**GC-MS Investigation of Volatiles from Honeybee Worker Larvae and Larval Food at Different Instars**

Hao-hao Zhang 1, Chun-sheng Hou 1, Yong-jun Liu 1, Ping-li Dai 1, Yan-yan Wu 1, Yong-gang Pang 2 and Qing-yun Diao 1,*

1 Key Laboratory of Pollinating Insect Biology, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing 100093, China; hhzhang83@163.com (H. Z.); houchunsheng@caas.cn (C. H.); liuyongjun@caas.cn (Y. L.); daipingli@caas.cn (P. D.); uyanyan@caas.cn (Y. W.); dqyun1@126.com (Q. D.)

2 Beijing Blooming Bio-tech Co., Ltd., Beijing 100079, China; pyg19831212@126.com

* Correspondence: dqyun1@126.com;

**Abstract:** (E)-β-ocimene was the only found volatile chemical emitted by whole, live worker larvae of *Apis mellifera* L. by sampling in the vapor phase. While in addition to (E)-β-ocimene, there is evidence for the existence of other volatiles; but the changes of their composition and contents remain unknown during larval development, as are their differences from larvae to larval food. This is the main purpose of the study. We investigated volatile components of worker larvae and larval food using solid phase dynamic extraction (SPDE) coupled with gas chromatography-mass spectrometry (GC-MS). Nine compounds were identified with certainty and six tentatively, consisting of terpenoids, aldehydes, hydrocarbons, ester and ketone. The contents of volatiles of the second-instar worker larvae differ greatly from larvae of other stages mainly attributable to terpenoids, which made the second-instar worker larvae had significantly higher amounts of overall volatiles. Larval food contained significantly higher amounts of aldehydes and hydrocarbons than the corresponding larvae from the fourth to fifth-instar. We discovered volatiles in worker larvae and their food which were never reported before; we also mastered the change of these volatiles’ contents during larval development.

**Keywords:** *Apis mellifera*; GC-MS; larva; terpenoids; volatiles

**1. Introduction**

Volatiles occur in honey bee colonies as a complex mixture, determined by pheromones produced by bees, and other chemicals emitted by beeswax, honey, pollen and larval food [1]. These volatiles have been identified as alcohols, aldehydes, benzenoid compounds, carboxylic acids, hydrocarbons, ketones and terpenoids [1].

Volatiles in honey bee colonies are easily dispersed and received by bees through antennal reception over long distance. Therefore, using volatiles are efficient means in moderating the bee social behaviors. With regard to the volatiles in maintaining social cohesion, most of them are found from adult worker bees, like alarm pheromone inducing defensive behavior of honeybees [2] Nasanov pheromone triggering aggregation [3], and other volatiles transmitting recognition cues; also a few of them are detected from queens specifically [4].

Very few efforts have been made to determine the volatiles emitted directly by intact and alive honey bee larvae. On the contrary, the non-volatile chemicals of honey bee larvae are widely studied based on the solvent extraction method [5-7]. (E)-β-ocimene is the only identified volatile chemical produced by alive worker larvae. There is evidence for the existence of other larval volatiles, because different chromatogram peaks in addition to (E)-β-ocimene were...
discovered [8], but their compositions are not clear. There is also a lack of information about how these unknown larval volatiles change with larval development. However, an understanding of the composition and content variation of volatiles during larval development is necessary for interpreting their functions.

Food provided to honeybees could directly or indirectly affect honeybee volatiles production. There is evidence that food shortages might stimulate worker bee larvae into releasing more (E)-β-ocimene [9]; and feeding honeybee worker larvae essential oils via diet supplements may change their volatiles[10]. However, the volatiles extracted from worker larval food have not been reported before. Only volatile carboxylic acids were identified in drone larval food, and other unidentified non-acidic volatiles were noteworthy [11]. If the volatiles of the worker larval food were analyzed combined with the volatiles analysis of the worker larvae, it would provide a deep insight into the relationship between volatiles in larvae and their food during the same larval instar.

In this paper, we analyzed the volatiles from worker larvae and their food at different instars, using solid phase dynamic extraction (SPDE) combined with high resolution gas chromatography tandem mass spectrometry (GC-MS). Through this research, we discovered some volatiles in worker larvae and their food that have been overlooked before. We believe that these discoveries are a first step towards determining the underlying important functions of volatiles within honey bee nests.

2. Materials and Methods

Three standard Chinese commercial strain of western honeybee (Apis mellifera ligustica L.) were used in this experiment. Large double-deep colonies (30,000 to 50,000 adult bees) located at our apiary in the Institute of Apicultural Research in Beijing (39° N, 116.2° E). Queens were caged within plastic controllers (interior dimensions: 457 × 50 × 245mm, only allowed passage of worker bees) to oviposit on empty new combs (without pollen and nectar stores) for 24 hours and were then removed to the outside of the controllers. Oviposition frames were reared in the controllers. At each caging interval, we obtained three combs with single-age cohorts of worker larvae for each replicate. Seven times caging were carried out, for a total of 21 combs.

After the combs were brought back to the laboratory, cells were randomly chosen to provide both the larval food and larvae samples for further analysis. Larvae and larval food was obtained with a small spatula. Larvae were inspected under the microscope, and only live and uninjured larvae were used for the study [9].

Treatments

The volatiles of grouped larvae at three development stages were monitored. Group sizes were 20 second-instar (2nd-instar) larvae, 10 fourth-instar (4th-instar) larvae, and 5 fifth- instar (5th-instar) larvae. The larval food volatiles were also detected at different stages. To monitor change in volatiles with ontogenetic development, the amount of volatiles released by individual larva was determined [8]. To compare volatiles content between larvae and food, the amount of volatiles released per unit weight of sample were calculated [12]. Larvae and food were carefully transferred into 20 mL glass vials. Before analysis, 2µL of 0.1 mg/L hexadecane hexane solution on a strip of filter paper was added as an internal standard for quantification. Clean empty vials were analyzed separately as controls to remove background interference.

SPDE System

Volatile extraction was performed using SPDE equipment installed in a CTC-Combi-PAL auto sampler (CTC Analytics, Zwingen, Switzerland), as described by Castro [13]. The SPDE needle (SPDE-01/AC-50-56, 50 µm × 56 mm), coated with 90% polydimethylsiloxane (PDMS) and 10% active charcoal (AC), and was preconditioned before use. Equilibration between sample and headspace lasted for 30 min at 35 °C in an incubation pool. After equilibration, the extraction procedure was operated as follows: extraction volume – 1 mL, agitator temperature – 35 °C,
headspace syringe temperature – 35 °C, number of strokes – 30, filling/eject speed – 25 µL/s. During the equilibration and extraction procedure, the larvae were alive and isolated from food for more than 45 min [9]. The needle was then withdrawn and introduced into the injection port of the gas chromatograph, and pumped with 1 mL nitrogen at 100 µL/s for desorption, at 250°C for 2 min in splitless mode.

GC-MS analysis was performed using a Shimadzu gas chromatograph-2010 equipped with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm; J&W Scientific, Folsom, CA, USA) coupled to a Shimadzu quadrupole-2010 mass spectrometer (Shimadzu, Kyoto, Japan). The oven program was as follows: 35 °C for 2 min, 35–200 °C at 5 °C/min, 200 °C for 2 min, 200–250 °C at 10 °C/min, then 250 °C for 3 min. Injector temperature was maintained at 250 °C, transfer line temperature was 250 °C, and ion source temperature was 200 °C. Helium was used as the carrier gas, at a flow rate of 1.7 mL/min.

Qualitative and quantitative analysis

The identification of the compound with authentic standards was performed by comparing the mass spectra (Wiley6 and NIST05) and retention times to those of authentic standards. Compounds without standards were identified by comparing the mass spectrum peaks with data system libraries (Wiley6 and NIST05) and other published spectra (Mass Spectrometry Data Centre 1974). Additionally, the linear retention indices (LRI) of the compounds were calculated by injecting a series of n-alkanes (C10–C25) (o2si Smart Solutions) into the GC-MS on two columns of different polarities under identical conditions. Authentic standards (listed in supplementary data) purchased from Alfa Aesar (Karlsruhe, Germany) were serially diluted with hexane to make standard solutions. The peak areas on the total ion chromatogram were used for quantification. The calibration curve derived from a step-series of standard compounds for individual target compounds was built by plotting the area ratio of target compounds to the internal standard against the concentration ratio. The concentrations of volatile compounds were calculated based on the corresponding calibration curves.

Statistical analysis

Principal component analysis (PCA) using a correlation matrix was applied to the data to establish relationships between the different samples and their volatile compounds. The SPSS software package Version 21 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis. A one-way ANOVA (p < 0.05) was used to test for significant differences in volatile compound concentrations among treatments. The SigmaPlot software package Version 12 for Windows (Systat Software Inc., California USA) was used to create the artwork.

3. Results

We detected fifteen compounds from the developing larvae and their corresponding food, which could be sorted into seven groups: three aldehydes, one ester, three hydrocarbons, one ketone, and seven terpenoids (Figure 1, Table 1).
Figure 1: Gas chromatogram of all identified volatiles reflected in the 2nd instar larvae (IS: internal standard).
Table 1: Volatile compounds tentatively identified from larvae and larval food at different larval instars.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Compound</th>
<th>Code</th>
<th>Peak</th>
<th>LRI Calc</th>
<th>LRI Lit</th>
<th>Identify</th>
<th>Quantifier ions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.75</td>
<td>(Z)-β-Ocimene</td>
<td>t1</td>
<td>2</td>
<td>104</td>
<td>1229</td>
<td>976</td>
<td>S. N. L</td>
</tr>
<tr>
<td>12.06</td>
<td>(E)-β-Ocimene</td>
<td>t2</td>
<td>3</td>
<td>105</td>
<td>1250</td>
<td>976</td>
<td>S. N. L</td>
</tr>
<tr>
<td>14.61</td>
<td>(E, Z)-Alloocimene</td>
<td>t3</td>
<td>5</td>
<td>113</td>
<td>1371</td>
<td>1088</td>
<td>N. L</td>
</tr>
<tr>
<td>16.56</td>
<td>α-Terpineol</td>
<td>t4</td>
<td>6</td>
<td>119</td>
<td>1143</td>
<td>S. N. L</td>
<td>59, 93, 121</td>
</tr>
<tr>
<td>22.62</td>
<td>α-Cedrene</td>
<td>t5</td>
<td>8</td>
<td>142</td>
<td>1556</td>
<td>1403</td>
<td>N. L</td>
</tr>
<tr>
<td>22.82</td>
<td>β-Cedrene</td>
<td>t6</td>
<td>9</td>
<td>142</td>
<td>1560</td>
<td>1403</td>
<td>N. L</td>
</tr>
<tr>
<td>27.34</td>
<td>Cedrol</td>
<td>t7</td>
<td>13</td>
<td>161</td>
<td>2112</td>
<td>1543</td>
<td>N. L</td>
</tr>
</tbody>
</table>

Terpenoids

|         |          |      |      |          |         |          |                |
|---------|----------|------|------|----------|---------|----------|                |
| 10.73   | Octanal   | a1   | 1    | 100     | 1005    | S. N. L  | 43, 56, 84    |
| 13.88   | Nonanal   | a2   | 4    | 110     | 1104    | S. N. L  | 57, 41, 70    |
| 16.91   | Decanal   | a3   | 7    | 121     | 1204    | S. N. L  | 43, 57, 70    |

Aldehydes

|         |          |      |      |          |         |          |                |
|---------|----------|------|------|----------|---------|----------|                |
| 24.80   | Pentadecane | h1  | 12   | 150     | 1498    | 1512     | S. N. L      | 57, 43, 71, 85 |
| 29.48   | Heptadecane | h2  | 14   | 170     | 1699    | 1711     | S. N. L      | 57, 43, 71, 86 |
| 31.77   | Octadecane | h5   | 15   | 181     | 1799    | 1852     | S. N. L      | 57, 71, 85, 43 |

Hydrocarbons

|         |          |      |      |          |         |          |                |
|---------|----------|------|------|----------|---------|----------|                |
| 24.03   | Ethyl 2(E)-decenoate | e1 | 11   | 148     | 1758    | 1389     | N. L         | 43, 55, 73     |

Ester

|         |          |      |      |          |         |          |                |
|---------|----------|------|------|----------|---------|----------|                |
| 23.65   | (E)-Geranylacetone | k1 | 10   | 145     | 1420    | N. L     | 43, 41, 69    |

Ketone

|         |          |      |      |          |         |          |                |
|---------|----------|------|------|----------|---------|----------|                |
|         |          |      |      |          |         |          |                |

*RT: Retention time (min).

b Code: abbreviation of the compound.

c LRI Calc: Linear retention index calculated through n-alkanes.

d LRI Lit: Linear retention index reported in the NIST Chemistry Web Book 2005.
The reliability of the identification or structural proposal is indicated by the following: (S) mass spectrum and retention time consistent with those of an authentic standard; (N) structural proposals given on the basis of mass spectral data (NIST98); (L) mass spectrum consistent with spectra found in literature.

3.1. Volatiles

3.1.1. Terpenoids

Terpenoids were the largest group of larvae except at 5th instar, accounting for 83.3%, and 73.7% of total larval volatiles at 2nd and 4th instar separately, calculated as ng/mg. The major terpenoids of food volatiles were \( \alpha \)-cedrene (t5) and cedrol (t7), accounting for 65.5–89.2% of total terpenoids, while the major terpenoids of larval was \((E)\)-\(\beta\)-ocimene (t2), and accounting for 60.9–93.8%.

When terpenoid amount was calculated as ng/individual larva while larval weight increased (Figure 2), the amount of all terpenoids (Figure 3, column St), \((E)\)-\(\beta\)-ocimene (Figure 3, column t2) and \(\alpha\)-terpinol (Figure 3, column t4) continuously decreased. The amount of \((Z)\)-\(\beta\)-ocimene (Figure 3, column t1) and \((E, Z)\)-alloocimene (Figure 3, column t3) disappeared at 5th instar. The amount of \(\alpha\)-cedrene (Figure 3, column t5) and \(\beta\)-cedrene (Figure 3, column t6) just fluctuated without significant difference by instar. The amount of cedrol (Figure 3, column t7) increased sharply from 2nd to 4th instar, and remained stable from 4th to 5th instar.

Figure 2. The change of larva weight during larval development

When terpenoid amount (St, t1, t2, t3, t4, t5, t6, t7) released by larva was calculated as ng/mg (Figure 3), there was a decreasing trend during larval development. The 2nd instar larvae always had significantly higher contents than larvae at other instars. When terpenoid amount in larval food was calculated as ng/mg, the detectable terpenoid amount changed insignificantly among instars.

When comparing the content in larvae and food at the same stage as ng/mg, the larvae had significantly higher terpenoid content than food at the 2nd instar, except \(\alpha\)-cedrene, \(\beta\)-cedrene and cedrol. At the 2nd instar, the contents of \(\alpha\)-cedrene and \(\beta\)-cedrene were insignificant between larva and food; the content of cedrol was significantly lower in larvae than in food. At the 4th instar, the larvae still had significantly higher content of \((E)\)-\(\beta\)-ocimene than food; the content of \((Z)\)-\(\beta\)-ocimene was insignificant between larva and food. As for other terpenoid compounds, larvae had substantially lower contents than food after the 2nd instar.

3.1.1. Aldehydes
Aldehydes had higher concentrations in food (25.4–59.6%) than in larvae (7.9–22.3%) when calculated as ng/mg. Nonanal (a2) was the most abundant aldehyde in each sample, accounting for 65.3–93.3% of total aldehydes, followed by decanal (a3) and octanal (a1).

Octanal (Figure 3, column a1) was only detected in larvae at the 2nd instar, but was present in food at every stage. Nonanal (Figure 3, column a2) and decanal (Figure 3, column a3) was detected in both larvae and food at every stage. When calculated by ng/individual larva, the content of each aldehyde and the total content of all aldehydes (Figure 3, column Sa) only changed slightly and non-significantly (p > 0.05) with the sharp increase in larval body weight (Figure 2). However, when calculated as ng/mg, both measures were significantly higher in larvae at the 2nd instar than at the other stages. When comparing both measures in larvae and food at the same stage as ng/mg, there was no significant difference at the 2nd instar, while at other stages food contained higher contents than larvae (p < 0.01). Trends in aldehyde content in food were similar. There were fluctuations during larval development, but no significant differences occurred.

3.1.1. Hydrocarbons

The amounts of hydrocarbons to total volatiles content in food were the third highest after terpenoids and aldehydes, when calculated as ng/mg. Pentadecane (Figure 4, column h1) and heptadecane (Figure 4, column h2) were the hydrocarbons that accounted for the major amount (69.7–88.1%).
**Figure 3.** Terpenoids and aldehydes extracted from larvae and larval food at different larval instars. N>10 for each group; error bars represent standard errors, different letters and ‘*’ on top of bars denote significant difference at the level of 0.05, ‘**’ at the level of 0.01 under Fisher’s PLSD test, after ANOVA showed a significant effect. Bars sharing a superscript letter are not significantly different.
Figure 4. Hydrocarbons and other minor volatiles extracted from larvae and larval food at different larval instars. N> 10 for each group; error bars represent standard errors, different letters and '*' on top of bars denote significant difference at the level of 0.05, '**' at the level of 0.01 under Fisher’s PLSD test, after ANOVA showed a significant effect. Bars sharing a superscript letter are not significantly different.
The amounts of hydrocarbons to total volatiles content in food were the third highest after terpenoids and aldehydes, when calculated as ng/mg. Pentadecane (Figure 4, column h1) and heptadecane (Figure 4, column h2) were the hydrocarbons that accounted for the major amount (69.7–88.1%).

When calculated by ng/individual larva, the contents of hydrocarbons generally increased during larval development. The 2nd instar larva had the lowest content of each hydrocarbon and the lowest total content of hydrocarbons. However, the situation was reversed when calculating as ng/mg. The 2nd instar larva had significantly higher content than other instars larva, and there was insignificant among other instars. Food at the 2nd instar also had higher content than food at other instars, h2 significantly, h1 and h3 insignificantly. When comparing the content in larvae and food at the same stage by ng/mg, the differences were always significant, except at the 2nd instar. Larvae had substantially lower content than food.

3.1.1. Ester

Ethyl 2(E)-decenoate (Figure 4, column e1) was the only detected ester present before the 5th instar. This ester was significantly higher in 4th than 2nd instar larvae when calculated as ng/individual larva (p = 0.02). The trend was reversed when it was calculated as ng/mg. There were few changes in food of different instars. At the 2nd instar, the larvae had significantly higher content than food, but significantly lower content at the 4th instar.

3.1.1. Ketone

(E)-Geranylacetone (Figure 4, column k1) was the only ketone detected in the materials of this experiment. When calculated as ng/individual larva, the 5th instar larvae had significantly higher content than larvae at other instars, which had comparable levels. When calculated as ng/mg, the 2nd instar larvae had significantly higher content than larvae at other instars. Food of various instars contained comparable levels of (E)-geranylacetone. When comparing the content in larvae and food at the same stage as ng/mg, larvae had substantially higher contents than food at the 2nd instar, while the opposite was true at the other stages.

3.2. Principal component analysis

PCA was performed to reveal relationships between the samples (scores) and their volatile compounds (loadings) (Figure 5). A total of 96 samples and 15 compounds were used (Kaiser-Meyer-Olkin Measure of Sampling Adequacy: 0.731; Bartlett’s Test of Sphericity: P < 0.01). The majority of information is contained in the first two PCs and accounts for 62.2 % of the explained variation. A scatter plot of the PCA scores (Figure 5A) shows the distribution of samples, and the corresponding loading plot (Figure 5B) shows the volatiles in larvae and food at different development stages. Combining these results allows interpreting the relationships between the samples and their compounds.

The analyses show clear differences between the volatile profiles of larvae and the volatile profile of food. The two kinds of samples were separated by PC2. The majority of food samples had positive PC2 scores, attributable to the contribution of all aldehydes (a1, a2, a3), hydrocarbons (h1, h2, h3), two of the terpenoids (t5, t7). Meanwhile, all the larvae samples had negative PC2 scores, driven by most of the terpenoids (t1, t2, t3, t4, t6), one ester (e1), one ketone (k1).

Samples of the same type were distinguished by PC1 according to development stages. Both larvae and food samples generally shifted from positive to negative along the PC1 axis, from the 2nd to 5th instar. Samples of 2nd larvae located at the fourth quadrant were essentially characterized by the compounds distributed in that quadrant, especially terpenoids. Larvae at other instars were located in the third quadrant, implying that the volatiles of these stages were highly conservative and had relatively low levels. Part of the 2nd and 4th instars food located at the first quadrant was mainly characterized by the compounds distributed in that quadrant,
especially aldehydes and hydrocarbons. The rest of the food samples were located in the third quadrant, with low PC scores.

Figure 5. Positions of PC scores of samples including larvae and larval food at different larval instars (2nd: 2nd instar, 4th: 4th instar, and 5th: 5th instar). (A): Scores scatter plot of PCA. (B): loadings plot of PCA. The codes in (B) correspond to the compound codes listed in Table 1.
4. Discussion

Terpenoids have previously been reported as characteristic products of the Nasanov gland of worker bees, and included nerol, geraniol, \((E)\) and \((Z)\)-citral, nerolic acid, geranic acid and \((E, E)\)-farnesol [14]. In the present study, we found that additional terpenoids are the major constituents of worker bee larvae. Larva and food at the 2nd instar was demonstrated to have greater similarity of dry substance weight per unit volume, compared to other stages (data not shown). Assuming that larvae and food at the 2nd instar had similar densities, larvae would have released significantly higher amount of terpenoids than food, thus, these terpenoids, such as \((Z)\)-\(β\)-ocimene (t1), \((E)\)-\(β\)-ocimene (t2), \((E, Z)\)-alloocimene (t3) and \(α\)-terpineol (t4), were likely produced by the larvae. Some terpene synthases have been identified for larvae [9]. Terpenoid concentration could be diluted in old larvae because body fluid accounts for most of their weight. Under these conditions, terpenoid contents in larvae were still significantly greater than in food, suggesting that compounds such as t1, t2 and t3 could be identified as larval volatiles.

\(t1, t2\) and \(t3\) were once reported as the components of Tagetes minuta essential oil, which is highly lethal to \(V. destructor\) [15]. \(T4\) was demonstrated to have a repellent effect on the mite in a laboratory assay, and may therefore repress its entry into the brood cells of hives [16]. These terpenoids might be noteworthy in \(V. destructor\) control. Cedrol is a sesquiterpene with tertiary alcohol functionality. This alcohol could easily eliminate to give mixtures of \(α\)- and \(β\)-cedrene. These sesquerpene alcohols might be gathered by bees from gum and pollen of Cedrus deodara.

All aldehydes detected in this study have been previously reported as the volatiles of hives. The aldehydes are emitted by adult worker bees [17] and virgin queens [18], present in enclosed brood combs containing active larvae and attending workers [1]. Our results found that aldehyde contents per unit weight were higher in larval food than in larvae. This supports the assumption that aldehydes are secreted by worker bees, because larval food is a material that is manipulated by worker bees, and is consistent with the findings of Torto et al. [19] who reported these aldehydes in pollen. The result also explains why aldehyde contents in food did not show significant fluctuations during larval development. Aldehyde contents in larvae also remained steady, regardless of larval growth, except that \(α1\) disappeared after the 2nd instar.

Hydrocarbons have transpiration-reducing functions in arthropods, including linear and branched, saturated and unsaturated hydrocarbons, with different numbers of carbon atoms ranging from 15 to 35 [20]. Some of these compounds function in nestmate recognition and social acceptance [21]. Pentadecane has been reported as a larval volatile [22] and was also detected in nurse bees, coupled with heptadecane [23]. Octadecane is found in comb wax, with worker bees treated with this compound becoming less acceptable to their untreated nestmates [24]. If we assume cuticular hydrocarbons have an even and constant distribution on the surface of larvae, hydrocarbon content should increase with larval volume. A previous report has indicated that the level of tricosane and pentacosane would increase with drone larval development [25]. In the present study, hydrocarbon content increased at different degrees in individual larva during larval development. To some extent, this provides another support for the hypothesis. In food, hydrocarbon content showed different degrees of decrease in per unit weight. This suggests that the production of hydrocarbons in food is independent from that in larvae. In other words, hydrocarbons in food are affected by the food provider, while hydrocarbons in larvae are affected by the larvae themselves.

Ethyl 2(\(E\))-decanoate is tentatively identified in honeybees for the first time in the present study. Aliphatic ester is another group released by honeybee which is ubiquitous in colonies. Decyl decanoate is secreted by virgin queens from the tergal gland; ethyl oleate is produced by forager bees, suppressing the onset of foraging among younger bees; and ethyl and methyl esters of palmitic, linoleic, linolenic, stearic, and oleic acids are brood pheromones mediating the
communication between brood and worker bees [14]. Ethyl 2(E)-decenoate might be a product of incomplete beta oxidation of longer fatty acids.

(E)-Geranylacetone has not been previously identified as a honeybee volatile. Concentrations of this compound in larvae did not show obvious changes with the growth of individual larva, and only displayed a dramatic increase during the capping stage (the 5th instar). Neither did the significant differences of the contents were found in food at different stages. This may suggest that the compound is released by larvae for a specific role in this particular period.

When tracing the roots of the volatiles of worker larvae and larval food, the factor of the adult worker bee also needs to be considered. Additionally, constancy of volatile components in samples from different localities has not yet been established. Finally, only a single extraction method was applied in this study, and use of more advanced extraction or detection methods for worker larva volatiles might yield additional compounds. The present study should provide some basis for further research into the molecular mechanism of the volatiles along these lines, and for verification of the role of the identified components

5. Conclusions

Our results show that more volatiles could be identified from honeybee worker larvae and their food, in addition to (E)-β-ocimene. We provide evidence that these volatiles change and have a set of rules during larvae development.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: Linearity of response for standards. Calibration fitting: \( y = kx + m \).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range</th>
<th>Slope Intercept</th>
<th>Regression</th>
<th>LO D(^{e})</th>
<th>LO Q(^{d})</th>
<th>Codes(^{e}) of the compounds calculated by this curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldehyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octanal</td>
<td>0.004-4</td>
<td>0.047±0.00</td>
<td>0.01±0.008</td>
<td>0.9998</td>
<td>0.00</td>
<td>0.008, 0.004</td>
</tr>
<tr>
<td>Decanal</td>
<td>0.0041-2</td>
<td>0.096±0.00</td>
<td>0.007±0.00</td>
<td>0.9974</td>
<td>0.04</td>
<td>0.008, 0.004</td>
</tr>
<tr>
<td>Ester</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl tridecanoate</td>
<td>0.0044-1</td>
<td>0.168±0.00</td>
<td>0.019±0.004</td>
<td>0.9994</td>
<td>0.048</td>
<td>0.008, 0.004</td>
</tr>
<tr>
<td>Hydrocarbon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetradecane</td>
<td>0.0017-2</td>
<td>0.097±0.00</td>
<td>0.0063±0.00</td>
<td>0.9997</td>
<td>0.039</td>
<td>0.008, 0.004</td>
</tr>
<tr>
<td>Ketone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Methyl-5-hepten-2-one</td>
<td>0.0038-2</td>
<td>0.277±0.00</td>
<td>0.08±0.0015</td>
<td>0.9992</td>
<td>0.036</td>
<td>0.008, 0.004</td>
</tr>
<tr>
<td>Terpenoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-β-Ocimene</td>
<td>0.005-5</td>
<td>0.282±0.00</td>
<td>0.080±0.008</td>
<td>0.9993</td>
<td>0.102</td>
<td>0.008, 0.004</td>
</tr>
</tbody>
</table>
In the regression equation \( y = kx + m \), \( y \) refers to the peak area ratio of target compound to internal standard, \( x \) is the concentration ratio of target compound to internal standard, \( r^2 \) is the correlation coefficient of the equation.

Standard deviation is abbreviated as SD.

Limit of detection, \( S/N = 3 \).

Limit of quantitation, \( S/N = 10 \).

The codes correspond to the volatile codes listed in Table 1.

Author Contributions: H.Z., C.H. and Q.D. conceived and designed the experiments; H.Z. performed the experiments; H.Z. analyzed the data; Y.L., Y.W., Y.P. and Q.D. contributed reagents, materials, and analysis tools; H.Z. and C.H. wrote the manuscript. Y.L., P.D. and Q.D revised it.

Funding: This research was funded by the Agricultural Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2017-IAR).

Conflicts of Interest: The authors declare no conflict of interest.

References


