Preliminary Investigation of Bee and Mite Volatiles for Possible Varroa Mite Control

Final Report

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Introduction

The honey bee mite, Varroa, is developing resistance to all the acaricides currently in use to treat it. A promising new strategy for Varroa control is to identify the chemical cues from honey bee larvae used by mites for host finding and reproduction. Chemical compounds are used by Varroa to distinguish the age and sex of bee larvae produced by both the larvae and pupae (De Guzman et al. 1995; Calderone et al. 2002, Rickli et al. 1994, Le Conte et al. 1989, Trouiller et al. 1992, Donzé et al. 1998). Four day-old and five day-old worker larvae attract older nurse bees and Varroa by producing different amounts of age-specific hydrocarbons such as alkanes, alkenes and methyl alkanes (Le Conte et al. 1999). The proportions of bee hydrocarbons vary with the age of the larvae and some larval compounds are also present on Varroa (Trouiller and Milani 1999, Martin et al. 2001b). Hydrocarbons from worker, drone and queen larvae are different from each other and might help Varroa to distinguish queens from workers, for example (Khodairy and Bruckner 1997; Aumeier et al. 2002). The methyl- and ethyl-esters from worker and drone larvae not only attract Varroa, but also stimulate mite reproduction (Milani 1990; Trouiller and Milani 1999) and can trigger Varroa egg-laying activities. These compounds can also influence the sequence in which male and female offspring are produced by the foundress mite (Garrido et al. 2000; Garrido and Rosenkranz 2003).

In addition to the volatile compounds from bee larvae, chemical cues from the mite itself appear to influence reproductive behaviors of Varroa. For example, mite-derived attractants within mite excreta contribute to the formation of mite aggregation and mating sites within the host cell (Donzé and Guerin 1994; Erickson et al. 1994; Yoder and Sammataro 2003). A fecal accumulation deposit by mites on the cell wall is where a multitude of mite-related activities occur, including mating, oviposition, maturation, protection from the active cocoon-spinning bee larva, and congregation in between blood meals (Donzé and Guerin 1994, 1997). If this signal can be disrupted, the Varroa life cycle could be compromised.

Since hydrocarbon compounds from immature bees have such a profound influence on Varroa's behavior, we concentrated our research to investigate if we could detect these compounds. First, we wanted to establish if chemical compounds from different honey bee lines could be demonstrated, then if volatiles from the mites could be seen, and finally, if differences in mature mites vs. immature mites and the mite feces left in the cell could be detected. Once we determine that we could document these different compounds, our next step will be to identify those that are used by Varroa for host-finding, mating and reproduction. If we can disrupt Varroa in these activities, then we can perhaps reduce mite population growth in honey bee colonies.

Objectives and Methods:

Objective 1. Identify volatile chemicals from different lines of mite-resistant and mite-susceptible bees.

Procedure: Queens of known mite-resistant honey bee lines (e.g. Russian and SMR queens) were obtained from the USDA Bee Breeding and Genetics Lab (Baton Rouge, LA). Hygienic queens (Glenn Apiaries, CA), reported to be mite-resistant, and local African stock (reported to have few mites) were also selected. Mite-susceptible, unselected stock from Hawaii (Big Island) was the control. All colonies were originally installed on new, unused equipment on 100% wax foundation mid-summer in 2003 and are now well-established in our apiary (USDA-ARS Carl Hayden Bee Research Center in Tucson, AZ). Colonies from each line have been periodically monitored for Varroa mite infestation levels via sticky board counts, to determine mite preferences and reproduction rates among the different bee lines (Ostiguy and Sammataro, 2000).

Volatile compounds were sampled from all stages from these five lines of bees (April 2004) of workers: eggs, larvae and pupae, and live adults. To obtain bee larvae of known ages, queens were caged on a frame for twelve hours to lay eggs (Boot and Calis 1991). Afterwards, the queen was released into the colony and the frame placed back in the cage and returned to the colony. The caging was made of metal queen excluder material that allows worker bees to pass through, but not the queen. The queen was caged twice, three days apart, to allow us to sample two different ages of bees from the same frame simultaneously.

Collecting Volatiles: Compounds were collected on SPME fibers (StableFlex™ #57326-U, Supelco, Bellefonte, Pa) and analyzed using gas chromatography. The SPME fiber was desorbed in a temperature programmable injector (Desorb 240° C for 3 min.) ramping from 40°C (3min hold) to 240° C min. @ 25°/min (He=1 ml/min). Desorbed compounds are focused on the column head (Column: Varian VF-5ms, 30m x 0.25mm, ID DF= 0.25) at 30° C and the components separated using a temperature program (40° – 240° C in 25 minutes at a flow rate of 1 ml/min.). Each day, a blank air sample was run to compare with the bee/mite samples. A fingerprint, in the form of a chromatogram plot, representing different volatile compounds, was generated for each sample. Samples were analyzed using a Varian Model 3800 Gas Chromatograph /Mass Spectrometer (GC/MS) to separate and identify the compounds (Model 2200 Ion trap MS). Some of the compounds have been tentatively identified using the on-line Mass Spectroscopy library (both Wiley and NIST).

Bee samples: Five eggs, larvae or pupae were placed in glass vials using cleaned steel forceps and the vial was then covered with aluminum foil. The SPME fiber was inserted through a hole in the foil and exposed for 10 minutes at a constant temperature (28.9°C [84°F], 30% RH) with minimum background contamination. Controls consisted of sampling empty vials for the same amount of time and under the same conditions as the

sample.

Objective 2: Identify volatile chemicals from Varroa.

In many instances, mite hydrocarbons mimic those found on bees, making the mites difficult for bees to locate (Martin et al. 2001). Our technique was designed to discover unique mite compounds. We first looked at mites (and bees) from local (Oracle, Arizona) Africanized bees (AHB). These bees were collected from a local beekeeper in Arizona who works with AHB and does not treat for mites. Our goal was to determine if we could separate out mite peaks from bees and from the beeswax comb. By sampling an empty honeycomb cell that had no mites or brood, those peaks of the wax hydrocarbons can be eliminated.

Procedure: In the late summer/early fall, when mite populations are normally high, mites were collected for sampling. As the various life stages from the different bee lines were collected and tested (as described above) concurrently, accompanying mites were collected and sampled.

Mite Volatiles: Several individual cohorts of mites found in each bee cell and in each of the queen lines were obtained. We also collected volatiles from the same infested pupa from which the mites were acquired. In general, five mites were placed in glass vials and a SPME fiber device was inserted (see above for bee volatiles). Controls and sampling conditions were the same as for the bee volatile collection.

Results

Volatiles from mite-resistant and mite-susceptible bee lines

We started by comparing the chemical profiles of worker larvae, pupae and adults from resistant and susceptible lines of honey bees sampled in April 2004. The work will continue by sampling bees later in the year (September) to determine if there is seasonal variation in chemical profiles. To eliminate the background fiber peaks and other noise, we selected particular ion fragmentation numbers that correspond to hydrocarbons and other chemical families. In this way, the chromatogram plots can be clarified, eliminating unwanted peaks such as fiber or machine material, and making the plots easier to read and analyze. Volatile compounds of worker bees from all bee lines were sampled: Day 1 (egg) to Day 21 (callow adult bee); the changes in weight and appearance over the life of each stage of the bee were also recorded.

Profiles from Day 1 eggs appear to have many of the same compounds for all bee lines (see Fig. 1). Compound A appears in all lines of bees but in different amounts, as seen in the different peak heights. Compound B, unique to the Russian and SMR lines, was not present in the other lines (see arrow Compound B). African bees appear to have fewer amounts of all compounds.

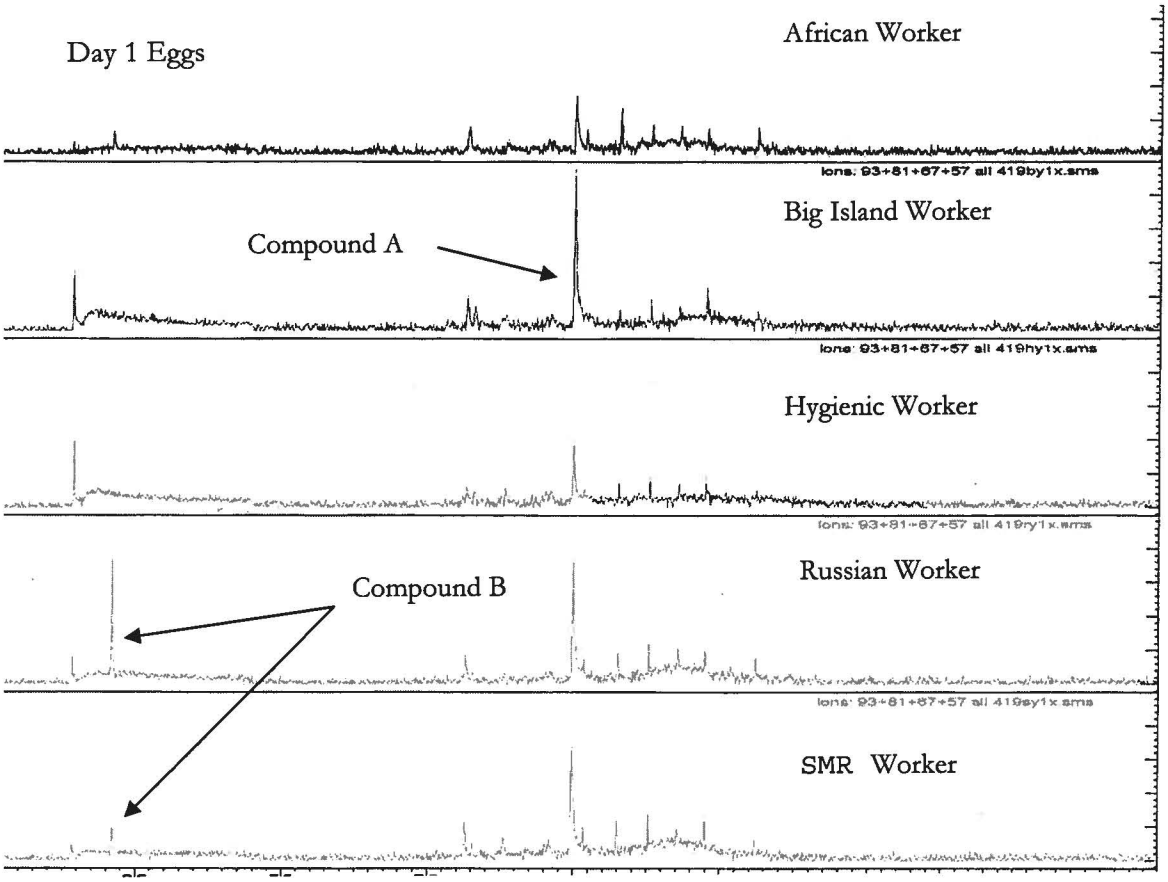

Figure 1. Day 1 eggs of five bee lines. Arrows indicate a unique compound B in the Russian and SMR lines. Compound A appears in all lines. All plots are at the same scale.

One day old larvae (4 days from the egg) have distinctive peaks in the SMR and the Big Island workers (see arrows in Fig. 2) that do not appear at any other time or in the other lines. A large peak is also evident in the Russian workers at this age (dashed arrow). Few peaks are prominent in the African line.

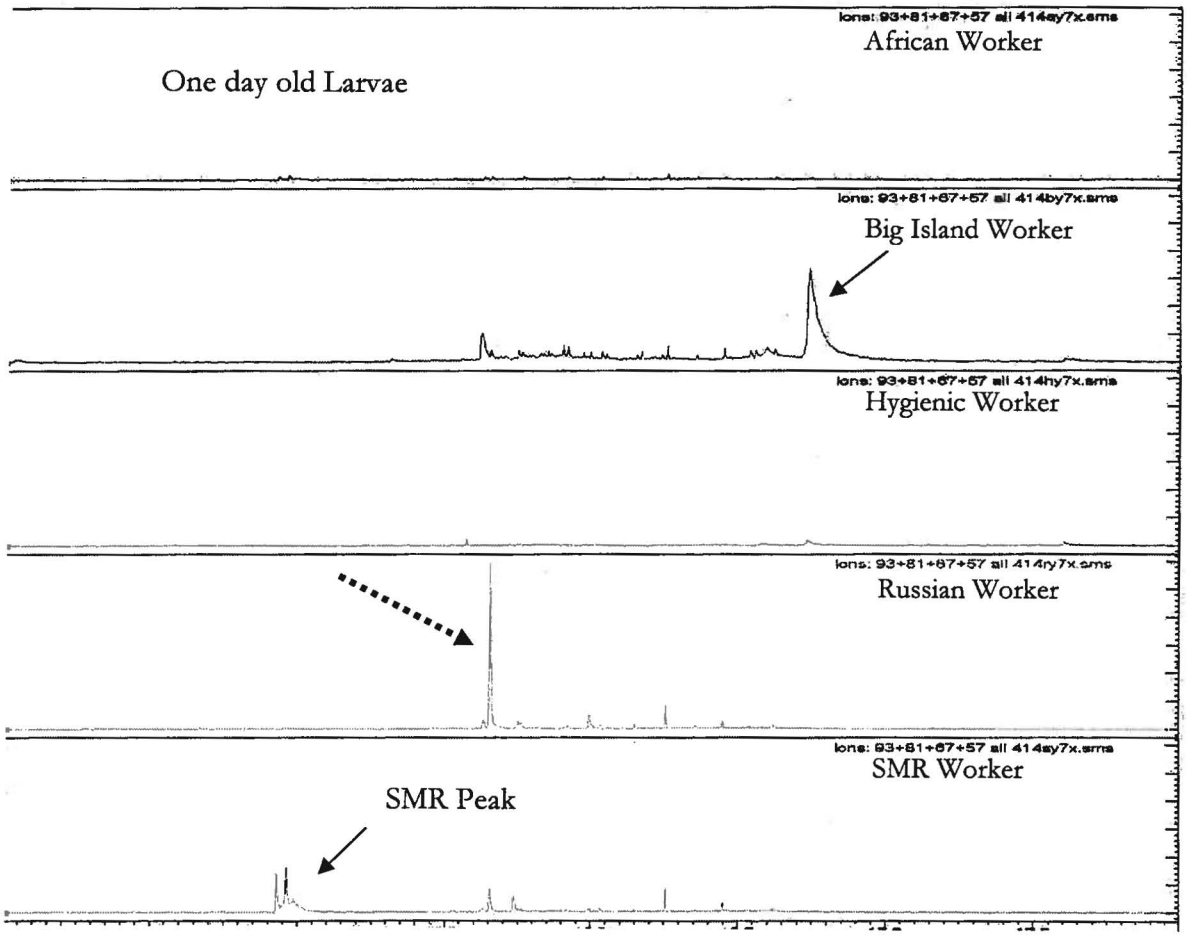

Figure 2. Chromatograms of volatile compounds collected from one day old larvae (four days from the egg). Arrows indicate unique compounds for the Big Island, Russian and SMR lines that will be investigated in future research. All plots are shown at the same scale.

The volatile profiles of Day 9 worker larvae, (6 day-old larva), are shown in the chromatogram plots in Figure 3. These larvae are beginning to spin their cocoons in preparation of being capped. This is the time when foundress mites have moved into the cell to wait for the larvae to finish spinning. The hygienic and Russian lines have a greater amount of the Compound C that is found in all the other lines (see dashed arrows). The African line has some unique compounds, (see solid arrows) not found in the other bee lines. Included with each plot is a photograph of the Day 9 larvae from each of the bee lines, corresponding to the volatiles they emit.

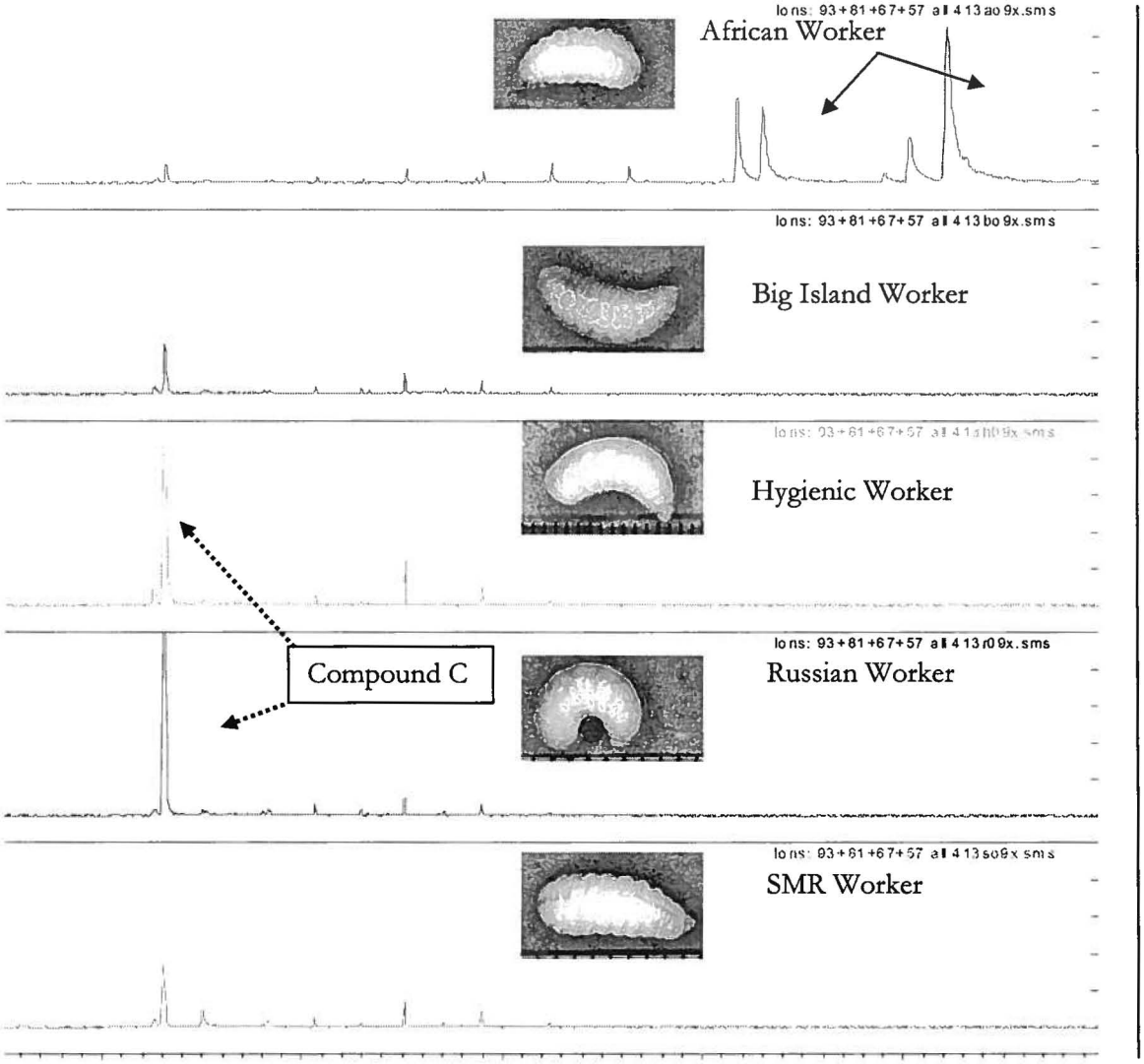

Figure 3. Day 9 worker larvae chromatograms and photos, all taken at the same scale. Host bee larvae are at the most attractive stage and the bee volatiles found at this time may be some of the best candidates for attracting mites. The large peak of Compound C in the Russian and Hygienic lines and the new peaks appearing in the African bees will be the focus of future work.

Mites begin feeding and laying eggs on their bee hosts when the larvae are 11 days old (from the egg); at this age the bees are white pupae. At least two compounds are present in resistant lines (Russian and SMR) but not in the susceptible line (Big Island). Some of the peaks present in Day 11 larvae, especially in the Russian line (Fig. 4 arrows) were not evident just two days previous (Fig. 3). The SMR a unique peak (dashed arrow) was not present in the Day 9 larvae as well.

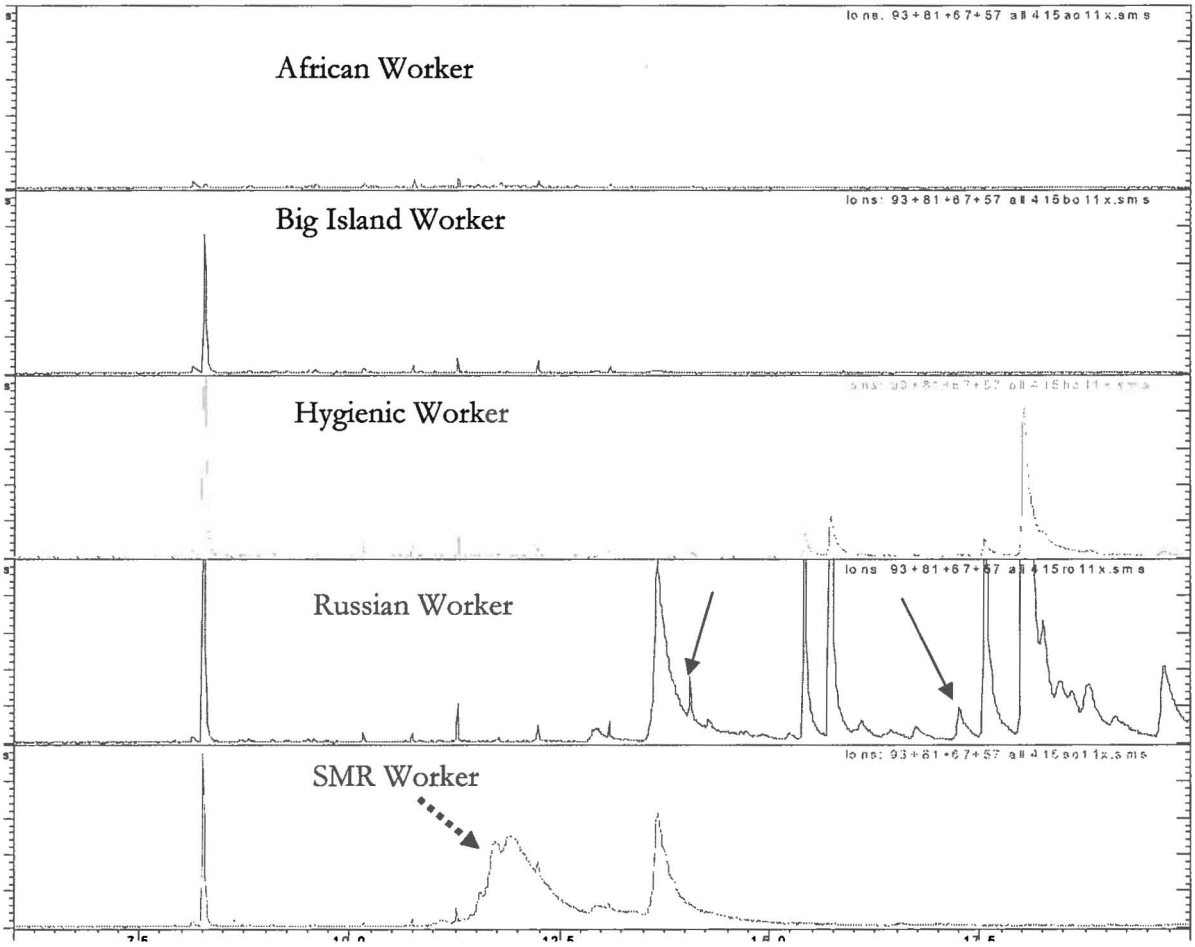

Figure 4. Gas chromatograms of Day 11 white pupae from five bee lines. At this stage of the bee, mites are feeding and reproducing. Arrows point to unique compounds in the Russian and SMR lines. All graphs at same scale.

Results of Mite Volatiles

Volatiles from mites and African honey bees were compared. Mites emit many of the same compounds as their bee hosts (Fig 5), since they feed off of bees. However, we detected some unique compounds from the mites (dashed arrows). Drone and worker larvae were also sampled. The peaks from a empty beeswax comb was also sampled, to eliminate wax-based peaks.

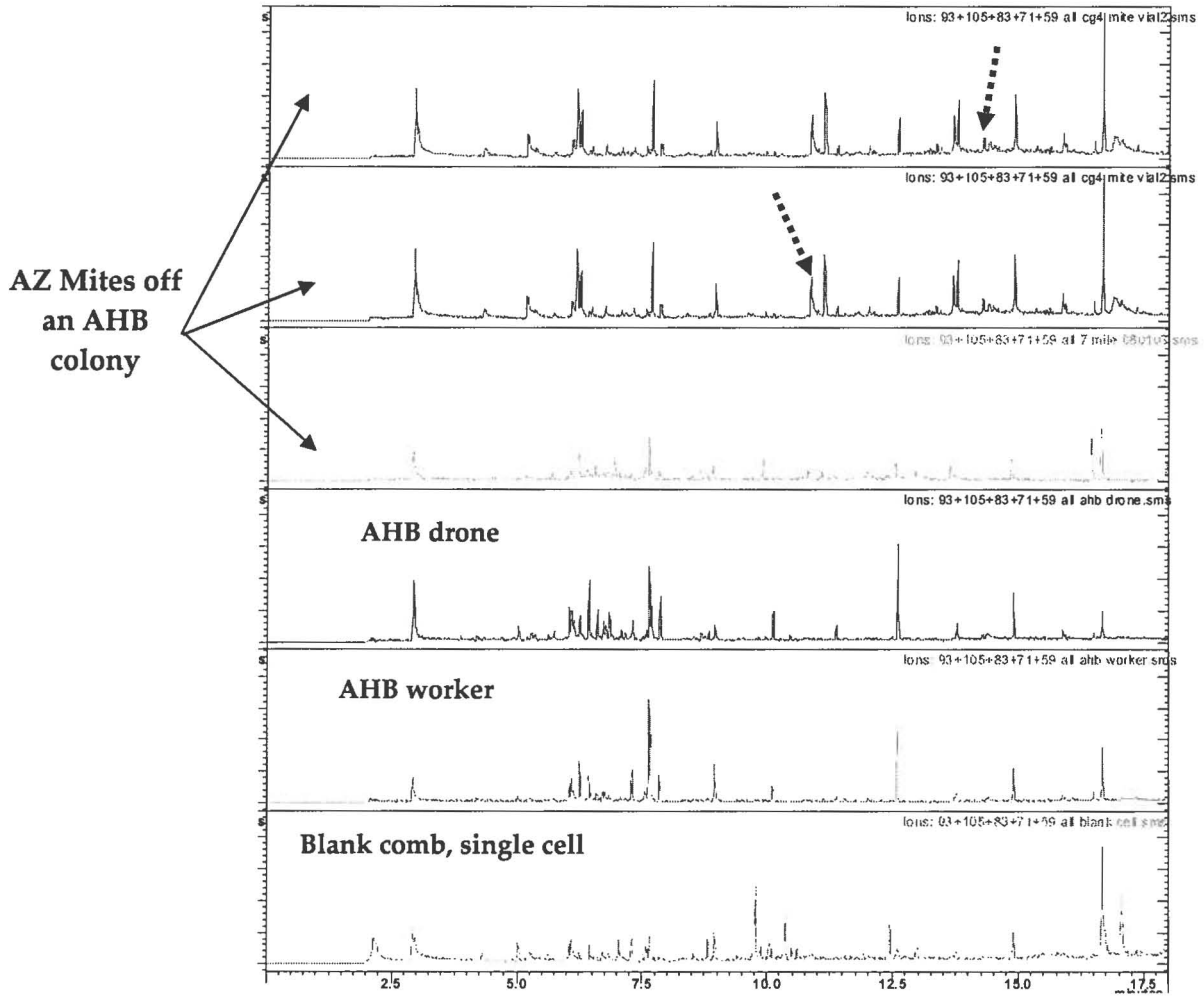

Figure 5. Chromatograms of drone and worker Africanized honey bee larvae and mites from the same colony. The plot of an empty honeycomb cell is also shown. Dashed arrows point to peaks that are unique to mites. All plots are shown at the same scale.

Next we compared profiles of mites removed from two ages of the Big Island (BI) drones. The plots in Figure 6 represent Day 12 (D12) drone prepupae and the older Day 20 (D20) pupae (four days before they emerge as adults). Older pupae are usually covered with mother mites and their offspring.

Infested D12 drone prepupae release volatile profiles that differ from the uninfested pupae of the same age (Fig. 6). In the top plot (A), the arrow points to some unique peaks in the infested bee. The infested older D20 drone (C) produce less amounts of volatiles compared with the uninfested D20 drone (E). The peaks in the uninfested D20 drone (dashed arrows) are much higher than those in the infested drone in C. We also compared the infested drone (C) with its mite cohort (D). Many of the mite peaks are the same as the drone peaks.

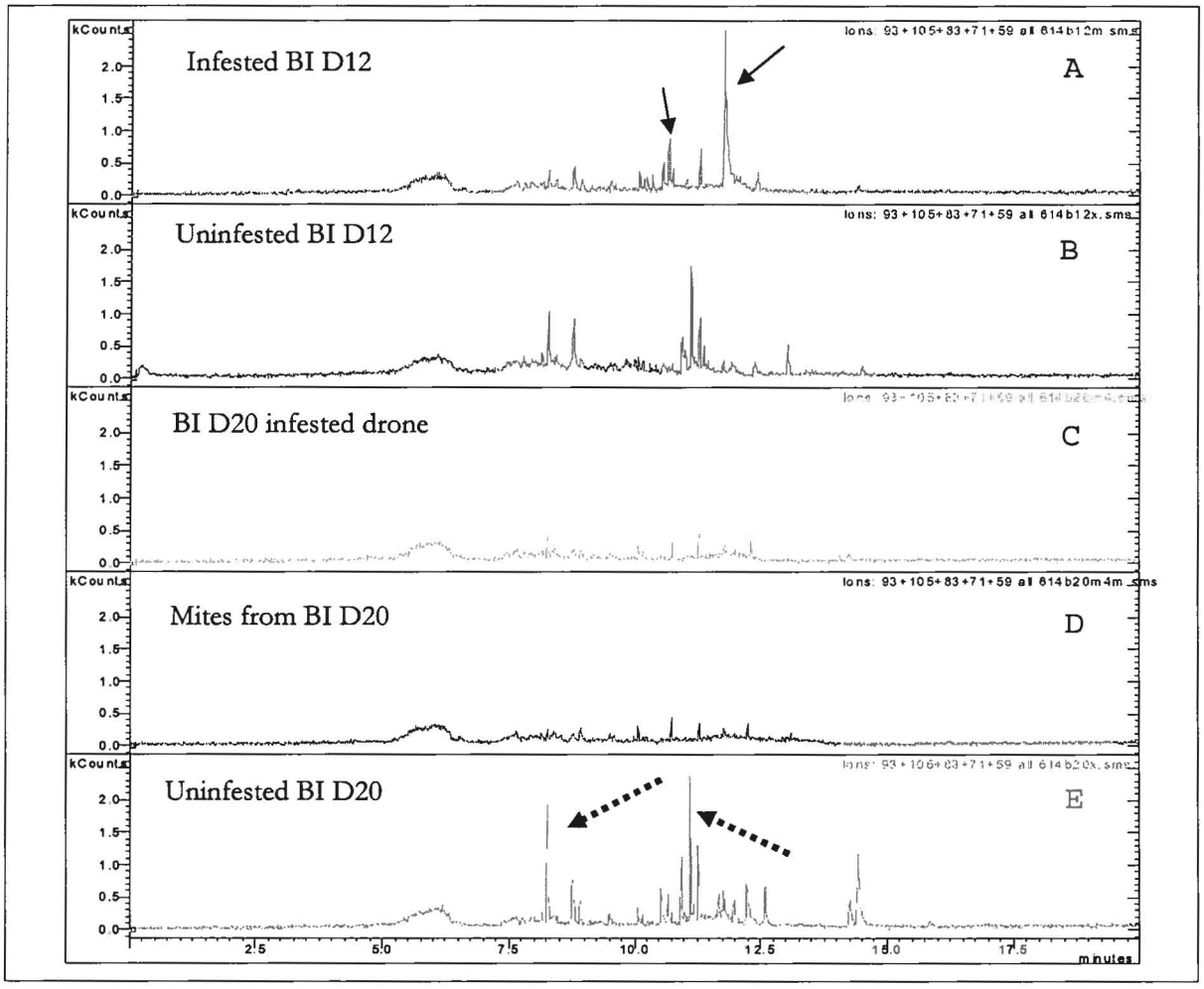

Figure 6. Chromatogram plots of Day 12 (A and B) and Day 20 (C and E) Big Island drone pupae. The Big Island drones are highly susceptible to Varroa infestation. Arrows in A indicates unique peaks from an infested pupa. Chromatogram D show volatiles from the mites parasitizing the D20 drone in plot C. Most of the peaks in C and D are similar. Peaks from an uninfested D20 drone (E, dashed arrows) are higher than in the infested drone. All plots are shown at the same scale.

Then we sampled a Day 12 (12 days old, from egg) Russian drone and compared its chemical profile with the mites infesting it (Fig 7). Most of the volatiles emanating from the mites are similar to the drone on which the mites were feeding (Fig. 7). However, the drone produced some unique peaks (dashed arrows) not present in the mite chromatogram. These compounds may be unique to the Russian bees. All peaks represent cuticular hydrocarbons.

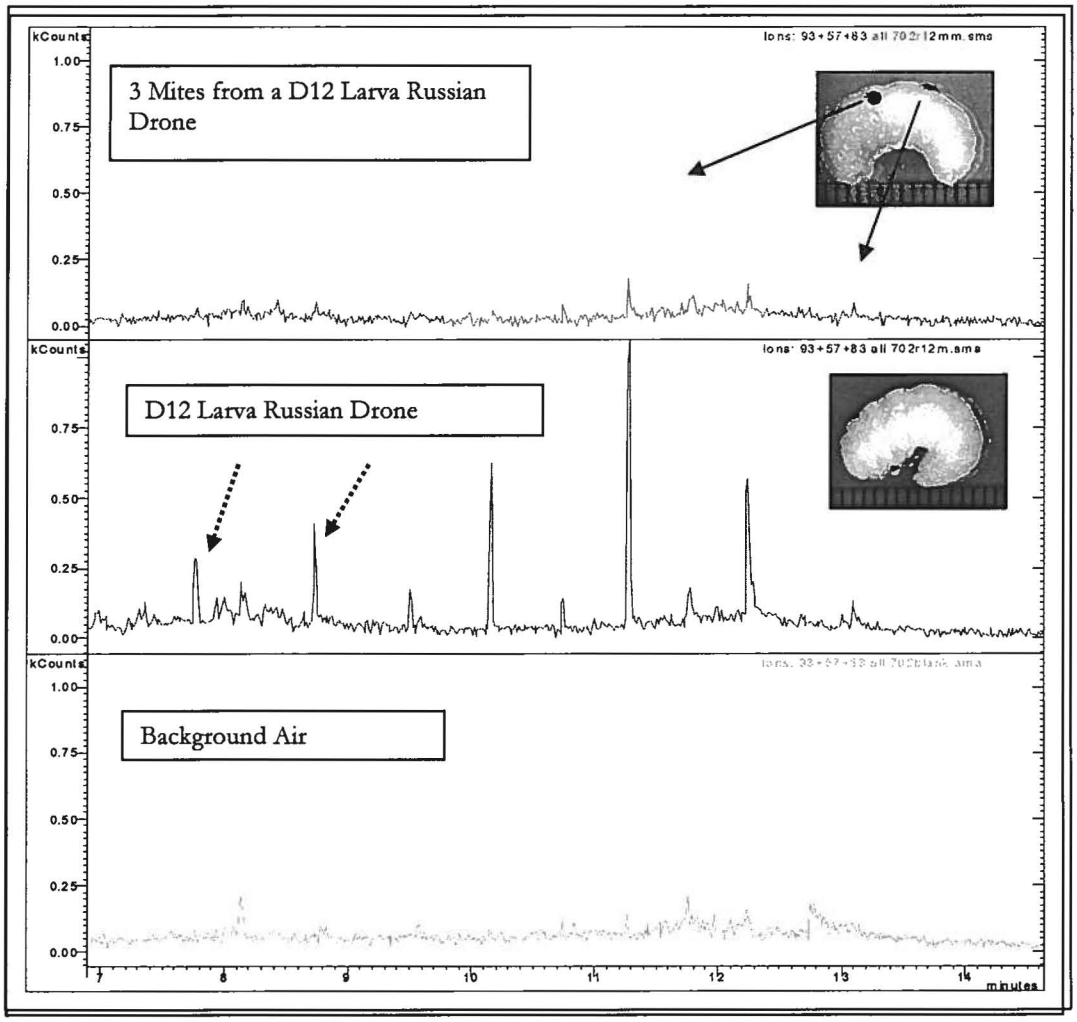

Figure 7. A 12 day old Russian drone (D12) and its mites, compared to the background air. Russian bees have some resistance to Varroa. The D12 drone larva is just beginning to spin the cocoon and would be most attractive to foundress Varroa mites. Distinctive drone peaks are indicated by the dashed arrows. All plots are shown at the same scale.

An 18-day old (D18) infested Russian drone pupa is old enough to have a mother Varroa mite and several of her offspring present in the cell. We sampled an infested drone and its mite cohort (Figure 8). Many of the peaks are similar, as mites feed on drone hemolymph (blood) and defecate in the cell. The SPME technique is sensitive enough to detect volatiles of the mature mites, immature mite offspring and the feces of the mites. The dashed arrows (A) indicate unique drone peaks that are not evident in the mites or the fecal material of mites.

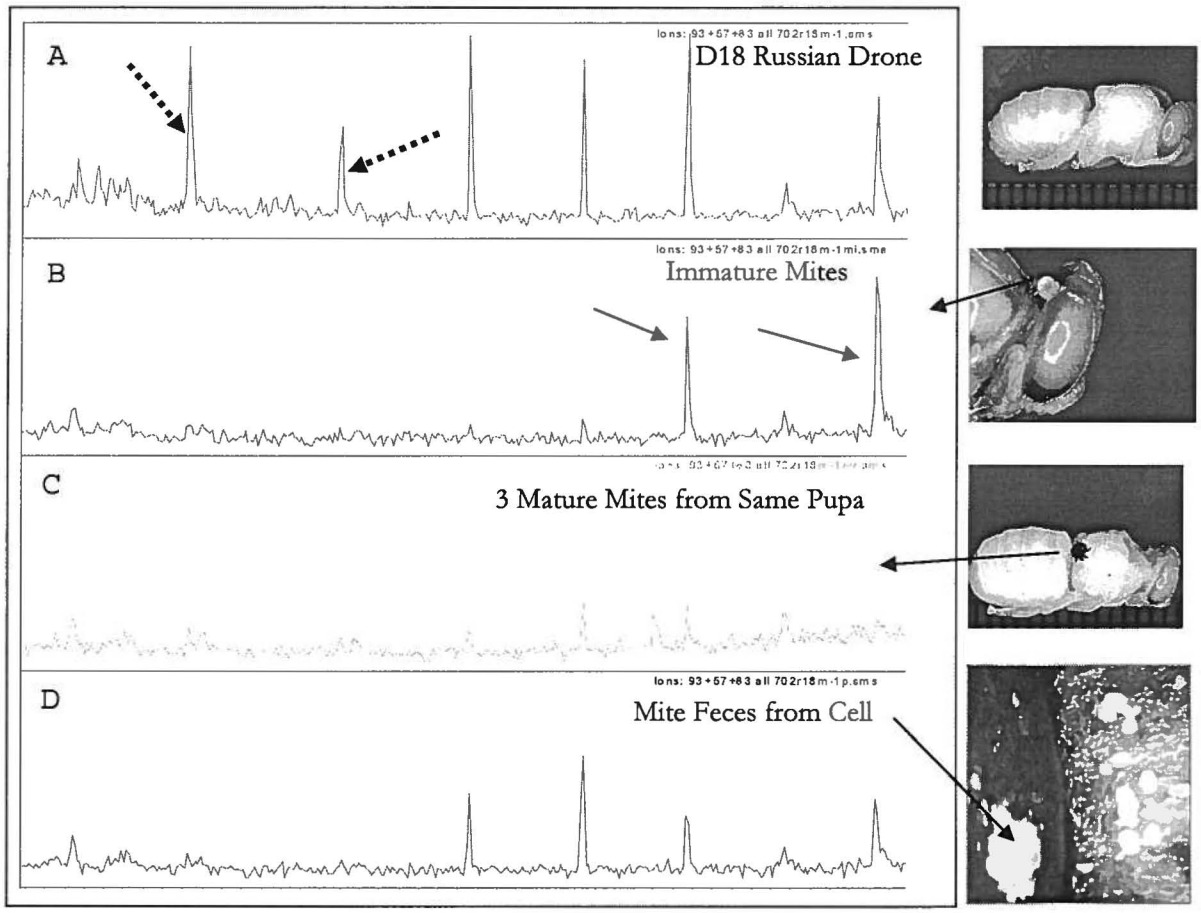

Figure 8. Chromatogram of a single Day 18 Russian pupa (D18) and its mites. All the mites were from a single drone, and we used the same cell from which the drone was pulled to sample the mite feces. Dashed arrows indicate peaks unique to the drone pupa (A). The mites were separated from the drone and sampled. The immature mites (B) were sampled separately from the mature females (C). Mite feces found inside the cell that housed this pupa was likewise sampled (D). All plots are shown at the same scale.

The volatile peaks from the immature mites (arrows, B) have higher molecular weight compounds than the mature mites (C), but did not have measurable amounts of the lower molecular weight compounds. The higher weight compounds are also present in the drone pupa, which in addition, have several peaks of lower molecular

weight (dashed arrows, A). Fecal material (D) had at least four peaks that were present in the drone pupa sample and two that were found from the mite sample.

Discussion

The preliminary investigation into volatiles collected from mite-resistant and mite-susceptible honey bee lines demonstrates that volatile compounds can be detected from different ages of bees, from different lines of bees and from different life stages of Varroa mites. At this point, most of the volatile compounds identified from the bees are hydrocarbons. This is consistent with what others have found (Nazzi et al. 2002a). However our work is different from other studies, in that we are examining bee lines known to have mite resistance (and local Africanized bees) simultaneously.

We have determined that there are differences in the composition of volatiles produced by all stages of African bees and the other 4 European lines we tested, including the egg stage (Figure 1), one day old larvae (Figure 2) and six day old larvae (Figure 3). Worker pupae from the different lines (Figure 4) have higher molecular weight compounds, indicating different types of hydrocarbons are given off. Drone volatiles differ from those of workers (Figures 5-8).

The method of using SPME for honey bee and varroa mite volatiles is a recent one, but has much potential in not only identifying unstable and transient compounds, but in helping researchers better understand the complex chemical relationship between and among bees and their parasitic mites. Now that we have established a baseline of information, we can begin to refine our experiment and isolate compounds that can be tested further. Not only are there compounds unique to the bee lines, but there are compounds that the mites themselves manufacture. Some compounds in the resistant bee lines may be responsible for confounding mite host finding or feeding. Other compounds from the mites could be cues that the bees use to detect infested cells (e.g. hygienic lines) and stimulate the workers to remove the larva or pupa. The process of peak identification has begun, and our next step will be to test individual compounds on mites.

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