Aphid-Ant Relationships: The Role of Cuticular Hydrocarbons and Different Chemical Stimuli in Triggering Mutualistic Behavior

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Abstract: In ant-aphid interactions, various aphid species offer honeydew to the ant partner and increase their density by ant attendance, whilst others never attend ant species, in which case ants tend to treat them as prey. In this regard, ants should have the ability to distinguish myrmecophile aphid species from non-mutualistic species, so that mutualistic aphids will be accepted as partners rather than prey. Although ant-aphid interactions are now the focus of chemical ecology studies, the role of the different chemical stimuli in determining mutualistic interactions has not been completely clarified. Therefore, we have investigated the cuticular hydrocarbon profile of four myrmecophiles aphid species using GC-MS. We also investigated the behavior of the worker-ants (Lasius niger L., Hymenoptera: Formicidae) to different chemical stimuli derived from aphids. We applied four treatments and found that the behavior of the ant workers varied depending on the source of the different treatments. In particular, the real aphid Aphis pomi and the sugar solution proved to be the most attractive to the ants, while the presence of pure extract of the individuals is enough to disturb the behavior of the ants. We provide evidence that the key stimuli of the tending behavior could be the CHC patterns of the aphids and the CHC profile of the aphids tends to be genus specific. This research will promote further investigations to test the behavior of ant workers towards other species of aphids and treatment combinations.

Keywords: cuticular hydrocarbons; chemical communication; chemical stimuli; ant-aphid interactions

1. Introduction

The relationship between aphids and ants is a mutualistic one. Mutualism is a common occurrence in the natural world, referring to interactions between diverse organisms that are mutually advantageous. In interspecific mutualisms, there are inherent conflicts of interest among the participating organisms. The interaction between ants and aphids is a well-known mutual model in which ants protect aphids from potential natural enemies such as predators and parasites while aphids in return provide honeydew to ants as a nutrient [1–18]. Ants employ various behavioral strategies when interacting with aphids, ranging from solitary foraging to specialized roles within organized work teams, each varying in complexity [19,20]. Aphid species are classified into two groups depending on
their relationship to ants: myrmecophile and non-myrmecophile species. Myrmecophilous aphid species may emit a chemical signal that ants like Lasius niger L., Hymenoptera: Formicidae can detect and use to distinguish between aphids that are potential sources of food (trophobionts) and those that are potential prey. The behavior of ants towards aphids can be of two types; attending behavior (touching behavior only) or predatory behavior [21]. Ant attendance can also have a negative effect on aphid colonies [15,22,23]. Depending on the ants’ available diet options [24], the mutualistic relationship might also turn into predation. Salazar et al. [25] reported a unique mutualistic interaction combined with aggressive mimicry occurring between the aphid species Paracletus cimiciformis and ants Tetramorium semilaeve, in which flat morphs of P. cimiciformis aphids imitate the cuticular hydrocarbons (CHCs) of ant larvae, gaining access to the host’s resources. This strategy likely evolved from a preexisting trophobiotic relationship between the aphids and ants, whereas the round morph of P. cimiciformis aphids exhibits behaviors typical of a trophobiotic relationship with ants T. semilaeve. In general, ants favor species that give large amounts of honeydew and that produce high quantities of amino acids with or without di- and tri-saccharides in it [26,27]. Attending and guarding large number of aphids is costly for individual ants [15,28], therefore it is adaptable for them to prey on the extra aphids as a protein source instead of attending them to acquire honeydew. Lang and Menzel [26] suggested that n-alkanes are the most likely candidates to be recognition signals because they are the main ingredient in aphid’s cuticular hydrocarbons (CHCs), and their comparative quantity varies between mutualistic and non-mutualistic aphid species. In contrast, hydrocarbons with more complex constructions are more easily detectable by ants when they are associated with sugar solutions [29]. Van Wilgenburg et al. [29] suggested also, that L. niger uses the cuticular hydrocarbon patterns of aphids to distinguish between different species. Quantitative differences in cuticular hydrocarbons are believed to have a significant role in nestmate recognition processes in ants. Examinations of the functional subcaste distinction in the ant species Camponotus vagus, assumed that the ants can differentiate foragers and brood-tenders by means of quantitative differences in their hydrocarbon profiles [30]. Therefore, in our study we began an optimization process of the CHC extraction and detection methods to gain more peaks on GC-MS (GC-MS refers to gas chromatography-mass spectrometry, which is a powerful analytical technique used in chemistry to separate and identify components within a sample mixture) which in turn can lead to determination of further components that might still be undetected.

The aim of this work was to identify the cuticular hydrocarbon patterns of the four aphid species that are the most notorious species in Central Europe causing damages to apple orchards. In particular, the aphids Dysaphis plantaginea, Dysaphis devecta, Aphis spiraecola, and different developmental stages of Aphis pomi feed on apple trees. Additionally, we examine the behavior of the ant species Lasius niger against different chemical stimuli, such as: aphid cuticular hydrocarbons extracted from aphids, sugar solutions. Our results will help to reveal the factors that determine the development and maintenance of the tending behavior of ants.

2. Materials and Methods

2.1. Plant Material

Apple trees (Malus domestica ‘Idared’) were grown from two year old seedlings in plastic pots in a climate-controlled room (24 ± 2 °C, 16 L: 8D) and were irrigated regularly, three times a week.

2.2. Study Organisms

Ant (L. niger) queens were collected from the Botanical Garden of the Hungarian University of Agriculture and Life Sciences (former SZIU) in Budapest (longitude N 47.480867 and latitude E 19.038809). The queen ant was reared in a climate-controlled room (24 ± 2 °C, 16 L: 8D). The same ant colony was used for all experiments: it had approximately 50 workers and the same number of offspring. The colonies were reared on
30 m/V% sucrose solution (as the carbohydrate source), and larvae of *Bombyx mori* (as a protein source). Ant colonies were kept in glass tubes (16 mm diameter, 150 mm long) in a dark carton case (240 × 175 mm, 100 mm high).

Aphid individuals for the production of clean, monoclonal strains of the four species (*Aphis. pomi*, *A. spiraecola*, *D. devecta* and *D. plantaginea*) were collected from the Research Farm of the Hungarian University of Agriculture and Life Sciences in Soroksár (longitude N 47.398575 and latitude E 19.149264). Aphids were reared on apple trees *Malus domestica* ‘Idared’ (pot dimensions; 25 mm diam., 20 mm deep). Aphids were kept in a climate-controlled room (24 ± 2 °C, 16 L: 8D). As predators and parasitoids might be present in the laboratory, the apple trees were enclosed in sticky barriers and insect exclusion nets.

2.3. Detection of the Chemical Composition of the Cuticular Extract of the Aphid Species by GC-MS

As the next step, we started an optimization process for the CHC extraction and detection methods, trialing different extraction periods to determine the optimal extraction period, in order to obtain the highest possible concentration of the CHC components from the aphids. The optimal duration of the CHC extraction from aphids is 30 min, and the optimal temperature is 70 °C (under elevated pressure). The optimal solvent/aphid individual ratio is 200 µL hexane to 25 aphid individuals. 25 aphid individuals (different species and developmental stages) were collected in clean GC-MS vials with a fine and clean paintbrush, then killed and kept in a freezer at −20 °C for five minutes. We added markings to the vials to identify the samples after the extraction. For the total CHC extraction we added 200 µL n-hexane (analytically pure) to the samples by micropipette. The micropipette tips were changed after every sample to prevent contamination from each other. We shook the vials for 1 min with a high-frequency mini shaker (IKA vortex). The extract (200 µL) was transferred by micropipette with clean micropipette tips to a GC vial equipped with a micro vial insert. The chemical analysis of the samples was conducted by GC-MS using an Agilent 6890N GC (Agilent Technologies, Santa Clara, CA, USA) with the 5973i MSD device. The GC-MS process had the following parameters: Oven temperature, 190 °C for 0.75 min (start temperature), then heating up with 8 °C/min to 320 °C and kept on the final temperature for 8 min. The GC device was equipped with an HP-1MS capillary column (DB-5ms 30 m × 250 µm × 0.25 µm: Cat. No.: J&W 122-5532). The volume of injection was 1 µL. The carrier gas was H2 at a constant flow of 2 mL/min. A split/splitless injector was set to splitless mode on 300 °C, with 10:1 split ratio. The solvent delay was three minutes. In the selected ion monitoring (SIM) acquisition mode, the selected ions were 55; 57; 71; 85. For data acquisition, analysis, and evaluation we used Agilent MSD ChemStation E.02 software. The molecular formula for the four standards of cuticle hydrocarbon components: pentacosane C25H52 (n-C25); heptacosane C27H56 (n-C27); nonacosane C29H60 (n-C29); hentriacontane C31H64 (n-C31). The GC-MS was calibrated based on the different concentration standard mixture solutions, and the retention times of the four standards (nC25 (pentacosane); nC27 (heptacosane); nC29 (nonacosane); nC31 (hentriacontane) [Figure S1 in Supplementary Materials (SM)]. The GC-MS detectable hydrocarbon components (comprising 16 peaks) of the cuticle extract of the four aphid species, were initially distinguished based on their mass spectra and retention index values [Figures 1–4; Tables 1 and 2]. The splitless injection method of GC showed significantly greater peak areas and lower background noise level, than the split method. The detecting method FID (flame ionization detector) showed more separated peaks and lower background noise compared to MS (mass spectroscopy).
Figure 1. The different components of the cuticle hydrocarbon profile of the adult stage of *A. pomi* (non-winged form). The different capital letters above peaks indicate to different compounds.

Figure 2. The different components of the cuticle hydrocarbon profile of the adult stage of *A. spiraeola* (non-winged form). The different capital letters above peaks indicate to different compounds.
Figure 3. The different components of the cuticle hydrocarbon profile of the adult stage of *D. devecta* (non-winged form). The different capital letters above peaks indicate to different compounds.

Figure 4. The different components of the cuticle hydrocarbon profile of the adult stage of *D. plantaginea* (non-winged form). The different capital letters above peaks indicate to different compounds.
Table 1. The amount (ng/individual) of the cuticular hydrocarbon standards (nC25, nC27, nC29, nC31) detected from the cuticular extracts of the four aphid species and the different developmental stages of *A. pomi* (nC31: a peak with close retention time to the standard).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ng/Individual Aphid</th>
<th>Ng/Individual Aphid</th>
<th>Ng/Individual Aphid</th>
<th>Ng/Individual Aphid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pentacosane nC25</td>
<td>heptacosane nC27</td>
<td>nonacosane nC29</td>
<td>hentriacontane nC31</td>
</tr>
<tr>
<td><em>Aphis pomi</em> 1_2.Nymphys</td>
<td>10.03</td>
<td>10.07</td>
<td>nd</td>
<td>24.00</td>
</tr>
<tr>
<td><em>A. pomi</em> 3_4.Nymphys</td>
<td>15.2</td>
<td>16.03</td>
<td>25.1</td>
<td>29.02</td>
</tr>
<tr>
<td><em>A. pomi</em> Adult (winged form)</td>
<td>13.08</td>
<td>25.2</td>
<td>28.04</td>
<td>73.2</td>
</tr>
<tr>
<td><em>A. pomi</em> Adult (non-winged form)</td>
<td>25.01</td>
<td>41.2</td>
<td>29.06</td>
<td>235.01</td>
</tr>
<tr>
<td><em>A. pomi</em> Skin</td>
<td>7.00</td>
<td>18.02</td>
<td>27.02</td>
<td>44.05</td>
</tr>
<tr>
<td><em>Aphis spiraecola</em> Adult (non-winged form)</td>
<td>12.01</td>
<td>39.07</td>
<td>35.05</td>
<td>119.3</td>
</tr>
<tr>
<td><em>Dysaphis devecta</em> Adult (non-winged form)</td>
<td>24.03</td>
<td>38.07</td>
<td>1735.1</td>
<td>24.2</td>
</tr>
<tr>
<td><em>Dysaphis plantaginea</em> Adult (non-winged form)</td>
<td>22.02</td>
<td>69.04</td>
<td>1336.03</td>
<td>58.09</td>
</tr>
</tbody>
</table>

Table 2. The amount (ng/individual) of the unidentified cuticular hydrocarbon peaks detected from the cuticular extract of the four aphid species and the different developmental stages of *A. pomi* (nd: not detectable).

<table>
<thead>
<tr>
<th>Retention Time/Min</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aphis pomi</em> 1_2.Nymphys</td>
<td>658.02</td>
<td>135.01</td>
<td>65.04</td>
<td>189.1</td>
<td>304.07</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>A. pomi</em> 3_4.Nymphys</td>
<td>1027.06</td>
<td>269.04</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>A. pomi</em> Adult (winged form)</td>
<td>2783.08</td>
<td>644.04</td>
<td>0.05</td>
<td>160.02</td>
<td>202.01</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>A. pomi</em> Adult (non-winged form)</td>
<td>6956.03</td>
<td>2673.1</td>
<td>146.00</td>
<td>2060.04</td>
<td>4704.04</td>
<td>260.08</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>A. pomi</em> Skin</td>
<td>1460.02</td>
<td>313.01</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>A. spiraecola</em> Adult (non-winged form)</td>
<td>11,577.2</td>
<td>1736.04</td>
<td>nd</td>
<td>300.01</td>
<td>7180.01</td>
<td>nd</td>
<td>242.04</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>840</td>
</tr>
<tr>
<td><em>Dysaphis devecta</em> Adult (non-winged form)</td>
<td>1313.06</td>
<td>nd</td>
<td>35,559.02</td>
<td>3083.1</td>
<td>448.07</td>
<td>nd</td>
<td>49,798.2</td>
<td>1318.3</td>
<td>596.1</td>
<td>729.2</td>
<td>1031.05</td>
<td>5839.02</td>
</tr>
<tr>
<td><em>D. plantaginea</em> Adult (non-winged form)</td>
<td>2357.05</td>
<td>nd</td>
<td>7875.02</td>
<td>182.1</td>
<td>nd</td>
<td>nd</td>
<td>39,768.02</td>
<td>143.02</td>
<td>857.01</td>
<td>934.1</td>
<td>1186.04</td>
<td></td>
</tr>
</tbody>
</table>

2.4. Behavioral Tests

Ant responses were recorded to different chemical stimuli in maze tests. The goal area of the tube was connected to the ant colony with a plastic tube (6 cm long, 5 mm in diameter). A cotton bud was used to prevent the ant escaping from the tube. The goal area was a one-centimeter-long, six-mm-diameter plastic tube closed with a small cotton wool plug. We carried out the treatments (chemical stimuli) on 3 mm × 3 mm cleaned filter paper pieces on the base of the goal area (6 cm long, 5 mm in diameter), ensuring that the base of the goal arena was clean after every observation to avoid contamination. The total duration of observation by video camera was two hours (120 min). We placed the maze in a closed room and fixed a high-speed camera above it. The sequence of the treatments was randomized. We started the different treatments at the same time on consecutive days, therefore the ant colony had a 22 h rest period between the observations. In total,
we performed three replicas of each treatment. The evaluation of the videotapes was performed manually. We distinguished three different types of the behavior of the ants: Touching; Around; Away [Figures S2–S4 in Supplementary Materials (SM)].

The treatments in the maze were as follows: (1) alive *A. pomi* aphids fixed to the arena (three individuals); (2) individuals (the solvent was n-hexane) adsorbed on filter paper; (3) 30m/V% sugar solution (imitating the aphid-extracted honeydew) adsorbed on filter paper; (4) untreated control (clean filter paper). The following protocols were used to obtain the full cuticular hydrocarbons (CHCs) of the aphids: First we washed the paintbrush with clean n-hexane then put 20 adults of *A. pomi* into a 1.5 mL clean Eppendorf tube, they were then killed through storing them in a freezer (−20 °C, for five minutes). We added 800 µL water to the tube and shook it for one minute (to remove honeydew remains), then we transferred the aphids to a new clean Eppendorf tube and threw away the previous one, and then added 100 µL n-hexane to the tube which contained the cleared aphids and shook the tube for one minute, then removed and discarded the aphids. Finally, we adsorbed the extracted CHC components to the clean filter paper quarters.

2.5. Statistical Analysis

We sent our observations to an excel database, second by second. We distinguished the beginning and the ending of every kind of behavioral state as well as the duration. For deeper analysis, we divided the total duration of observation (120 min) into four 30-min-long quarters to detect the potential behavioral differences within the longer (120 min) period. For the statistical analysis of the data, we used robust mixed two-way ANOVA: Welch’s test with Games-Howell pairwise comparison to compare the effect of the different treatments (touching, turning around, moving away), and the Geisser-Greenhouse test with Games-Howell pairwise comparisons to compare the effect of the different quarters within the observation period and to analyze the interaction of the two explanatory variables.

3. Results

Three of the four standards (pentacosane (n-C25), heptacosane (n-C27), nonacosane (n-C29), and hentriacontane (n-C31)) were detectable from the cuticle of the four of aphid species of non-winged form (*A. pomi*, *A. spiraecola*, *D. devecta*, *D. plantaginea*), and from the different developmental stages of *A. pomi* including 1-2 nymphs, 3-4. nymphs, winged adults, non-winged adults, and the skin), whilst in the case of n-C31 we found a detectable peak with close retention time to the standard, which was chemically not identical with the standard [Table 1].

Above the four hydrocarbon standards, we could detect 12 more hydrocarbon peaks from the cuticle extract of the four aphid species [Figures 1–4; Table 2]. Some of the peaks appeared for all of the aphid species [Table 2; *Aphis pomi*, *A. spiraecola*, *Dysaphis devecta*, *D. plantaginea*: A,D], while some of them seemed to be genera-specific [Table 2; specific for *Aphis* spp.: A,B,D,E; specific for *Dysaphis* spp.: A,C,D,G,H,J,K,L]. Within the different developmental stages of *A. pomi* we could detect no qualitative differences, but did find quantitative differences [Tables 1 and 2]. On the other hand, qualitative and quantitative differences appeared between the different genera [Figures 1–4; Tables 1 and 2]: the cuticular hydrocarbon profile of the two species from the genus *Aphis* (*A.pomi* and *A. spiraecola*) were very similar to each other, but differed significantly from the profile of the two *Dysaphis* species (*D. plantaginea* and *D. devecta*), suggesting that the CHC patterns of the aphids are genera-specific.

3.1. Behavioral Assays

Behavioral State ‘Touching’

The different chemical stimuli treatments (real, alive aphid *A. pomi*. Adult individuals (non-winged form) fixed in the goal arena, extract of the cuticular hydrocarbon profile of *A. pomi*, (30m/V% sucrose solution and untreated control) affected the activity of the *Lasius niger* ant workers differentially. Significantly different activity occurred for the different
chemical stimuli, as indicated by the touching period of the ant workers for the different treatments [Table 3; Welch-corrected df = (3; 4); \( f = 7854; p = 0.0383 \)]. The activity of ants had the highest level towards dummies treated with sucrose solution and real aphids, nevertheless they did not differ significantly from the extract and untreated control. The only significant difference appeared between the untreated control and the extract, thus the total CHC extract of *A. pomi* can influence the ant behavior significantly. There were no significant differences in the ant activity between the different quarters within the observation period (two hours) [Table 3; Geisser-Greenhouse test df (3.0; 24.0); \( f = 0.923; p = 0.4447 \)].

**Table 3.** Mean period in seconds (standard deviation) of behavioral state ‘Touching’ toward different chemical stimuli in the four-time quarters.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Size</th>
<th>Unit</th>
<th>Quarter 1</th>
<th>Quarter 2</th>
<th>Quarter 3</th>
<th>Quarter 4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 Sec</td>
<td>33 (57)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>45 (77)</td>
<td>19.33 (A)</td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>3 Sec</td>
<td>348 (232)</td>
<td>173 (134)</td>
<td>34 (58)</td>
<td>16 (26)</td>
<td>142.67 (B)</td>
<td></td>
</tr>
<tr>
<td>Aphid (<em>Aphis pomi</em> Adult_non-winged form)</td>
<td>3 Sec</td>
<td>751 (603)</td>
<td>1300 (1133)</td>
<td>1322 (1174)</td>
<td>1026 (1393)</td>
<td>1100.1 (AB)</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>3 Sec</td>
<td>785 (744)</td>
<td>2267 (2906)</td>
<td>1115 (1253)</td>
<td>1243 (1111)</td>
<td>1352.9 (AB)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>Sec</td>
<td>479.42 (A)</td>
<td>935.25 (A)</td>
<td>617.83 (A)</td>
<td>582.5 (A)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different capital letters between treatments in the table indicate significant differences (\( p < 0.05 \)) between treatments; The same capital letters between quarters in the table indicate no significant differences (\( p > 0.05 \)) between quarters.

### 3.2. Behavioral State ‘Around’

In the case of the behavioral state ‘Around’ (when the ant worker is in the arena, but it is not touching the stimuli) there was no significant difference in the ant activity against different chemical stimuli [Table 4; Welch-corrected df = (3; 3.3); \( f = 1800; p = 0.3072 \)]. There was no significant difference between the ant activity towards the different quarters within the observation period (two hours) [Table 4; Geisser-Greenhouse test df (3.0; 24.0); \( f = 0.175; p = 0.9125 \)].

**Table 4.** Mean period in seconds (standard deviation) of behavioral state ‘Around’ toward different chemical stimuli in the four-time quarters.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Size</th>
<th>Unit</th>
<th>Quarter 1</th>
<th>Quarter 2</th>
<th>Quarter 3</th>
<th>Quarter 4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 Sec</td>
<td>4.667 (8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>18 (32)</td>
<td>5.75 (A)</td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>3 Sec</td>
<td>250 (251)</td>
<td>219 (158)</td>
<td>49 (85)</td>
<td>41 (67)</td>
<td>140 (A)</td>
<td></td>
</tr>
<tr>
<td>Aphid (<em>Aphis pomi</em> Adult_non-winged form)</td>
<td>3 Sec</td>
<td>3673 (4731)</td>
<td>3600 (4340)</td>
<td>1808 (1529)</td>
<td>2561 (2711)</td>
<td>2910.8 (A)</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>3 Sec</td>
<td>144 (221)</td>
<td>451 (391)</td>
<td>1368 (2147)</td>
<td>1312 (1600)</td>
<td>819.17 (A)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>Sec</td>
<td>1018.2 (A)</td>
<td>1067.2 (A)</td>
<td>806.58 (A)</td>
<td>983.25 (A)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The same capital letters between treatments in the table indicate no significant differences (\( p > 0.05 \)) between treatments. The same capital letters between quarters in the table indicate no significant differences (\( p > 0.05 \)) between quarters.

### 3.3. Behavior State ‘Away’

For the behavioral state ‘Away’ (the ant not in the arena) no significant difference was found in the ant activity against the different chemical stimuli [Table 5; Welch-corrected df = (3; 4.4); \( f = 0.681; p = 0.6050 \)]. However, the ant activity was significantly different during the observation period (two hours): in quarters three and four ants spent significantly longer time away from the goal arena compared to the first quarter, with the second
quarter in between the first and third periods [Table 5; Geisser-Greenhouse test df (3.0; 24.0) \( f = 4682 \) \( p = 0.0103 \)].

Table 5. Mean period in seconds (standard deviation) of the behavioral state ‘Away’ toward different chemical stimuli in the four time quarters.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Size</th>
<th>Unit</th>
<th>Quarter 1</th>
<th>Quarter 2</th>
<th>Quarter 3</th>
<th>Quarter 4</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>Sec</td>
<td>2072 (3589)</td>
<td>0 (0)</td>
<td>2072 (3589)</td>
<td>2279 (3947)</td>
<td>1605.9 (A)</td>
</tr>
<tr>
<td>Extract</td>
<td>3</td>
<td>Sec</td>
<td>2966 (3033)</td>
<td>4899 (4532)</td>
<td>2542 (2794)</td>
<td>5543 (4139)</td>
<td>3987.8 (A)</td>
</tr>
<tr>
<td>Aphid (Aphis pomi Adult_non-winged form)</td>
<td>3</td>
<td>Sec</td>
<td>3548 (5545)</td>
<td>2946 (4345)</td>
<td>3965 (4619)</td>
<td>3278 (2399)</td>
<td>3434.8 (A)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3</td>
<td>Sec</td>
<td>925 (1550)</td>
<td>3691 (1244)</td>
<td>8651 (4201)</td>
<td>7152 (39610)</td>
<td>5105.3 (A)</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>2378.3 (A)</td>
<td>2884.3 (AB)</td>
<td>4307.8 (B)</td>
<td>4563.4 (B)</td>
<td></td>
</tr>
</tbody>
</table>

The same capital letters between treatments in the table indicate no significant differences (\( p > 0.05 \)) between quarters; Different capital letters between quarters in the table indicate significant differences (\( p < 0.05 \)) between quarters.

4. Discussion

Our objectives were to determine the CHC patterns of the four aphid species and the different developmental stages of \textit{Aphis pomi}, and to examine the behavior of ants towards different chemical stimuli that could help us understand more deeply the nature of ant-aphid mutualism in an economically important group of pests. Our results indicate that the CHC profile of aphids tend to be genus specific, and show that the two tested aphid genera (\textit{Aphis} and \textit{Dysaphis}) have a different level of ant attendance (Csaba Nagy, personal observation). We concluded that ant attendance might be partly driven by the quality and quantity of the CHC components of the different aphid species or genera, and the behavior of ant workers (\textit{Lasius niger}) varied depending on the chemical stimuli derived from the aphids. The real aphid and sugar solution were the most attractive to the ants, while the presence of pure extract of the individuals disturbed their behavior. Ant attendance influences the diversity and composition of bacterial symbionts of the aphid genus \textit{Dysaphis} Börner. Aphids attended by ants have fewer but more diverse bacterial symbionts compared to unattended aphids [31]. Ants should have the ability to recognize mutualistic aphid species from not mutualistic species, and accordingly mutualistic aphids have to be recognized as partners instead of prey. The capability of aphids to identify the presence of ants decisively was shown by the repetition of excretion behavior, which can notably increase under ant attendance [32]. Cherix [33] and Yao and Akimoto [32] found that four species of aphids were attended by \textit{Formica yessensis} on the Ishikari Coast. The nourishment of \textit{F. yessensis} depended essentially on aphid honeydew instead of protein sources, meaning that these aphid species might compete for ant attendance [33]. Notably, Danielsson [34] showed that \textit{A. fabae} is a better ant-mutualist partner than \textit{D. plantaginea}, and artificial introduction of this aphid species into apple orchards with ground cover vegetation might increase the impact of biological control by natural enemies of \textit{D. plantaginea} colonies.

The cuticular hydrocarbon profile of the two species from the genus \textit{Aphis} was very similar but differed significantly from the profile of the two \textit{Dysaphis} species, which also matched each other. In the CHC profile of the four species, we found 16 detectable peaks, where two coincided with the normal alkane standards (nC25 and nC27). On the other hand, simply showing the resemblances among species in their cuticular hydrocarbons is not sufficient to prove that the cuticular hydrocarbons operate as a disguise or that they describe species- or colony-specific recognition signals [35]. Due to the importance of the different compounds, we were able to detect three (pentacosane nC25, heptacosane nC27, nonacosane nC29) of the four examined standards in the CHC profile of the four examined aphid species. More investigations are necessary to identify the other 12 detectable chemical components and their biological roles. For hentriacontane (nC31), we could detect a peak with close retention time to the standard, but it has a chemically different structure.
Cuticular hydrocarbons might be gained by a myrmecophile aphid easily as a by-product of living in an ant colony but might not transmit any signal to the ant. Therefore, identification bioassays are necessary to prove that chemical mimicry or camouflage happens when the cuticular hydrocarbons of a myrmecophile aphid are similar to its host’s CHCs [35]. Our findings in the behavioral tests revealed that *L. niger* ants show the highest preference towards sucrose solution. The reason for the high ant activity can be based on the biological role of the sugars: ants use sugar solutions as a primary food source [24,36]. The second highest ant activity was detectable on real aphids, then on the total extract of the CHC patterns of *A. pomi*, while only a very weak action was detectable toward the untreated control. Additionally, the total duration of observation by video camera was two hours, and we found that the behavior of the ants did not differ significantly between the quarters (30 min long periods).

5. Conclusions

For chemical analysis, we investigated the total cuticular hydrocarbon (CHC) patterns of four aphid species feeding on apple (*A. pomi*, *A. spiraecola*, *D. devecta* and *D. planatginea*). We can state that the CHC patterns of the aphids are genera-specific. For behavioral tests, we can state that the sucrose solution and real aphids had remarkably higher effects on the ants’ behavior than the other two treatments (CHC extract of the *A. pomi* and untreated control). The CHC extract of the *A. pomi* had a significant effect to the behavior of the ants (compared to the untreated control), but it did not reach the level of the real aphids and the sugar solution. Our results suggest that the ant-aphid mutualisms are not based exclusively on cuticular hydrocarbons, we need further investigations to clarify the role and importance of the different factors on ant attendance of aphids such as CHC patterns and its concentration, honeydew, the shape of the different aphid species, behavior of the different aphid species, and different treatments (chemical stimuli).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14040529/s1, Figure S1: The GC-MS chromatograms (total ion counts) of the four standards (nC25, nC27, nC29, nC31). The arrows below the X axes indicate the retention times of odd-numbered n-alkanes for comparison; Figure S2: Behavioural state ‘Touching’ (Ant is in the arena and touching the stimuli chemical); Figure S3: Behavioural state ‘Around’ (Ant is in the arena and not touching the chemical stimuli); Figure S4: Behavioural state ‘Away’ (Ant is not in the arena). The online version contains supplementary material available.

Author Contributions: Conceptualization, A.E.-H. (Amged El-Harairy); methodology, A.E.-H. (Amged El-Harairy) and J.K.J.; formal analysis, A.E.-H. (Ahmed El-Harairy); investigation, A.E.-H. (Amged El-Harairy), J.K.J. and A.M.; data curation, A.E.-H. (Amged El-Harairy) and J.K.J.; writing—original draft preparation, A.E.-H. (Amged El-Harairy) and J.K.J.; writing—review and editing, A.E.-H. (Amged El-Harairy) and J.K.J. and A.M.; visualization, A.E.-H. (Amged El-Harairy); supervision, A.E.-H. (Amged El-Harairy); All authors have read and agreed to the published version of the manuscript.

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References


22. Offenberg, J.; Nielsen, J.S.; Damgaard, C. Wood ant (*Formica polyctena*) services and disservices in a danish apple plantation. *Sociobiology* 2019, 66, 247–256. [CrossRef]


26. Lang, C.; Menzel, F. *Lasius niger* ants discriminate aphids based on their cuticular hydrocarbons. *Anim. Behav.* 2011, 82, 1245–1254. [CrossRef]


31. Kaszyca-Taszakowska, N.; Depa, L. Microbiome of the Aphid Genus Dysaphis Borner (Hemiptera: Aphidinae) and Its Relation to Ant Attendance. *Insects* 2022, 13, 1089. [CrossRef]


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