Aphid-ant Relationships: The Role of Cuticular Hydrocarbons and Different Chemical Stimuli

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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 are never attended by ant species, in which case ants tend to treat them as prey. In this regard, ants should have the capability to distinguish myrmecophile aphid species from not mutualistic species, and consequently mutualistic aphids have to be accepted as partners rather than prey. Based on previous studies it is known that ants use the cuticular hydrocarbons (CHCs) pattern of aphids to distinguish mutualistic and non-mutualistic species, but it is unclear, whether the capability to recognize their partners from mutualistic aphid species is innate or depends on learning. Therefore We investigated the cuticular hydrocarbon profile of four myrmecophile aphid species (*Aphis pomi* , *Aphis spiraeicola* , *Dysaphis devecta* , *Dysaphis plantaginea*) using GC-MS. Then we investigated the behaviour of the ants workers (*Lasius niger* L., Hymenoptera: Formicidae) towards the different chemical stimuli deriving from the aphids. We applied four treatments: real *Aphis pomi* individuals; total extract of the CHC profile of *Aphis pomi*; sucrose solution, and untreated control. The behaviour of the ant workers was different towards the source between the treatments: the real aphid and the sugar solution proved to be the most attractive to the ants, and the presence of pure extract of the individuals is enough to disturb the behaviour of the ants. We provide evidence that the key stimuli of the tending behaviour could be the CHC patterns of the aphids and the CHC profile of aphids tend 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.

Introduction

The behaviour of ants towards aphids can be of two types; attending behaviour (touching behaviour only) and predatory behaviour(H. Sakata 1994) Ant-aphid interaction is a well-known model of mutualism: ants protect the aphids against potential natural enemies such as; predators and parasitoids while in return aphids provide honeydew to ants as a nutrient. (Addicott 1978; Banks 1962; Breton and Addicott 1992; Bronstein 1998; BRONSTEIN and BARBOSA 2002; Buckley 1987; Carver et al. 2003; Choe and Rust 2006; Dixon 1998; Floate and Whitham 1994; Hayashi et al. 2014; Hayashi, Nakamuta, and Nomura 2015; Hölldobler and Wilson 1990; Horstmann 1972; Ito and Higashi 1991; Johnson 2008; Kaplan and Eubanks 2005; Nielsen, Agrawal, and Hajek 2009; I. Sakata, Hayashi, and Nakamuta 2017; Schwartzberg et al. 2010; Skinner and Whittaker 1981; Bernhard Stadler and Dixon 2005; Styrsky and Eubanks 2006; W Völkl and Kroupa 1997; Way 1963; Whittaker and Warrington 1985; Xu and Chen 2021; Youngsteadt and Devries 2005), prevent potential competitors (Ando and Ohgushi 2008); An aphid's longevity was significantly correlated with the daily mean number of workers tending it and increase the fitness of the attended aphids through matured earlier, lived longer, a higher expected number of offspring than aphids not tended by ants (Ando and Ohgushi 2008), and had a higher rate of reproduction (Flatt and Weisser 2000). In contrast,(Billick et al. 2014) observed that aphid management by ants is considered to be a less specific relationship than fungus-farming by ants and relationship among ants and aphids extend from mutualistic to antagonistic, depending on aphid species and environmental

conditions. Ant attendance can also have a negative effect on aphid colonies (J. Offenberg, Nielsen, and Damgaard 2019; B Stadler and Dixon 1999; Bernhard Stadler and Dixon 2005). Depending on the ants' available diet options (Joachim Offenberg 2001), mutualism relationship might also turn over into predation. In general, ants favour to species that give large amounts of honeydew, and which produce high quantities of amino acids and/or di- and tri-saccharides in the composition of honeydew (Lang and Menzel 2011; Wolfgang Völkl et al. 1999). Attending and guarding large number of aphids is costly for individual ants (Endo and Itino 2012; Bernhard Stadler and Dixon 2005), therefore it is adaptable for them to prey on the extra aphids as a protein source instead of attending them to assemble honeydew. Differences in ant attendance can be a consequence of either the different amino acid composition of the honeydew (Lanza and Krauss 1984), or of the differences in frequency and volume of honeydew production (Fischer, M. K., & Shingleton 2001; Wolfgang Völkl et al. 1999) provided by the aphid partner. Seasonal changes can affect the concentration of sugars and amino acids of honeydew of aphids, consequently can affect the behavior of red wood ants (*Formica rufa*) (N. E.L. Madsen and Offenberg 2020). (Fischer et al. 2002) found that amino acids (especially asparagine and glutamine) are less important for ant resolutions than the honeydew quantity. (Glinwood, Willekens, and Pettersson 2003; H. Sakata 1994) noted that the behaviour of *Lasius niger* L. (Hymenoptera: Formicidae) might be changed from attending to predation when ants meet a rising proportion of aphids which have not been attended by the ants' colony before. There is a hypothesis that aphids perhaps favour the rapid growing plants, as their sap carries higher quantities of nitrogen and sugars. This can affect the quality and quantity of the honeydew-rewards excreted by the aphids to the ants, and therefore it can increase the degree of protection (number of ants) (Baylis and Pierce 1991; Hansen, Hattendorf, and Nentwig 2006; Messina 1981; Bernhard Stadler and Dixon 2005) .

The major sugar components existing in the honeydew of *Aphis fabae* are sucrose and fructose, showing the highest concentrations (14.3 and 8.1 g L⁻¹) as well as the highest molarities (approximately 0.04 M) among all the specified sugars (Detrain et al. 2010). In a maze test, time spent by ants in the foraging area after drinking the sugar solution (glucose, melezitose, raffinose and sucrose) was significantly lower, than after drinking pure water (Detrain et al. 2010). Outcomes of (Natalia E L Madsen, Sørensen, and Offenberg 2017) revealed that ants favoured disaccharides over monosaccharides, however the presence of trisaccharides also increased the attractiveness of the sucrose solutions. (Endo and Itino 2012; H. Sakata 1994) revealed that ants are able to mark the aphids they attend somehow to facilitate the decision to prey on or not. (H. Sakata 1994) observed that the ants were less aggressive towards previously marked aphids which provided honeydew earlier, compared to the aphids which have not attended.

Ants stopped attacking when the aphids provide honeydew. Recognition of potential partners mostly depends on chemical cues between connected living organisms, particularly insects that have developed extremely advanced ways to produce and reveal semiochemicals for interspecific and intraspecific interactions (Brezolin et al. 2018). (Lang and Menzel 2011) suggested that n-alkanes are the first nominees as recognition signals, because they are the main ingredient in aphid CHCs, and their comparative quantity varies between mutualistic and non-mutualistic aphid species. In contrast,

hydrocarbons with more complex constructions are more simply detectable for the ants when they are connected with sugar solution (van Wilgenburg et al. 2011). However, methylalkanes might also be the key stimuli. Nevertheless, there is no definite proof, as yet, to demonstrate which structural category of aphid CHCs might operate as partner identification signals for the ant partners (I. Sakata, Hayashi, and Nakamuta 2017). In addition, aphid species are classified into two groups based on their relationship with ants: myrmecophilous and nonmyrmecophilous species. Myrmecophilous aphid species might use a shared cuticular hydrocarbon signal with the partner ants, which can be used by *L. niger* to classify aphid individuals into probable trophobiont or probable prey groups. (van Wilgenburg et al. 2011) suggested also, that *L. niger* uses the cuticular hydrocarbon patterns of aphids to distinguish between different species. Cuticular hydrocarbons of aphids in general are formed mainly of n-alkanes, with fewer amounts of branched alkanes and n-alkenes. Therefore, it is suggested that ants might detect aphid colonies based on (E)-b-farnesene (aphid alarm pheromone), but thereafter they use their CHC pattern to recognise the trophobiotic species (van Wilgenburg et al. 2011).

The aim of this work was to identify the cuticular hydrocarbon patterns of the four aphid species which are the most famous species in Central Europe that causes damages to apple orchards (*Aphis pomi*, *Aphis spiraecola*, *Dysaphis plantaginea*, *Dysaphis devector*) feeding on apple; and to examine the behaviour of *L. niger* against different chemical stimuli, such as; aphid, cuticular hydrocarbon extracted from aphids, sugar solution, and untreated paper control) to reveal the factors playing role in the development and maintenance of the tending behaviour of the ants. There are three circumstances in which ants choose to attend aphids or to assault them: (1) ants prey less on aphid that they attended previously, even though they are myrmecophilous aphids; (2) ants are less probable to assault individual aphids which their nestmates have attended, even within the same aphid species; and finally (3) ants are less likely to assault the aphids that provide honeydew to their nestmates.

Material And Methods

Plant Material

Apple trees (*Malus domestica* 'Idared') were grown from two years old seedlings in plastic pots under a climate-controlled room ($24 \pm 2^\circ\text{C}$, 16 L: 8D) and were irrigated regularly, three times a week (Fig. 1 in SI)

Study Organisms

Ant *Lasius niger* queens were collected from the Botanical Garden of Hungarian University of Agriculture and Life Sciences (vot SZIE) in Budapest (longitude N 47.480867 and latitude E 19.038809). The queen ant was reared under a climate-controlled room ($24 \pm 2^\circ\text{C}$, 16 L: 8D). The same ant colony was used for all experiment: it had approximately 50 workers and the same number of offspring. The colonies were reared on 30 m/V% sucrose solution 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 bringing up clean, monoclonal strains from the four species (*A. pomi*, *A. spiraecola*, *D. devector* and *D. plantaginea*) were collected from the Research Farm of Szent István University 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 under a climate-controlled room ($24 \pm 2^\circ\text{C}$, 16 L: 8D). Predators and parasitoids might be present in the laboratory, so apple trees were enclosed by sticky barriers and insect exclusion nets.

Detection of the chemical composition of the cuticular extract of the aphid species by GC-MS

25 aphid individuals (different species and developmental stages, see Table 1) were collected in clean GC-MS vials with fine and clean painting brush, and killed keeping them in a freezer on -20°C for five minutes. We added markings to the vials to identify the samples easily 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 the contamination from each other. As next step, we were shaking the vials for 1 minute with a high-frequency mini shaker (IKA vortex). The extract (200 μL) was transferred by a micropipette with clean micropipette tips separately to a GC vials equipped with micro vial insert. The chemical analysis of the samples was conducted by GC-MS using an Agilent 6890N GC with 5973i MSD device. The GC-MS process had the following parameters: Oven temperature: 190°C for 0.75 minutes (start temperature) then heating up with $8^\circ\text{C}/\text{minutes}$ to 320°C , and kept on the final temperature for 8 minutes. The GC device was equipped with an HP-1MS capillary column (DB-5ms 30 m x 250 μm x 0.25 μm ; Cat. No.: J&W 122-5532). The volume of injection was 1 μL . The carrier gas was H_2 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 SIM acquisition mode the selected ions were 55; 57; 71; 85. For the data evaluation we used Agilent MSD ChemStation E.02 software. The samples in the order of injection shown in ESI Table. 1.

Behavioural tests

Ant responses were recorded to different chemical stimuli in maze tests. A goal area of the tube was connected to the ant colony with a plastic tube (6 A goal area of the tube was connected to the ant colony with a plastic tube (6 cm long, 5 mm in dia.). Cotton bud was used to avoid the escaping of the ant from the tube. The goal arena was a one-centimeter-long, 6 mm in diameter plastic tube closed with a small cotton wool plug. We carried out the treatments (chemical stimuli) on a 3 mm x 3 mm cleaned filter paper pieces on the base of the goal area cm long, 5 mm in dia.), considering the base of the goal arena was clean after every observation to avoid contamination. The duration of the observation was 120 minutes. We placed the maze to a closed room, and fixed a high speed camera over it. The sequence of the treatments was randomised. We started the different treatments at the same time on following days, consequently the ant colony has 22 hours rest period between the observations. Totally we performed 3 replicas from all treatments. The evaluation of the videotapes was made manually. We distinguished three different types of the behaviour of the ants (Fig. 2, 3 and 4 in SI).

The treatments in the maze were the following (1) Alive *A. pomi* aphids fixed to the arena (3 individuals); (2) Extract of the full CHC pattern of 10 *A. pomi* 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 protocol to get the full cuticular hydrocarbons (CHCs) of Aphids First we washed the painting brush with clean n-hexane then put 20 adults of *A. pomi* into a 1.5 ml clean Eppendorf tube then were 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 replaced 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 the aphids (and threw away). Finally, we adsorbed the extracted CHC components to the clean filter paper quadrants.

Statistical analysis

We fixed our observations to an excel database second by second. We distinguished the beginning and the ending of every kind of behavioural state as well as the duration. For deeper analysis, we divided the total duration (120 minutes) into four 30-minute-long quarters to detect the potential behavioural differences within the longer (120 minutes) 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, around, away), and Geisser-Greenhouse test with Games-Howell pairwise comparison to compare the effect of the different quadrants within the observation period and to analyse the interaction of the two explanatory variables.

Results

Chemical Analysis

The GC-MS was calibrated based on the different concentration standard mixture solutions, and the retention times of the four standards (nC25; nC27; nC29; nC31) (Fig. 1.). 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. (Fig. 2, 3, 4, 5; Table 1, 2).

peak with close retention time to the standard, which was chemically not identical with the standard (Table 2.). In the case of *A. pomi* we measured three replicates from the adult stage to estimate the standard division of the GC-MS device (Table1, 2).

Three of the four standards (pentacosane (n-C25), heptacosane (n-C27), and nonacosane (n-C29), hentriacontane (n-C31)) were detectable from the cuticle of the four aphid species (*A. pomi*, *A. spiraceloa*, *D. devacta*, *D. plantingeinea*), and from the different developmental stages of *A.pomi* including 1-2 nymphs, 3-4. nymphs, winged nymphs, non-winged adults, and the skin), whilst in the case of n-C31 we found a detectable

Above the four hydrocarbon standards, we could detect 12 more hydrocarbon peaks from the cuticle extract of the four aphid species (Fig.2, 2, 4, 5; Table 2). Some of the peaks appeared by all of the aphid species (Table 2; A,C,D,E), while some of them seemed to be genera-specific (Table 3; specific for *Aphis* spp.: B,F; specific for *Dysaphis* spp.: H,I,J,K). Within the different developmental stages of *A. pomi* we could detect no qualitative, but quantitative differences (Table 2, 3.). On the other hand, qualitative and quantitative differences appeared between the different genera (Fig.2, 3, 4, 5; Table 1, 2): the cuticular hydrocarbon profile of the two species from the genus *Aphis* (*A.pomi* and *A. spiraecola*) was very similar to each other, but significantly differed from the profile of the two *Dysaphis* species (*D. plantaginea* and *D. devector*), suggesting that the CHC patterns of the aphids are genera-specific.

Behavioural Assays

Behavioural state 'Touching'

The different chemical stimuli treatments (real, alive *A. pomi* individuals 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 differently. Significantly different activity occurred against different chemical stimuli as indicated by the touching period of the ant workers in the different treatments [Table.3, blue; Fig. 6, 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 behaviour significantly. There was no significant differences in the ant activity between the different quadrants within the observation period (two hours) [Table.3, green; Fig. 7, Geisser-Greenhouse test $df (3.0; 24.0)$; $f = 0.923$; $p = 0.4447$].

Behavioural state 'Around'

In the case of the behavioural 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, blue; Fig. 8, Welch-corrected $df = (3; 3.3)$; $f = 1,800$; $p = 0.3072$]. There was no significant difference between the ant activity towards the different quadrants within the observation period (two hours) [Table.4, green; Fig. 9, Geisser-Greenhouse test $df (3.0; 24.0)$; $f = 0,175$; $p = 0.9125$].

Behaviour state 'Away'

For the behavioural state "Away" (the ant is not in the arena) no significant difference was found in the ant activity against the different chemical stimuli [Table 5, blue; Fig. 10, 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 the quarters three and four ants spent significantly longer time away from the goal arena

compared to the first quarter with the second quarter between the first and third periods [Table.5, green; Fig. 11, Geisser-Greenhouse test $df(3.0; 24.0) = 4682$ $p = 0.0103$].

Discussion

Aphids and ants are one of the most studied models of mutualistic associations in the animal kingdom. Aphids produce honeydew, the waste product of their sugar rich but amino acid poor diet, plant sap. After probing by ant antennae, aphids excrete honeydew droplets without dumping them to simplify their collection and assimilation by ants. In exchange for this sugar supply, ants not only protect aphids against natural enemies, but also supply them with hygiene by cleaning the colony from aphid exuviae and honeydew, and decrease the hazards of the fungal contagion (Buckley 1987; Detrain et al. 2010; El-Ziady 1960; EL-ZIADY and Kennedy 1956; Van Emden 2007; Hölldobler and Wilson 1990; Pontin 1959; Samways 1983; Way 1963; Yao, Shibao, and Akimoto 2000). Cuticular hydrocarbons have a substantial function in the chemical communication of social insects such as ants (Blomquist and Bagnères 2010; I. Sakata, Hayashi, and Nakamuta 2017). In the nestmate recognition in ants for instance, some studies have revealed that methyl-alkanes and -alkenes are the main signal components, whereas other studies have showed that n-alkanes play also an important role (Sturgis and Gordon 2012). In such situations where a complex cuticular hydrocarbon pattern is involved, it is not easy to determine which compounds and/or in what quantity are adequate to inform the signal receiver (Liepert and Dettner 1996). In our research, we focused on the n-alkane components of the n-hexane extract of the CHC profile of the aphids. On the other hand, methyl alkanes- which also appeared in our samples as detectable, but not identified components- have been shown to be physiologically effective in several contexts of insect synergy (Howard 1993). We examined the cuticular hydrocarbon profile of four aphid species feeding on apple (*A. pomi*, *A. spiraecola*, *D. devector*, *D. planatiginea*) using GC-MS. For the further studies, we attempted to optimize the extraction methods of the CHC-s from the cuticle of the aphids using different extraction periods to gain the optimal extraction period to get the possible highest concentration of the CHC components from the aphids. The optimal extraction period proved to be 30 minutes, and the optimal extraction temperature is 70°C (under raised pressure). The splitless injection process of GC show remarkably greater peak areas and less background noise level, than the split method. Accordingly, the optimal solvent/ aphid individual ratio is 250 µl hexane to 25 aphid individuals. As for detecting method, FID (Flame Ionization Detector) gave more separated peaks and lower background noise level compared to the MS (Mass Spectroscopy). The cuticular hydrocarbon profile of the two species from the genus *Aphis* was very similar but differed significantly from the profile of the two *Dysaphis* species, which also matched to each other. In the CHC profile of the four species, we found 16 detectable peaks, where two coincided to the normal alkane standards (nC25 and nC27). Our results approved that the CHC profile of aphids tend to be genus-specific. On the other hand, simple exposing resemblances among species in their cuticular hydrocarbons are not sufficient to prove, that the cuticular hydrocarbons operate as a disguise or they describe species or colony-specific recognition signals (Allan et al. 2002). It is very probable that Me-11 heptacosene and Me-11 nonacosene compounds promote the ants to prey on *Trioxys angeticae*. Preliminary bioassays, in which the *T. angelicae*- specific amounts of these

compounds were applied on dead, hexane-washed parasitoids, and then this dummies were offered in ant-attended *A. fabae* colonies, found that *L. niger* responded very aggressively towards such test objects (Liepert and Dettner 1996). Due to the importance of the different compounds, although we were able to detect three (pentacosane nC25, heptacosane nC27, nonacosane nC29) of the examined four 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 and 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 (Allan et al. 2002). Other studies suggest that chemical mimicry or chemical camouflage of ants is also involved in the deception of ants and that is through investigated the morphological or behavioural characterisation of the ants (Allan et al. 2002; Breed et al. 1992; Feener 1995; Garnett, Akre, and Sehlike 1985; W Völkl and Mackauer 1993; Wolfgang Völkl 1995; Weissflog et al. 1995). Therefore, we investigated the behavioral characterisation of the ants towards different chemical stimuli. Quantitative differences in cuticular hydrocarbons are supposed 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 are able to differentiate foragers and brood-tenders by means of quantitative differences in their hydrocarbon profiles (Bonavita-Cougourdan, Clement, and Lange 1993). Nevertheless, it is still not clear whether such quantitative variations between models and mimics are important in chemical mimicry systems. For instance, (Howard, Stanley-Samuelson, and Akre 1990) stated variations in relative abundances of cuticular hydrocarbons in the mimicry system of the syrphid *Microdon albicomatus* and its host ant *Myrmica* incomplete. Therefore, we began an optimisation process of the CHC extraction and detection methods to gain more peaks on GC-MS which in turn can lead to determination of further components that might be still undetected. As a result of this study, we detected 16 CHC components in different amounts from the four aphid species with different retention time. As we showed that the CHC pattern of the four species is genera-specific, and the tested two aphid genera (*Aphis* and *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. (Paris and Espadaler 2009) assumed that aphid abundance and honeydew production would rise when the aphids are tended by *L. neglectus* ants, as this effect appeared by other invasive ant species as well (Ness and Bronstein 2004). In this investigation the density of ant-attendance to the honeydew by the invasive ant *L. neglectus* was remarkably higher than that of the native ant *L. grandis*. Aphid honeydew carries a mixture of saccharides: monosaccharides (glucose, fructose), disaccharides (sucrose, trehalose, maltose), and trisaccharides (melezi- tose, raffinose, fructomaltose) (Auclair 1963; Hendrix, Wei, and Leggett 1992; Mittler 1958; Němec and Starý 1990; Wolfgang Völkl et al. 1999; Walters and Mullin 1988; Yao and Akimoto 2001), as well as several kinds of amino acids (Barlow and Randolph 1978; Maltais and Auclair 1962; Mittler 1953, 1958; Sasaki et al. 1990; Yao and Akimoto 2001). attending ants (*Formica yessensis*) completely changed the sugar composition of the honeydew of the aphid (*Tuberculatus querciuola*)

partner: the honeydew under ant attendance composed of a noticeable lower ratio of glucose and noticeable higher ratios of trehalose and sucrose in a comparison with sugar composition under the absence of ants (Yao and Akimoto 2001). Sucrose in honeydew is considered as an undigested part of the overflowing sugar sucked from the host plant (Srivastava 1987). In this case a raised ratio of sucrose in honeydew means that the effectiveness of its hydrolysis in the alimentary canal is reduced under ant attendance. In this regard, we examined the behaviour of workers of *L. niger* ants towards different chemicals connected to aphids: real *A. pomi* individuals; total extract of the CHC profile of the aphids; 30 m/V% sucrose solution and untreated control. Our results revealed that *L. niger* ants show the highest preference towards sucrose solution. The reason of the high ant activity can be based on the biological role of the sugars: ants use sugar solutions as a primary food source (Beattie 1985; Joachim Offenberg 2001). The second highest ant activity was detectable on real aphids, then on the total extract of the CHC pattern of *A. pomi*, and just 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 behaviour of the ants did not differ significantly between the quarters (30 minutes long periods).

Conclusions

we can state that the sucrose solution and real aphid had remarkably higher effect on the ant behaviour than the other two treatments. Further investigations are needed to clarify if high ant activities in these two different treatments (sugar solution and real aphid) are similar or different behaviours (just feeding on the sugar solution, and tending/protecting behaviour on real aphids). The CHC extract of the *A. pomi* had significant effect to the behaviour 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 (CHC pattern and its concentration, honeydew, shape of the aphid, behaviour of the aphid) as determinants of ant attendance of aphids.

Declarations

Conflicts of interest/Competing interests

The authors declare no competing financial interest.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

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Tables

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* (nd: not detectable, red colour: a peak with close retention time).

Samples	Ng/individual aphid Pentacosane nC25	Ng/individual aphid Heptacosane nC27	Ng/individual aphid Nonacosane nC29	Ng/individual aphid Hentriacontane nC31
<i>Aphis pomi</i> 1_2.Nymphys	10	10	not detected	24
<i>A. pomi</i> 3_4.Nymphys	15	16	25	29
<i>A. pomi</i> Adult (winged form)	13	25	28	73
<i>A. pomi</i> Adult _replicate1	23	41	53	139
<i>A. pomi</i> Adult _replicate2	27	44	30	259
<i>A. pomi</i> Adult _replicate3	25	41	29	235
<i>A. pomi</i> Skin	7	18	27	44
<i>Aphis spiracelo</i> Adult	12	39	35	119
<i>Dysaphis devacta</i> Adult	24	38	1735	24
<i>D. plantinginea</i> Adult	22	69	1336	58

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, red colour: the retention time of the component).

	A	B	C	D	E	F	G	H	I	J	K	L
Retention time / min	12.55	14.15	19.19	21.47	24.63	21.87	10.87	17.05	17.91	19.75	20.69	26.01
<i>Aphis pomi</i> 1_2.Nymphys	658	135	65	189	304	nd	nd	nd	nd	nd	nd	nd
<i>A.pomi</i> 3_4.Nymphys	1027	269	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>A.Pomi</i> Adult (winged form)	2783	644	0	160	202	nd	nd	nd	nd	nd	nd	nd
<i>A.Pomi</i> Adult	2196	650	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>A.Pomi</i> Adult	7417	2804	184	2329	6002	nd	77	nd	nd	nd	nd	nd
<i>A.Pomi</i> Adult	6956	2673	146	2060	4704	260	nd	nd	nd	nd	nd	nd
<i>A.Pomi</i> Skin	1460	313	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>A. spiraecola</i> Adult	11577	1736	nd	300	7180	nd	242	nd	nd	nd	nd	840
<i>Dysaphis devacta</i> Adult	1313	nd	35559	3083	448	nd	49798	1318	596	729	1031	5839
<i>D. plantinea</i> Adult	2357	nd	7857	182	nd	nd	39768	143	nd	857	934	1186

Table 3: Mean period (standard deviation) of behavioural state 'Touching' toward different chemical stimuli in the four time quarters

Group	Quarter 1	Quarter 2	Quarter 3	Quarter 4	Average
Control	33 (57)	0 (0)	0 (0)	45 (77)	19.33 (A)
Extract	348 (232)	173 (134)	34 (58)	16 (26)	142.67 (B)
Aphid	751 (603)	1300 (1133)	1322 (1174)	1026 (1393)	1100.1 (AB)
Sucrose	785 (744)	2267 (2906)	1115 (1253)	1243 (1111)	1352.9 (AB)
Average	479.42 (A)	935.25 (A)	617.83 (A)	582.5 (A)	

Table 4: Mean time period (standard deviation) of behavioural state 'Around' toward different chemical stimuli in the four time quarters.

Treatments	Size	Quarter 1	Quarter2	Quarter 3	Quarter 4	Average
Control	3	4.667 (8)	0 (0)	0 (0)	18 (32)	5.75 (A)
Extract	3	250 (251)	219 (158)	49 (85)	41 (67)	140 (A)
Aphid	3	3673 (4731)	3600 (4340)	1808 (1529)	2561 (2711)	2910.8 (A)
Sucrose	3	144 (221)	451 (391)	1368 (2147)	1312 (1600)	819.17 (A)
Average		1018.2 (A)	1067.7 (A)	806.58 (A)	983.25 (A)	

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

Treatments	Size	Quarter 1	Quarter 2	Quarter 3	Quarter 4	Average
Control	3	2072 (3589)	0 (0)	2072 (3589)	2279 (3947)	1605.9 (A)
Extract	3	2966 (3033)	4899 (4532)	2542 (2794)	5543 (4139)	3987.8 (A)
Aphid	3	3548 (5545)	2946 (4345)	3965 (4619)	3278 (2399)	3434.8 (A)
Sucrose	3	925 (1550)	3691 (1244)	8651 (4201)	7152 (39610)	5105.3 (A)
Average		2378.3 (A)	2884.3 (AB)	4307.8 (B)	4563.4 (B)	

Figures

Figure 1

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 2

The different components of the cuticle hydrocarbon profile of the adult stage of *A. pomi*

Figure 3

The different components of the cuticle hydrocarbon profile of the adult stage of *A. spiraeicola*.

Figure 4

The different components of the cuticle hydrocarbon profile of the adult stage of *D. depecta*.

Figure 5

The different components of the cuticle hydrocarbon profile of the adult stage of *D. plantaginea*.

Figure 6

The period of behavioural state 'Touching' toward the different chemical stimuli.

Figure 7

The period of behavioural state 'Touching' in the different quarters.

Figure 8

The period of behavioural state 'Around' toward the different chemical stimuli.

Figure 9

The period of behavioural state 'Around' in the different quarters.

Figure 10

The period of behavioural state 'Away' toward the different chemical stimuli.

Figure 11

The period of behavioural state 'Away' in the different quarters.

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