPME-GC-EAD. The MS source and interface temperatures were 200˚C and 275˚C, respectively. The MS was operated in electron impact mode (EI+) at 70 eV, and scanned from 33-350 amu once per second. EAD active volatiles were identified by comparing their mass spectra with the mass spectrometer NIST library and by EAD with standards.

- Source of test Chemicals

Unnamed in this report to protect potential IP.

- EAD with standards

Individual compounds that were identified as being electrophysiologically active by GC-EAD were tested either to confirm their electrophysiological activity or to determine which of a number of overlapping compounds elicited an electrophysiological response in SHB. The extracts were tested on at least five male and five female insects using gross EAD.

- Presenting the compounds to the insects

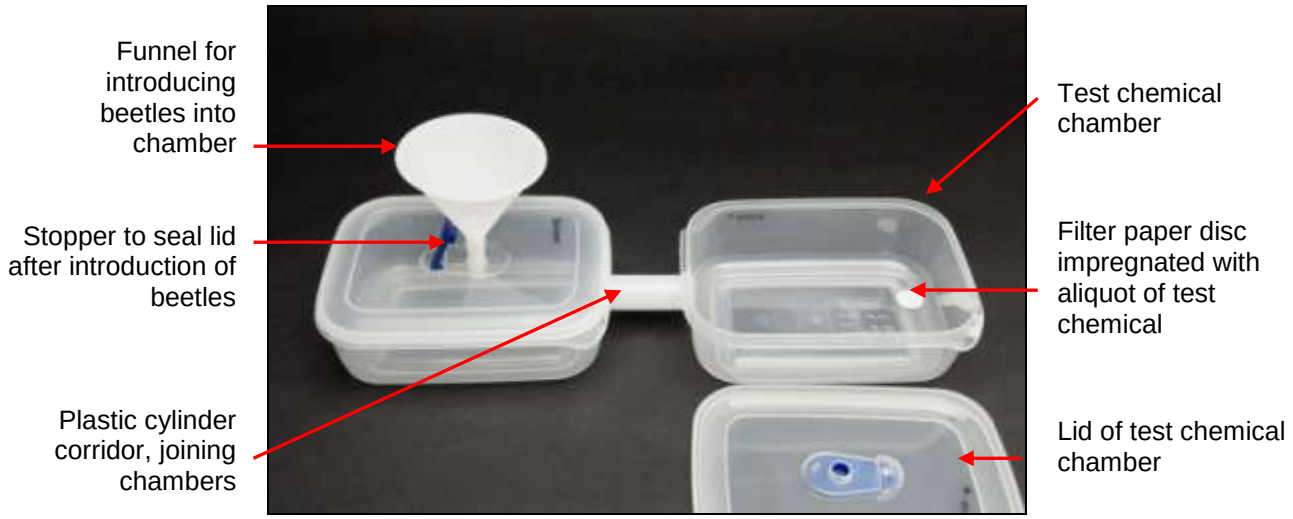
Each compound was presented at a concentration of 10 µg/µl in hplc grade pentane. Isopentyl acetate (bee alarm pheromone) was presented in the same amount as the test compounds as the positive control. EAD recordings were made as described above. All materials were presented in a 10 µl volume to give a total amount of 100 µg applied to the filter paper. The amount of material presented is expressed in terms of the amount that was applied to the filter paper prior to insertion in the test cartridge. Solvent was allowed to evaporate before placing the filter paper strip in the test cartridge. The test material was released into a continuous flow of humidified air, which was passed over the antenna (1000 ml/min) with a pulse duration of 1 sec (500 ml/min plus 500 ml/min continuous flow to give a total of 1000 ml/min), regulated by a Syntech Stimulus Controller. Intervals of 120s were left between presentations of each test substance. Pentane (hplc grade) was used as the control to take into account any response to the solvent or to mechanical disruption of the airflow. A response was measured as the maximum amplitude of depolarisation elicited by a stimulus. The response to the control was subtracted from the response to the test materials. The gender of each insect was determined after the head had been removed. Five insects of each gender were tested with each of the 41 test compounds

The above trials identified 16 individual chemical components as potential attractants for adult SHB (see results). Of these, the ten that elicited the greatest EAG responses were then subjected to further behavioural bioassays. These chemicals are hereafter referred to as chemicals T1 to T10, respectively.

3.2) The effect of individual, potentially attractive, chemicals on the behaviour of adult SHB

In order to ascertain the relative “attractiveness” of the ten individual chemicals that had been identified as being of interest, a further series of behavioural bioassays was performed. Experimental design was as follows: double-chambered bioassay boxes were constructed such that two plastic boxes (dimensions 19x14x6 cm) were co-joined by a plastic cylinder (dimensions 7cm long; diam. 3cm), to create a dumbbell-shaped structure (Figure 2): Sources of each test chemical were prepared by pipetting 10µl of the substance in question, as supplied, onto a small disc of white filter paper (diameter 20mm). Each disc was immediately placed into the distal end of a dumbbell box, and the lid to the chamber replaced. Using a plastic funnel, adult SHB were then introduced, in batches of 10 beetles/box, into the other half of the dumbbell, through a small aperture in the box lid.

Figure 2. Double-chambered arrangement for behavioural bioassays



Sealed dumbbells were then placed into a large, clear, perspex observation chamber (internal dimensions 56cm (at highest point) x 50cm x 64cm; total volume 160,000cm^3). Behaviour of the beetles was subsequently continuously monitored for 20min. The following information was recorded:

- The total number of behavioural events observed throughout the 20minutes, as defined by the number of entries / exits from the chamber with the chemical;
- The total number of beetles in the chamber with the chemicals after respectively 5, 10, 15 and 20minutes;
- The maximum number of beetles in the chamber with the chemicals at any one time.

(All trials were conducted at 20°C, 65% r.h. Light intensity was reduced to simulate dawn/dusk-like conditions, as the times of day at which SHB are known to be most active). Preliminary observations revealed that chemicals T9 and T10 were extremely volatile, and their presence in the observation chamber, even when contained in sealed plastic boxes, interfered with other test replicates. For this reason trials with T9 and T10, respectively, were conducted separately. To ensure that robust comparisons could be made between the behaviour of beetles exposed to each of the remaining test substances, reactions to these eight chemicals (and a blank control) were observed in blocks of four substances at any one time. The experiment was designed as an incomplete block design with 56 blocks, 21 replicates of all treatments (excluding the control) and four units within each block (note that the control was replicated in all blocks, hence 56 replicates). Also, the design was produced such that any two treatments (excluding the control) appeared together in a same block six times.

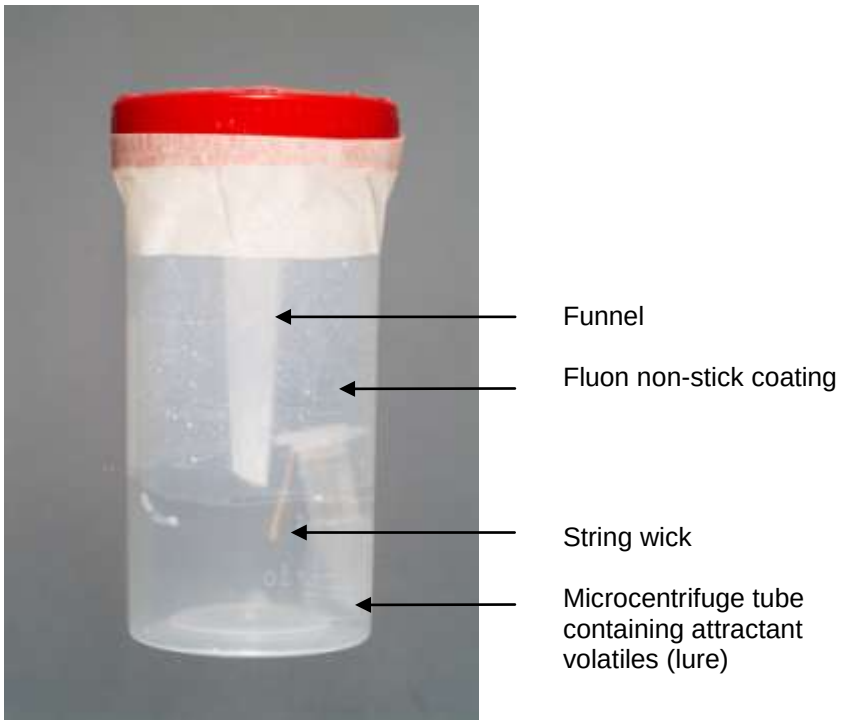
Method 4. Establishing use of pheromone communication by SHB

Many different species of insect use pheromones for communication. Pheromones may be released by males or females, and may attract the opposite sex for the purposes of mating (sex pheromones) or both sexes (aggregation pheromones). There are also other types of pheromone e.g. alarm pheromones but these tend not to result in attraction of beetles to the source. Pheromones have been identified for other Nitidulidae species and are used in attract and kill systems[15]. Therefore the possibility of a pheromone communication system between adult *A. tumida* was investigated. Male and female SHB were extracted in distilled pentane. The responses of male or female SHB (24-72 h old) to solvent extract of male or female beetles (20 μl) were determined using a static four-way olfactometer as described previously. The EAG response of male and female SHB to these extracts was also assessed using the methodology described previously. Extract from bees was used as a positive control. Solvent extracts were examined by GC-MS to determine whether there were differences in the volatile profiles for male and female beetles. SPME sampling was also used to investigate production of volatiles by adult SHB. For the purposes of statistical analysis, the total time spent in the quadrant above the test odour source was expressed as a percentage of the total time, and an arcsine transformation was used to transform the percentage time spent in this quadrant. A comparison with the expected time spent in the quadrant (25%) was made using a single sample Student t test.

Method 5. Development of trap incorporating attractant lure, and protocol for use

Prior to developing a prototype trap and lure, experiments were conducted to determine a practical temperature at which adult SHB will be reliably observed to fly, based on a design developed by Cox[16]. The number of SHB taking flight over a 24hour period was established at a range of temperatures, using beetles derived from two different geographical locations (USA & South Africa). Preliminary trials confirmed that SHB (male and female) readily flew at both 20^{o}C and 25^{o}C. On the strength of these observations, the lure and trap was tested at the lower temperature of 20^{o}C. A prototype trap and lure for adult SHB was evaluated in laboratory studies using a method that enabled the beetles to enter the trap either by crawling up the trap or by flying on to the trap. Based on the results of the laboratory bioassays of the individual chemicals, just two were chosen for inclusion in the prototype lure (test chemicals T1 and T8. See Methods and Results 3, and Annex 2). Since no correlation was found between the concentration of either chemical and their effects on adult SHB's behaviour, these substances were subsequently used undiluted. The lure consisted of a 1.5 ml microcentrifuge tube to which were added equal volumes (0.5 ml) of 100% T1 and 100% T8, respectively. A short length of string (approx. 50 mm) was inserted into the microcentrifuge tube such that one end of the string was at the bottom of the tube and the other end protruded approx. 15 mm from the sealed lid of the microcentrifuge tube. The prototype trap consisted of a plastic bottle (200 ml; 5.5 cm diameter, 11.5 cm high), adapted to permit entry by the beetles by cutting a 4.5 cm diameter hole in the lid and inserting a plastic funnel. The funnel was sealed to the lid using a hot glue gun. The inner surface of the funnel was coated with Fluon (Whitford Plastics, UK). The lure was placed in an upright position at the base of the trap (Figure 3). The trap was placed at the centre on the floor of a perspex insect handling cage. Separate cages were used for the trap with the lure and a control trap, which contained a microcentrifuge tube with a string 'wick' only.

Figure 3. Completed trap for adult SHB, baited with prototype lure mixture of attractant volatiles



Adult SHB between 2 and 5 days old were batched into groups of 50 insects 24 h prior to testing. The beetles had access to water and were maintained at 20°C prior to the test. The beetles were chilled briefly prior to introduction to the test chambers and were left for a period of 24 h at 20°C, 16:8 L:D. The percentage of beetles in the trap was recorded at the end of the 24 h test period. Control and test traps were examined simultaneously and the experiment was repeated ten times. The percentage of beetles caught was subjected to an arcsine transformation and a comparison between the control traps and traps with a lure was made using a two-sample t test of the transformed data.

Method 6. Validation of monitoring system for SHB in field trials

In order to validate the prototype traps and lure developed above, it was necessary to carry out testing at sites where SHB adults are known to be flying. In spring of 2009, three consignments of 30 traps each (15 for use with lures; 15 for use with blank controls) were dispatched to two separate field test recipients; one in South Africa, and one in the USA. These recipients were: Dr. Mike Allsop (Head of Honey Bee research Programme *ARC*-PPRI South Africa); Dr. Jeff Pettis (Research Leader USDA-ARS Bee Research Laboratory, Beltsville); Dr. Anika Lowe (Research student USDA-ARS Bee Research Laboratory, Beltsville). Each recipient was supplied with instructions for trap deployment in small-scale field trials. The following recommendations were made for trap placement:

- Traps should be placed in a variety of contrasting locations, including places where beetles are known to be active (e.g. close to bee colonies), and at other sites where adults are believed to be emerging.
- Traps should not be placed in very strong, direct sunlight.
- Traps should be placed at a range of elevations, between ground level, and higher e.g. on tree branches.
- Baited traps and controls should not be placed immediately next to each other. However, it was important to record what unbaited traps catch when sited in areas similar to those where baited traps are placed, so field collaborators were invited to identify analogous sites to those used for baited traps, in which to place controls.
- All traps (baited and controls) must be checked at least once every 24 hours.
- All incidences of SHB trapped must be counted.
- Any other invertebrate species found in either baited or control traps should also be identified.

Traps tested in South Africa were placed as follows:

The 30 prototype traps dispatched to South Africa were deployed in six different apiary sites. Traps were placed in a variety of paired positions (trees, under bushes, on window ledges, on roofs). All traps (baited with lure and blank controls) were monitored at 24 h intervals over a 2week period (March 2009). During this time, lures were replenished once.

Traps tested in USA (Florida were placed as follows:

Table 2 summarises the placement of the prototype traps and lure in a variety of apiary sites in Florida.

Table 2. Description of USA trap sites (all data supplied by Anika Lowe, USDA Florida)

Name of site	Location	Temp. & r.h. at time of placement	Weather conditions at time of placement	Description of site	Vegetation type	Soil type
Biology Unit of the University of Florida (Bee Yard)	N: 29° 37.632 min W: 82° 21.389 min	25°C r.h. 86 %	Light rain	21 colonies; light shadow, no direct sun, surrounded by trees	Red Cedar, Live Oak, Slash Pine	Sandy
Beef Unit of University of Florida (Boston Yard)	N: 29° 55.550 min W: 82° 29.045 min	35°C r.h. 49 %	Sunny	4 colonies in front of a tree line on a mowed field (cow pasture)	Oak trees, China Berry, Pea Field	Not recorded
Beef Unit of University of Florida (Boston Yard)	N: 29° 55.536 min W: 82° 29.045 min	34°C r.h. 52%	Sunny	5 colonies on a meadow in front of a swamp and surrounded by trees	Slash Pine, Sweet Gum, Wax Myrtle, Red Maple, Live Oak	Not recorded
Dadant Bee Yard	N: 29° 48.671 min W: 82° 32.690 min	33°C r.h. 87 %	Sunny/ light raining	Randomly distributed hive boxes ≥21 colonies in front of a tree line (little forest)	Pine trees and Laurel Oaks	Not recorded
Planted Pine Tree Farm	N: 29° 43.121 min W: 82° 24.836 min	31°C r.h. 71 %	Raining	20 colonies in front of a tree line and blackberry bushes and pine trees	Fetter Bush, Sweet gum, elderberries, and Passion Fruit	Not recorded

Method 7. Examining behaviour of larval SHB to identify possible chemical cue to influence behaviour.
During routine culturing of the SHB it was noted that once a proportion of the larval population had reached the 'wandering' stage other younger larvae would also leave the available food source and 'follow' the larger larvae. The possibility that wandering larvae laid down some form of trail that other larvae followed was investigated. Wandering or non-wandering larvae were confined to glass Petri dishes for a period of 24 h. The surfaces of the Petri dish were rinsed with distilled pentane. The larvae were also extracted in pentane as described above for adult SHB (see Method 4). Pentane extracts were concentrated under a stream of argon. The samples were analysed using GC-MS to determine differences in the profiles between wandering and non-wandering larvae.

Method 8. Investigating novel control methods for the SHB that could be used in conjunction with the attractants/monitoring systems, or larval chemical cues if found.
Many insect-derived peptides have high potency and specificity of action towards invertebrates, and as such are ideal candidate novel insecticides. Although such substances are typically ineffective when administered orally, fusion protein technology enables the oral delivery of insecticidal peptides to their site of action. The current project sought to examine the effect of one such fusion protein, in which a toxic peptide was linked to the carrier molecule *Galanthus nivalis* agglutinin (GNA) (snowdrop lectin), on larval SHB. Materials were supplied by the University of Durham:

Larval SHB susceptibility to insecticidal fusion proteins
Fusion protein and recombinant GNA were each tested at a concentration of 300 ppm. The fusion protein, or recombinant GNA, was suspended in 50% aqueous honey solution (1 ml) and then mixed with 2.5 g of crushed bee pollen. This quantity of diet was divided into two and held in a small plastic weigh boat. For each replicate, a single weigh boat containing the diet was placed in a 355 ml ventilated, tissue-lined plastic pot (Solo Cup Company, UK). The tissue was dampened with tap water to prevent desiccation of the larvae. Ten SHB larvae (0-24 h post-emergence) were transferred from the egg slide to the edge of the weigh boat in the plastic pot using a fine paintbrush. The larvae were left to feed for 3 days. After this time all pots were placed in a freezer at -18°C for a minimum of 48 hours. Individual larvae were carefully removed from the pot and rinsed in a small quantity of water to remove traces of diet. Each larva was then air-dried and weighed. Survival was also assessed.
Examination of the presence of the fusion protein in larval SHB

The presence of the fusion protein in SHB larvae as a result of ingestion was examined by immunoassay (Western blotting) using the enhanced chemiluminesence (ECL) method (Amersham). This was carried out as previously described by Fitches *et al*.[17]. Larvae that had fed on the diet containing the fusion protein for 3 days were extracted in 50 mM Tris-HCl pH 7.5 (+ 1% fresh 36 mg/ml PMSF in ethanol), prepared for SDS-PAGE on 17.5% gels and proteins were subsequently transferred to nitrocellulose filters. Blots were probed with anti-GNA antibodies. This permitted detection of the GNA component of the fusion protein. Antibodies for the venom component of the fusion protein were not available.

Statistical analyses

A Generalized Linear Model (GLM) (using a Bernoulli distribution and a logit link function) was used to compare the survival between treatments. Where a significant difference was indicated, pair wise comparisons between treatments were done (on the logit-transformed data) using the least significant difference. Differences in the weight of the larvae between treatments were compared using one-way ANOVA.

Method 9. Dissemination of information to stakeholders, beekeepers and inspectors.

Throughout the duration of this project, information generated has been disseminated to interested parties via the bee health inspectorate (RBIs) and the project steering group. In addition, valuable feedback on experimental techniques which could add value to the project and encourage uptake by the Bee Industry was offered by the RBI representative. Additional details of specific dissemination processes are presented in Results 9., below.

Results

Results 1. Establishing project steering group and project team meetings

A project steering group was established, comprising Defra, NBU, RBI's and Fera representatives, to allow discussion of project research. The first Steering Group Meeting was held on 13th February 2007 at which members received a short overview from the project leader of the potential impact SHB may have on the UK bee industry should it become established. Presentations were also made by the relevant scientists concerning the various aspects of the experimental work outlining progress to date. Further project group meetings were convened throughout the course of the project.

Results 2. Establishing and maintaining SHB culture

A viable culture of SHB was successfully established within Fera's quarantine entomology unit. This culture was routinely maintained under the conditions described by Cuthbertson *et al*.[11], and yielded ample insects for the purposes of the proposed research. (Female:male ratio 1.7:1. Adult beetles sexed using genitalial features as described by Schmolke[18]). All routine culturing was carried out under full quarantine containment, because *Aethina tumida* is an exotic pest and notifiable in Europe under EC honeybee legislation. Maintenance of the licensed facility was in accordance to FERA SOP's and protocols. Procedures detailed in the following FERA SOP: EFF/408: Working with quarantine (licensed) organisms at FERA (Block 29 and 02F01) were strictly adhered to. Published quarantine insect rearing protocols within peer-reviewed journals were also used as standards for maintenance of the SHB culture[11, 19, 20]. During the culturing of the beetles we have gained several interesting observations in regard to the beetle's lifecycle and behaviour. Of particular note is the observation that, even without access to a food source, 'wandering larvae' can survive for up to 48 days: Although larvae that had been maintained on moist laboratory roll alone for 7 weeks did appear very sluggish in their movement, when put into sand they once again burrowed down and were seen to form their pupation chambers as normal. Over time, "starved larvae" developed through to viable adults. These adults were smaller in size to the original imported beetles from the USA. It is known that nutrition plays a role in beetle development and adult size. However, when offered the opportunity to breed they produced eggs that went on to produce viable offspring[11].

Results 3. Identifying natural cues to attract SHB

3.1) The identification of electrophysiologically active compounds by SPME-GC-EAD

Sixteen different compounds were found to elicit EAG responses in both male and female SHB that were greater than or equal to the isopentyl acetate (IPA) positive control. Of these, only the ten that elicited the greatest EAG responses were subjected to further behavioural bioassays. Hereafter they are referred to as test chemicals T1 to T10.

3.2) The effect of individual, potentially attractive, chemicals on the behaviour of adult SHB

3.2.1) Statistical analyses

Experiments completed as described in Method 3.2 yielded the following data regarding the behaviour of groups of SHB when separately exposed to each of the 10 test chemicals:

- The total number of behavioural events observed throughout the 20minutes, as defined by the number of entries / exits from the test chemical chamber;
- The total number of beetles in the test chemical chamber after 5, 10, 15 and 20 minutes, respectively;
- The maximum number of beetles in the test chemical chamber at any one time.

For the purposes of statistical analyses, when looking at either the maximum number of beetles in the chemical chamber, or the total number of behavioural events, Generalized Linear Models (GLM) were fitted to the data. This was because data were either counts (number of behavioural events) or proportions (number of beetles, out of ten, in the chamber with the chemicals) and were not, therefore, normally distributed. When considering the number of behavioural events (counts), a logarithm link function was used; when considering the maximum proportion of beetles in the chemical chamber, a logit link function was used. On those occasions where data was more variable than what would be expected from Poisson (count) or binomial (proportion) data, the standard error of the parameter estimates were inflated accordingly (using the dispersion parameter, as estimated by the residual mean deviance). When looking at the proportion of beetles in the chamber with the chemicals over time (at 5, 10, 15 and 20 minutes), for the same distributional reasons as above, as well as to account for the possible autocorrelation in time of the observations (for any single treatment in any given block, the repeated observations are likely to be correlated with each other), Generalized Estimating Equations (GEE) were used. Similarly to what is done when looking at the maximum proportion of beetles in the chamber with the chemicals at any one time, a logit link function was used. As before, the dispersion parameter was estimated from the residual mean deviance. GEE analyses were done using SPSS 15.0; all other analyses were done using Genstat 11.1.

3.2.2) The effects of components T1 to T8 on the behaviour of adult SHB

Behavioural events: With respect to the first eight chemicals tested with SHB, analysis of deviance confirmed that they had a very strong effect ($p < 0.001$) on the number of behavioural events observed over the 20 minute period (Table 3). Figure 4 presents fitted values (and 95% confidence intervals) for the GLM of the number of behavioural events over the 20 minute period for all chemicals. Table 4 shows the parameter estimates from the regression (on the log scale). At the 5% significance level, four treatments (T1, T2, T6 and T8) showed significantly different numbers of behavioural events compared to the control. T1 and T8 showed significantly more events than the control (See Table 4, positive estimates), whereas T2 and T6 showed fewer (See Table 4, negative estimates).

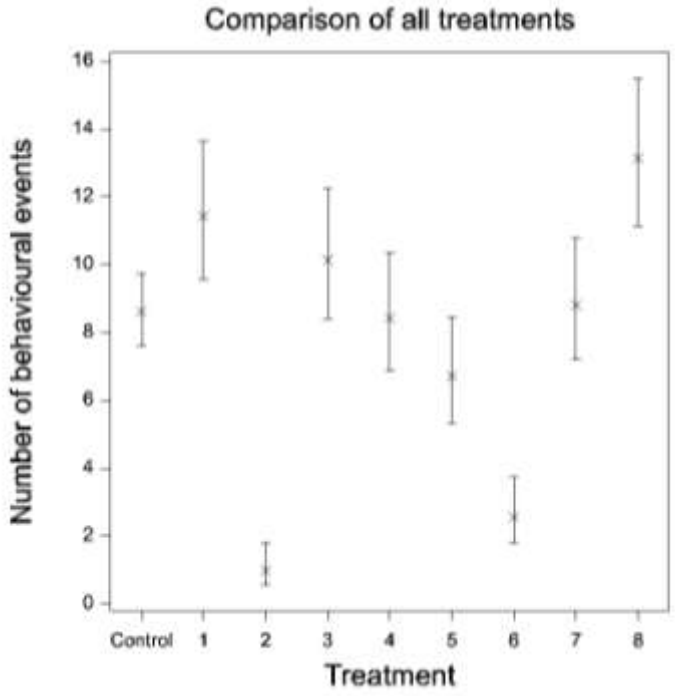
Table 3. Analysis of deviance table for the number of behavioural events in all chemicals tested

Source	d.f.	Deviance	Mean deviance	Deviance ratio	Approximate F probability
Treatment	8	417.7	52.21	27.15	< 0.001
Residual	215	413.5	1.92		
Total	223	831.1	3.73		

Table 4. Parameter estimates from the analysis of the number of behavioural events in all chemicals tested. [1]Note that "Control" is used as the reference level

Parameter[1]	Estimate	s.e.	t-value	t probability	Antilog of estimate
Constant	2.15	0.06	34.08	< 0.001	8.61
T1	0.28	0.11	2.59	0.01	1.33
T2	-2.15	0.31	-6.99	< 0.001	0.12
T3	0.16	0.11	1.44	0.15	1.18
T4	-0.02	0.12	-0.17	0.86	0.98
T5	-0.25	0.13	-1.87	0.06	0.78
T6	-1.21	0.20	-6.07	< 0.001	0.30
T7	0.02	0.12	0.19	0.85	1.02
T8	0.42	0.11	4.04	< 0.001	1.53

Figure 4. Fitted values (95% confidence intervals) for the GLM of the number of behavioural events over the 20minute period of the experiment for chemicals T1-T8



Maximum number of beetles (out of ten) in the test chemical chamber at any one time: Chemicals were found to have a highly significant (p < 0.001) effect on the maximum proportion of insects found in the test chemical chamber at any time (Table 5). Five treatments were found to be significantly different from the control at the 5% significance level (Table 6). Two of these (T1 and T8) had a significantly higher (positive estimates in Table 6) proportion of insects in the chamber with the chemicals than the control, whereas the other three (T2, T5 and T6) had a significantly lower (negative estimates in Table 6) proportion than the control. See also Figure 5.

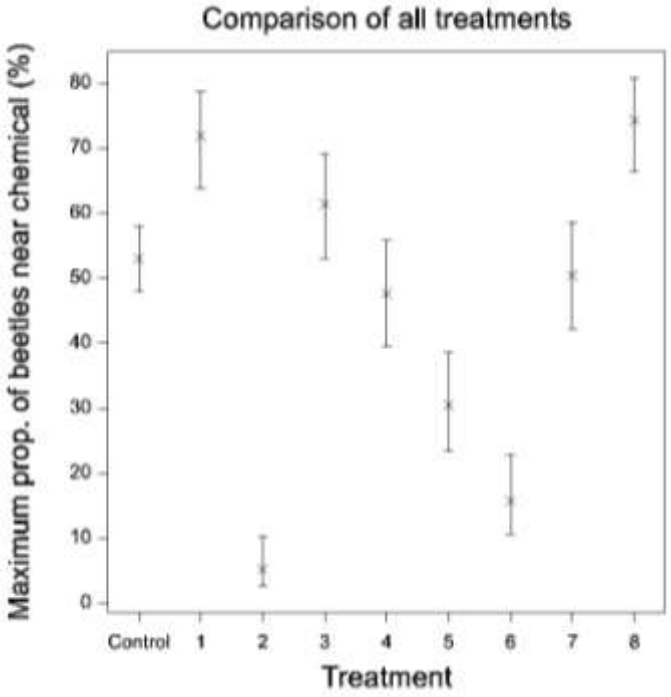
Table 5. Analysis of deviance for the maximum proportion of beetles in the chamber containing the chemical at any time for all chemicals tested

Source	d.f.	Deviance	Mean dev.	Dev. ratio	Approximate F probability
Treatment	8	443.7	55.46	37.39	< 0.001
Residual	215	318.9	1.48		
Total	223	762.6	3.42		

Table 6. Parameter estimates from the analysis of max. proportion of SHB in the test chemical chamber at any time for 8 different volatiles. [1]Control was used as the ref. level

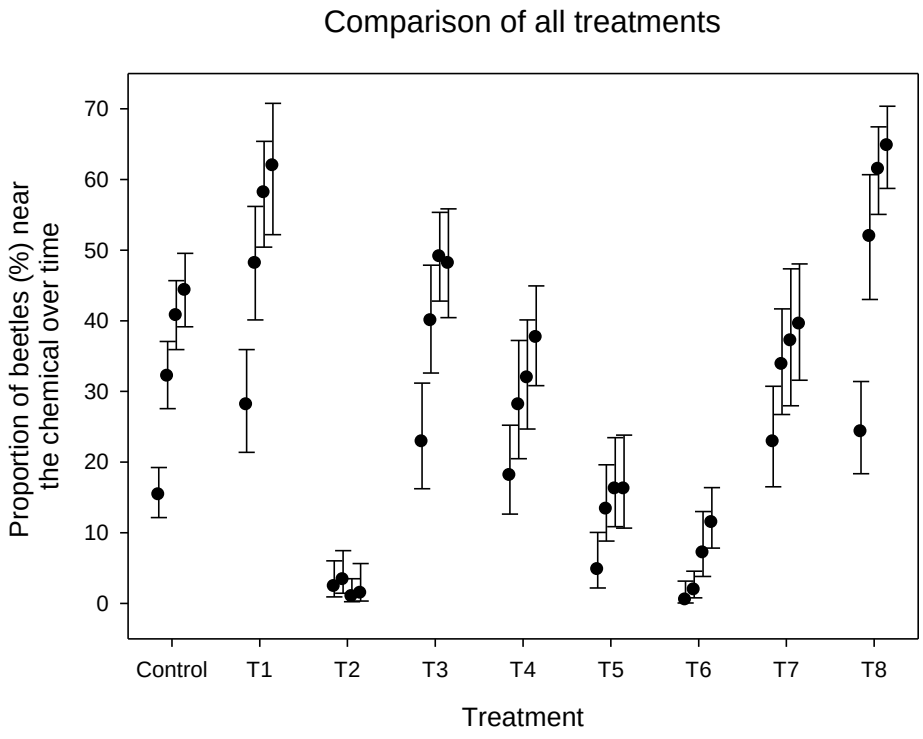
Parameter[1]	Estimate	s.e.	t-value	t probability	Antilog of estimate
Constant	0.12	0.10	1.18	0.24	1.13
T1	0.82	0.21	3.83	< 0.001	2.27
T2	-3.02	0.39	-7.72	< 0.001	0.05
T3	0.34	0.20	1.71	0.09	1.41
T4	-0.22	0.20	-1.10	0.27	0.81
T5	-0.95	0.21	-4.51	< 0.001	0.39
T6	-1.80	0.25	-7.12	< 0.001	0.17
T7	-0.10	0.20	-0.52	0.60	0.90
T8	0.94	0.22	4.30	< 0.001	2.56

Figure 5. Fitted values (95% confidence intervals) for the GLM of the max. proportion of SHB in the test chemical chamber over a 20minute period, for all chemicals.



Location of the beetles over time (5, 10, 15 and 20 minutes): There was clear evidence that treatment (Change of deviance (C.o.d.) = 296.0; Degrees of freedom (D.o.f). = 8; $p < 0.001$), time (C.o.d. = 60.2; D.o.f. = 3; $p < 0.001$), and also the interaction between treatment and time (C.o.d. = 48.5; D.o.f. = 24; $p = 0.002$) were all significant at the 5% significance level. (Regression parameters not presented in this report). The autocorrelation matrix (also not presented here), showed a very strong autocorrelation (r = 0.65) between consecutive points. Fitted proportions for the GLM, together with their 95% confidence intervals are presented in Figure 6.

Figure 6. Fitted values (and 95% confidence intervals) for the Generalized Estimating Equations of the proportion of beetles near the chemical at 5, 10, 15 and 20 minutes for all chemicals.



Summary of Results for test chemicals T1 to T8: On the strength of the above findings, two test chemicals were selected for use in prototype lures. These were substances identified as T1 and T8.

3.2.3) The effects of components T9 and T10 on the behaviour of adult SHB

As stated in Method 3, preliminary observations revealed that components T9 and T10 were extremely volatile, and their presence in the observation chamber, even when contained in sealed plastic boxes, interfered with other test replicates. For this reason, trials with T9 and T10, respectively, were conducted separately from the trials with the other 8 chemicals.

Behavioural events: Figure 7 presents the fitted values (and 95% confidence intervals) for the GLM of the number of behavioural events over the 20minute period of the experiment for both chemicals (plus control) tested. Analysis of deviance (Table 7) showed that exposing adult SHB to T9 and T10 had a highly significant effect ($p = 0.002$) on the activity of the beetles, as measured by the number of behavioural events recorded in these trials. However, closer examination of the data revealed that this was attributable to T9, which, on average, was associated with significantly fewer behavioural events than the control (negative estimate, Table 8), whereas no significant difference was observed between the control and T10.

Figure 7. Fitted values (and 95% confidence intervals) for the GLM of the number of behavioural events over the 20min period of the experiment for all chemicals.

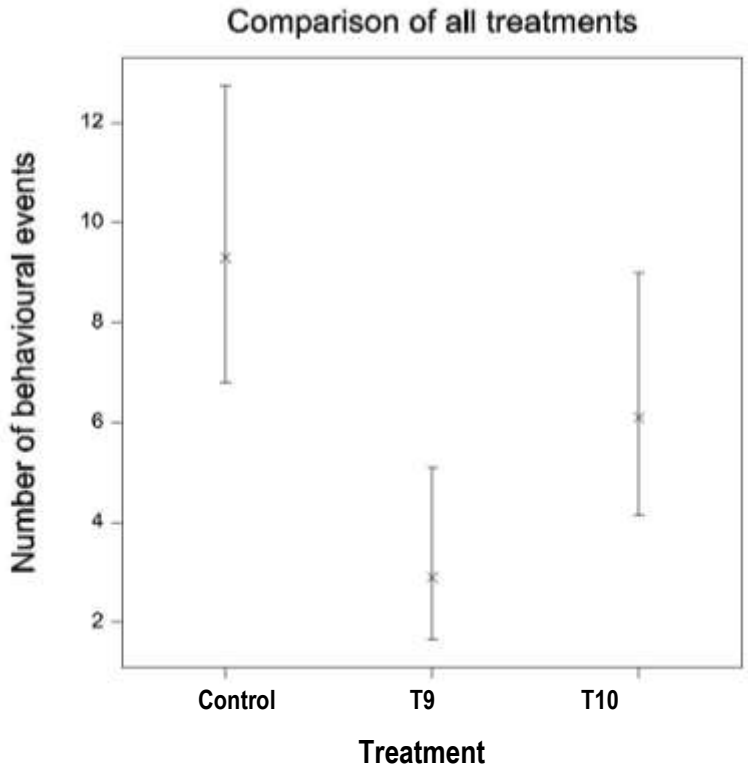


Table 7. Analysis of deviance table for the number of behavioural events with both T9 and T10, respectively

Source	d.f.	Deviance	Mean deviance	Deviance ratio	Approximate F probability
Treatment	2	35.31	17.66	8.09	0.002
Residual	27	58.96	2.18		
Total	29	94.28	3.25		

Table 8. Parameter estimates from the analysis of the number of behavioural events in both chemicals (plus control) tested. [1]Note that "Control" is used as the reference level

Parameter[1]	Estimate	s.e.	t-value	t probability	Antilog of estimate
Constant	2.23	0.15	14.55	< 0.001	9.30
T9	-1.17	0.31	-3.71	< 0.001	0.31
T10	-0.42	0.24	-1.73	0.10	0.66

Maximum number of beetles (out of ten) in the test chemical chamber at any one time: Figure 8 shows fitted values (and 95% confidence intervals) for the GLM of the maximum proportion of beetles near the chemical over the 20minute period of the experiment for T9 and T10 (plus control). When looking at the maximum proportion of beetles found in the test chemical chamber with either of these two test chemicals at any time, although T9 showed fewer beetles than either the control (negative estimate in Table 9) or T10 (smaller estimate for T9 than for T10 in Table 9), there was no evidence of any significant effect of the chemicals (p = 0.08) at the 5% significance level.

Table 9. Analysis of deviance table for the maximum proportion of beetles in the chamber containing either T9, T10 or the control

Source	d.f.	Deviance	Mean deviance	Deviance ratio	Approximate F probability
Treatment	2	12.29	6.15	2.86	0.08
Residual	27	57.98	2.15		
Total	29	70.28	2.42		

Figure 8. Fitted values (and 95% confidence intervals) for the GLM of the maximum proportion of beetles near the chemical over the 20min period of the experiment for T9, T10 and control.

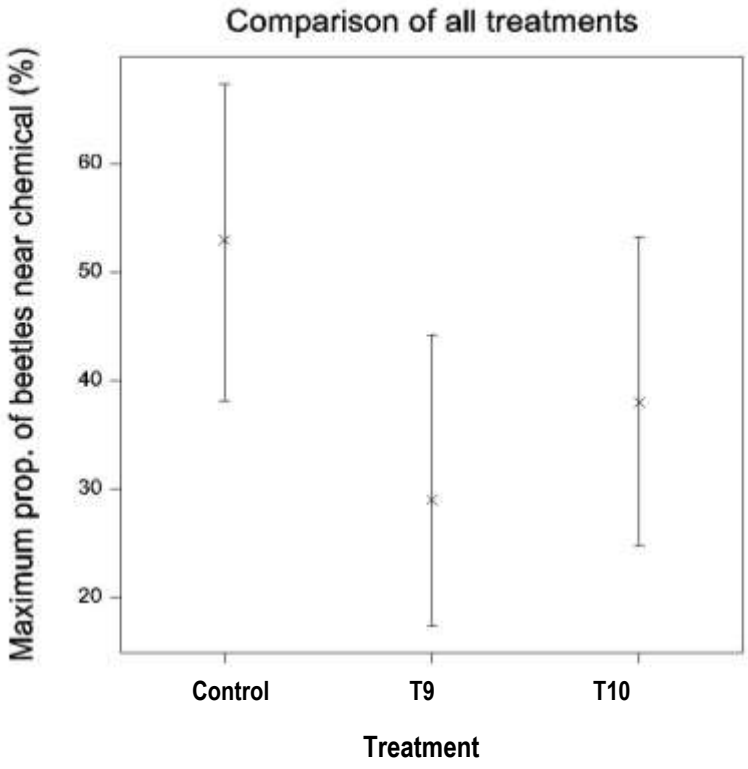
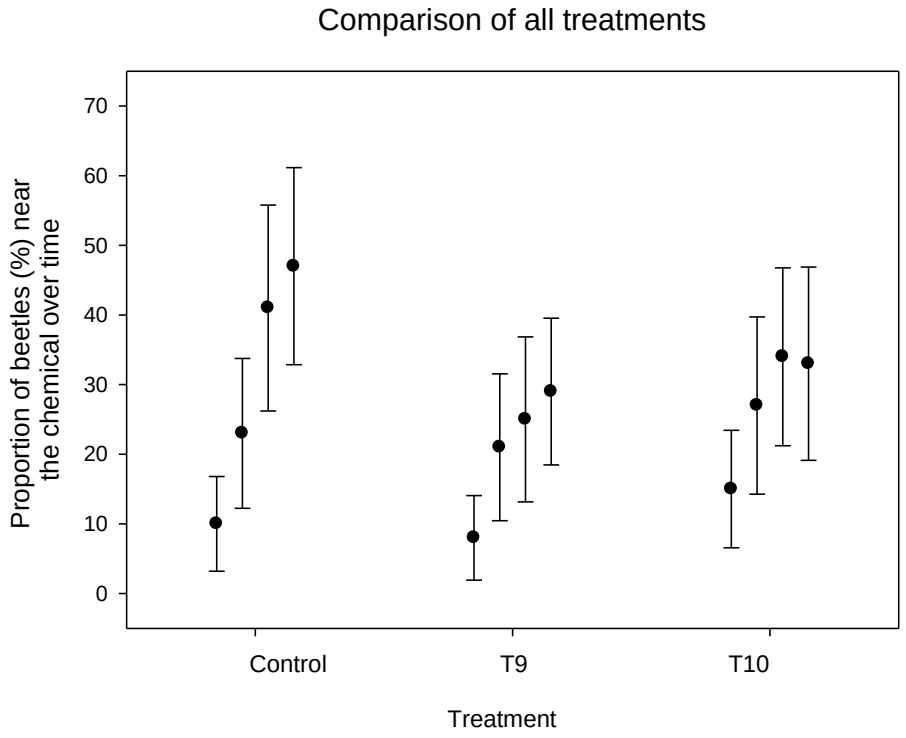


Table 10. Parameter estimates from the analysis of the maximum proportion of beetles in the chamber containing T9, T10 or the control at any time. [1]Note that "Control" is used as the reference level					
Parameter[1]	Estimate	s.e.	t-value	t probability	Antilog of estimate
Constant	0.12	0.29	0.41	0.69	1.13
T9	-1.02	0.44	-2.33	0.03	0.36
T10	-0.61	0.42	-1.45	0.16	0.54

Location of beetles over time (5, 10, 15 and 20 minutes): Neither T9 or T10 had any effects on the location of adult SHB over time (C.o.d. = 1.57; D.o.f. = 2; p = 0.46). Although there was no significant interaction between chemical treatment and time (C.o.d. = 9.40; D.o.f. = 6; p = 0.15), there was, however, very strong evidence of an effect of time (C.o.d. = 68.80; D.o.f. = 3; p < 0.001), with the number of beetles in the chamber with either T9, T10 or the control increasing over the course of each experiment, irrespective of the substance in question. The autocorrelation matrix (not presented), showed a very strong autocorrelation (r = 0.87) between consecutive points. Fitted proportions for the GLM, together with their 95% confidence intervals are presented in Figure 9.

Figure 9. Fitted values (and 95% conf. int.) for the Generalized Estimating Equations of the proportion of beetles near the chemical at 5, 10, 15 and 20 minutes for T9, T10 and the control.



Summary of Results for test chemicals T9 and T10: On the strength of the above findings regarding test chemicals T9 and T10, it was evident that neither of these two chemicasl was attractive to adult SHB, and indeed there was some data to suggest that T9 may actually act as a behavioural arrestant or a repellent. Although these results are interesting, the remit of the current project, as stated in the objectives, was to "*Identify sources of natural cues to **attract** the SHB, establish the chemical identity of the attractants and the feasibility of their use in attractant lures*". For these reasons, chemicals T9 and T10 were not investigate any further.

Results 4. Establishing use of pheromone communication by SHB

Male SHB spent a greater amount of time in the quadrant with female SHB extract compared to how long they spent in the control quadrants. However, duration of stay was not significantly longer than the proportion of time that would be spent there by chance alone (i.e.25%) (t=1.54, P=0.15) (Figure 10). Male SHB spent less time in the quadrant with male SHB extract but, again duration was not significantly shorter than expected by chance (t=1.95, P=0.07) (Figure 10). The proportion of time spent by female SHB in the quadrant with female SHB extract was significantly less than expected (t= 2.63, P=0.02), but their response to male extract was not significantly different to expected (t=0.63, P=0.54) (Figure 11).

Figure 10. Mean time (secs) (± SE) spent in quadrants of a static four-way olfactometer by a male *A. tumida* (N=15). Three quadrants acted as controls and the other quadrant contained 20 μl of extract from male or female *A. tumida*. Total test duration was 300 secs.

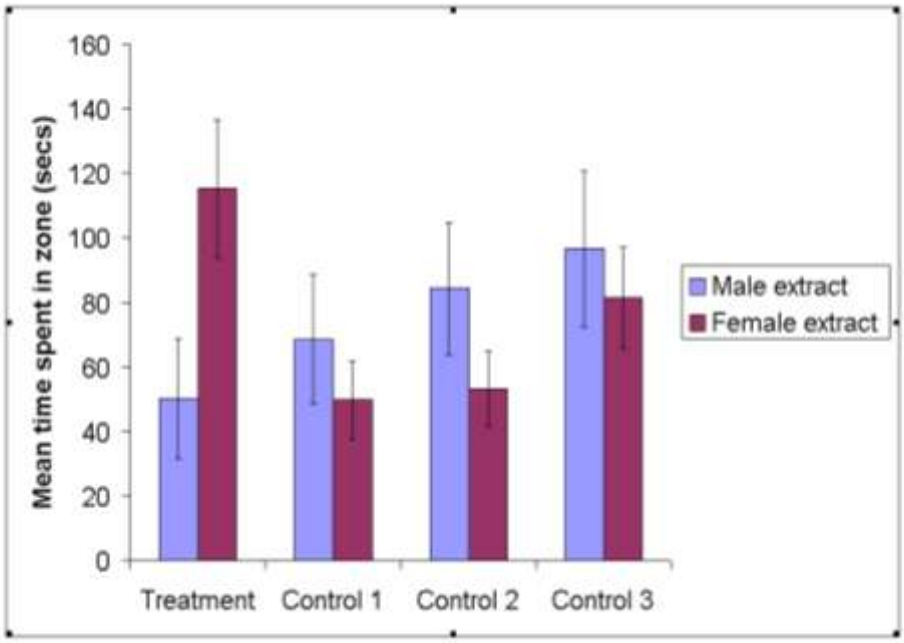
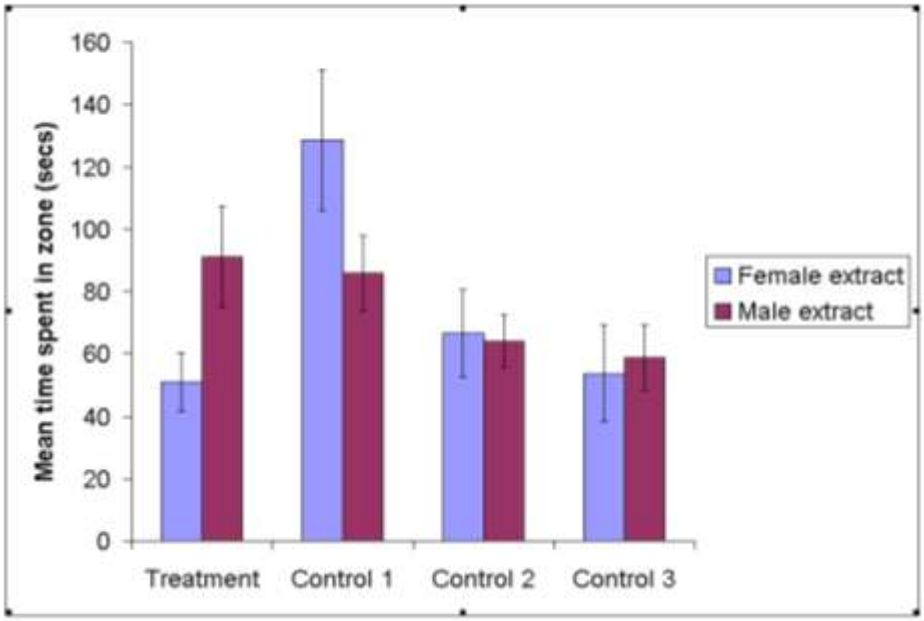


Figure 11. Mean time (secs) (± SE) spent in quadrants of a static four-way olfactometer by a female *A. tumida* (N=15). Three quadrants acted as controls and the other quadrant contained 20 μl of extract from male or female *A. tumida*. Total test duration was 300 secs.



Male and female SHB gave an EAG response to solvent extracts from male and female beetles. As was observed for the responses to volatiles from hive-associated components, the EAG response of female beetles was greater than that of male beetles for all sources tested (Table 11). Analysis of solvent extracts of adult male and female SHB by GC-MS did not reveal any consistent observable differences between the profiles. SPME-GC-MS did not show a difference between volatiles released by male and female beetles.

Table 11. Mean (± SE) EAG responses (mV) by male and female SHB to extracts of male and female SHB (one beetle equivalent) after subtraction of the solvent control

	Source of extract				
	Males <24 h	Females <24 h	Males 24-48 h	Females 24-48 h	Bees (+ve control)
Males	0.59 ± 0.16	0.58 ± 0.13	0.62 ± 0.33	0.67 ± 0.35	0.72 ± 0.25
Females	0.84 ± 0.24	0.78 ± 0.11	0.80 ± 0.23	0.84 ± 0.1	1.18 ± 0.28

Results 5. Development of trap incorporating attractant lure, and protocol for use

The percentage of beetles caught in the traps with a lure was significantly greater than the percentage of beetles caught in the control traps (t = 1.73, d.f = 18, *P*<0.001) (Table 12).

Table 12. Derived mean percentage of adult SHB (n=10) caught in a trap with or without the prototype lure (composed of T1 & T8). Means followed by different letters are significantly different (t test, *P*<0.001)

	Control trap	Trap with lure
Derived mean % of beetles caught	7.6 [a]	31.4[b]

Results 6. Validation of monitoring system for SHB in field trials

South African field data

Neither unbaited traps nor lures caught any SHB adults. At this point, the project team concluded that a lack of flying adult beetles was the most likely explanation, given an observation that earlier in the year, beetle numbers were markedly reduced in comparison with the same time the previous year: December 2007 saw >1,000 beetles/colony in affected apiaries; December 2008 yielded a total of 21 beetles from 36 colonies.

American Field data

No Small Hive Beetles were trapped, although a number of ants and flies were collected. During the trial it was lightly or heavy raining every day. If numbers of flying beetles were very low, this is likely explanation for lack of data.

Summary

Given the above results, further, more robust trap/lure validation needs to be undertaken. It is also possible that traps may be ineffective when deployed out of doors. Several other potential SHB attractants have failed to perform in the field when in competition with live bee hives for migrating beetles in the wild (Dr. Jeff Pettis, *pers. comm*.). It is important to explore how the traps and lures perform if placed directly inside SHB-infested hives. For this reason, the project team has supplied prototype lures to Dr. Lilia De Guzman (USDA Research Entomologist, Louisiana). She will compare the performance of these kits witha range of other known/potential attractants (Results available 2010).

Results 7. Investigating larval SHB to identify the possibility of a chemical cue to influence behaviour.

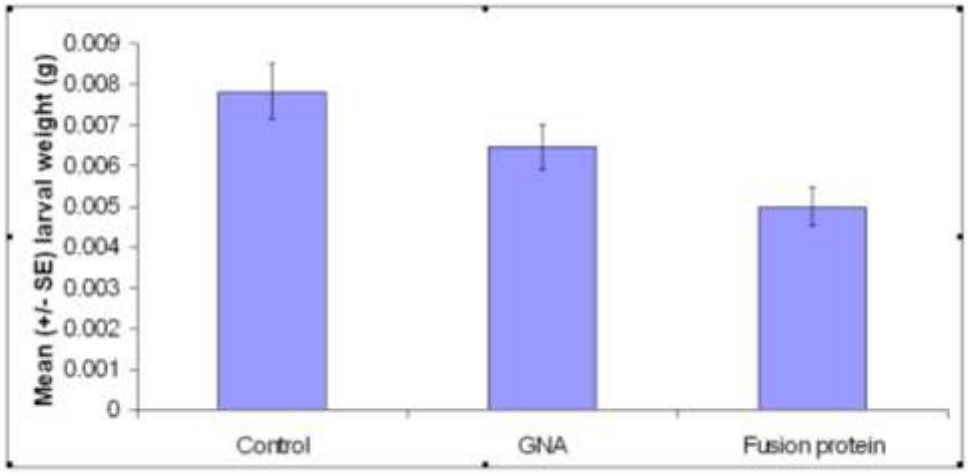
GC-MS analysis of solvent extracts from wandering and non-wandering SHB larvae did not reveal any consistent differences between profiles of the larvae themselves or surfaces over which the larvae had crawled.

Results 8. Investigating novel control methods for SHB that could be used in conjunction with the attractants/monitoring systems, or larval chemical cues if found.

Larval SHB susceptibility to insecticidal fusion proteins

Ingestion of the fusion protein by larval SHB over a period of 3 days resulted in a significant decrease in their mean weight compared to that of controls (P<0.05) (Figure 12). There was no significant difference in the mortality of larvae between the three treatments (See Annex 1).

Figure 12. Mean % mortality (± SE) of SHB larvae feeding on control diet or diet treated with 300 ppm recombinant GNA or fusion protein, for 3 days.



Results 9. Dissemination of information to stakeholders, beekeepers and inspectors.
As well as reporting project activities via the Bee Inspectors and the project steering group, information has also been disseminated via BeeBase (see https://secure.Fera.gov.uk/beebase/pdfs/shbResearch.pdf). In addition, project members have made a number of presentations, notably at recent Bee Inspectors conferences and Bee Farmers Forums. Several manuscripts detailing the scientific findings have been prepared for publication within international peer-reviewed journals, two of which have already been accepted for publication. See also Section 9 of this report for full list of project outputs.

Conclusions/Further Research

- A viable culture of SHB has been successfully established within Fera's Plant Health-licensed quarantine entomology unit. This culture is now routinely maintained under closely controlled conditions, and provided ample insects for the purposes of this project (in full accordance with Fera's SOP's and protocols, which reflect *A. tumida's* status as an exotic pest, notifiable in Europe under EC honeybee legislation). This culture is, in its own right, an extremely valuable resource, being the only live population of laboratory-reared SHB in the EU.
- In accordance with its primary aims, this project has clearly identified two volatile chemicals (T1 and T8), which each elicit highly significant ($P<0.001$) positive behavioural responses in adult SHB. These have been incorporated into a lure and trap, which proved to be effective at trapping adult beetles when deployed under controlled laboratory conditions. This system is currently being field tested in both South Africa and the USA. There is a need to undertake further laboratory and field trials to refine the trap and lure design. This project also identified certain volatiles (T9 & T10) that have significant ($P<0.001$) negative effects on adult SHB. These have the potential to be exploited as repellents, and require further investigation.
- For the purposes of this project, bioassays were deliberately conducted under a very limited range of controlled environmental conditions, chosen to ensure that adult SHB were behaviourally active. There is a pressing need to ascertain the biology (development and behaviour) of *A. tumida* under a range of environmental conditions that are more representative of the UK (i.e. likely survival, activity and fecundity of adult SHB at cooler temperatures). Such information is not only important in elucidating the efficacy of the lure components under UK conditions, but also crucial to our understanding of the true threat (likely establishment and spread) posed by SHB in the event of its introduction to the UK. Furthermore, the incidental observation made during this project, that starved SHB larvae can still complete their development and emerge as viable adult beetles[11], has important implications in the development of containment/eradication strategies. The survival, development and subsequent reproductive success of nutritionally-compromised SHB larvae requires further research.
- Behavioural bioassays of adult SHB, and also their electrophysiological responses, both indicate that male and female adult beetles respond to conspecifics to varying degrees. The electrophysiological response indicates that male and female SHB are able to perceive components in solvent extracts from both male and female adults. The adults used to prepare the extracts had not been exposed to a food source and therefore the response cannot be due to any food material e.g. pollen or honey present on the beetles. Data presented in this report suggest that adult female SHB are particularly sensitive to extracts derived from their own sex, although any chemical(s) that might be responsible for this (repellent) effect have yet to be identified. It has previously been shown that adult SHB carry a yeast, *Kodamaea ohmeri*, which in the presence of pollen, produces isopentyl acetate amongst other compounds. This is the same chemical found in alarm pheromone components of honeybees and it has been shown that this acts as an attractant for adult SHB[21]. Both male and female SHB have been shown to carry this yeast. It is not known whether this yeast species is present in the beetle populations used in these studies. However, as the yeast itself is not an attractant, it is unlikely to have caused the observed responses. (Analysis of

solvent extracts of male and female SHB did not indicate the presence of isopentyl acetate). GC-MS analysis did not reveal any consistent differences in the profiles of male and female beetles. It is possible that chemical derivitization could result in differences being observed. In spite of extensive studies, Torto[22] reported that pheromone communication in *A. tumida* could not be demonstrated for SHB that had been isolated from a food source. I.e. unfed beetles may not produce or respond to pheromones. However, the extracts in the present study were from beetles not exposed to a food source, and yet evidence of both electrophysiological and behavioural activity was recorded.

- The observation that in their final stages of larval development, immature "wandering" SHB cause other younger larvae to follow them away from their shared food source (bee brood) requires further investigation. While the current study was unable to determine any specific chemical characteristic(s) underlying this phenomenon, such a marked behavioural trait must have some physiological basis that, if properly understood, has clear potential to be exploited as control method.
- In this project, experiments to ascertain the effects of an insecticidal fusion protein on the growth and survival of larval SHB were inconclusive. Although the potential of this technology in SHB control has been investigated more thoroughly elsewhere (Defra project PH0505), there is still a need to improve the stability of fusion proteins within appropriately modified beetle diet.
- Given the outstanding status of SHB as a potential threat to the UK/EU beekeeping sector, findings of this and other recent R&D projects that examine the biology, behaviour and control methods for this beetle must be used to inform appropriate contingency plans to be implemented in the event of an incursion. It is anticipated that the existing *draft England and Wales Contingency Plan for Exotic Pests and Diseases of Honey Bees* will be updated and agreed by spring 2010.

Benefits to the Customer

- The outputs generated by this project enhance our understanding of existing and potential threats posed to UK bee stocks by the exotic pest SHB, and thus contribute to Defra's aim to achieve healthy and sustainable bee populations. In particular, they will help to improve biosecurity measures, inform contingency planners and compliment the aims of the Government's 10-year Healthy Bee plan. Specific benefits are as follows:
- This project has successfully identified at least two volatile substances that, together, serve as an attractant to adult SHB under laboratory conditions. The inclusion of this chemical formulation in a prototype lure-and-trap is currently being subjected to small scale field testing in South Africa and the USA. Early development of detection methods, prior to the arrival of the SHB will have enormous benefits: Detection of the presence of SHB in the UK upon its very first arrival will be vital for key decisions that must be made on the occurrence, degree and significance of the problem; early detection will also be critical for any attempts at successful eradication; it will also facilitate a more precise application of appropriate control responses and form the basis for further work on integrated control measures, for example, by providing a platform for development of lure-and-kill techniques.
- Development of detection methods will assist the apiculture industry by providing tools to effectively monitor for the presence of the SHB. Early detection is key in eradication and will reduce its impact, and therefore maintain healthy colonies to provide essential pollination services to agriculture and horticulture and sustain the beekeeping industry.
- Technology transfer is an important element of the work of the NBU and the NBU inspectorate will deliver this element of the project. Once validated, methods can be incorporated within the extension programmes delivered by the NBU, and will provide for rapid dissemination of advice on how to detect and control the SHB to beekeepers in the UK. Advisory materials/information along with research and development information will also be provided to beekeepers via BeeBase online.

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References to published material

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

Peer-reviewed publications

Cuthbertson, A.G.S. & Brown, M.A.(2006). Vital pollinators: honey bees in apple orchards. *Biologist*, **53:** 78-81.

Cuthbertson, A.G.S., Mathers, J.J., Blackburn, L., Wakefield, M.E., & Brown, M.A. (2008). Maintaining *Aethina tumida* (Coleoptera:Nitidulidae). under quarantine laboratory conditions in the UK and preliminary observations on its behaviour. *Journal of Apicultural Research & Bee World*, **47**(3): 192-193.

Manuscripts in submission/in press

Cuthbertson, A.G.S. & Brown, M.A..(200-) Environmental issues affecting British honey bee biodiversity. *International Journal of Environmental Science and Technology*, In press.

Fitches, E. C., Bell, H. A., Powell, M.E., Back, E., Sargiotti, C., Weaver, R. J. & Gatehouse, J. A. (2009) Insecticidal activity of insecticidal toxin and snowdrop lectin (GNA)- containing fusion proteins towards pest species of different orders. *Accepted Pest Management Science*.

In draft

Collins, L.E., Wakefield, M., Cuthbertson, A.G.S., Mathers, J.J., Blackburn, L. & Brown, M.A. (200-). Small Hive Beetle (*Aethina tumida*) electrophysiological and behavioural responses to volatiles collected from beehive material by solid phase microextraction. Draft.

Wakefield, M.E., Marris, G.C., Collins, L.E., Cuthbertson, A.G.S., Mathers, J.J., Blackburn, L. & Brown, M.A. (200-). Development of an attractant lure for monitoring of the small hive beetle. Draft.

Presentations and Conferences

Cuthbertson, AGS. (2007). Maintaining and culturing small hive beetle (*Aethina tumida*). National Bee Inspectors Conference, 11-12th April 2007. Central Science Laboratory, York.

Wakefield, ME. (2007). Detection and monitoring of the small hive beetle (*Aethina tumida*). National Bee Inspectors Conference, 11-12th April 2007. Central Science Laboratory, York.

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Various presentations to beekeepers by NBU staff as part of extension programme.