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Why do ants shift their foraging from extrafloral nectar to aphid honeydew?

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Running title: Rewards of extrafloral nectar and honeydew

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1 **Abstract**

2 When aphids parasitize plants with extrafloral nectaries (EFNs) and aphid colony size is small,
3 ants frequently use EFNs but hardly tend aphids. However, as aphid colony size increases, ants
4 stop using EFNs and strengthen their associations with aphids. Although the shift in ant
5 behavior is important for determining the dynamics of the ant-plant-aphid interaction, it is not
6 known why this shift occurs. Here, we test two hypotheses to explain the mechanism
7 responsible for this behavioral shift: (1) Extrafloral nectar secretion changes in response to
8 aphid herbivory, or (2) plants do not change extrafloral nectar secretion, but the total reward to
9 ants from aphids will exceed that from EFNs above a certain aphid colony size. To judge which
10 mechanism is plausible, we investigated secretion patterns of extrafloral nectar produced by
11 plants with and without aphids, compared the amount of sugar supplied by EFNs and aphids,
12 and examined whether extrafloral nectar or honeydew was more attractive to ants. Our results
13 show that there was no inducible extrafloral secretion in response to aphid herbivory, but the
14 sugar concentration in extrafloral nectar was higher than in honeydew, and more ant workers
15 were attracted to an artificial extrafloral nectar solution than to an artificial aphid honeydew
16 solution. These results indicate that extrafloral nectar is a more attractive reward than aphid
17 honeydew per unit volume. However, even an aphid colony containing only two individuals can
18 supply a greater reward to ants than EFNs. This suggests that the ant behavioral shift may be
19 explained by the second hypothesis.

20

21 **Key words:** HPLC; indirect interaction; mutualism; plant-animal interaction; sugar

1 **Introduction**

2 Mutualisms are reciprocal interspecific interactions in which interacting species
3 provide net benefits to each other. Mutualisms are ubiquitous in nature (Boucher et al. 1982;
4 Herre et al. 1999), and various types of mutualism are known, for example, those in which
5 resources are exchanged (e.g., mycorrhizal fungi and plants) (Smith and Read 1997), those in
6 which services are exchanged (e.g., sea anemones and anemonefish) (Allen 1972), and those in
7 which services are exchanged for resources (e.g., ants and aphids) (Way 1963). The ecological
8 and evolutionary importance of mutualisms is well accepted since mutualisms are strong driving
9 forces for community organization (Wimp and Whitham 2001; Ohgushi et al. 2007) and may
10 also play a role in speciation (Thompson 2005). However, mutualisms have been traditionally
11 studied as pair-wise interactions, and our knowledge of how third-party species in a community
12 influence the dynamics of mutualisms is limited. To investigate the effects of third species on
13 mutualisms is important because such knowledge deepens our understanding of temporal and
14 spatial variation in interaction strength, which is necessary to elucidate mutualism dynamics
15 (Strauss and Irwin 2004; Thompson 2005).

16 Both some plants and some hemipteran insects have mutualistic associations with ants
17 (Bentley 1977; Koptur 1992; Dixon 1998; Linsenmair et al. 2001). They provide sugar-based
18 rewards (i.e., extrafloral nectar or honeydew) to ants in return for protective services by the ants
19 (Bentley 1977; Koptur 1992; Katayama and Suzuki 2002, 2003b, 2004, 2005). Since
20 hemipteran insects frequently parasitize plants with extrafloral nectaries (EFNs) (Becerra and
21 Venable 1989), ant-mediated interactions occur between them (Buckley 1983; Gaume et al.
22 1998; Offenberg 2000; Engel et al. 2001). Ant-plant-hemipteran interactions are excellent
23 systems to investigate the dynamics and complexity of multi-species interactions (Becerra and
24 Venable 1989; Sakata and Hashimoto 2000; Engel et al. 2001) because the competition between
25 plants and hemipteran insects for shared partners (i.e., ants) and their association with ants are

1 related to the stability and fitness outcomes of these mutualistic interactions (Buckley 1987;
2 Suzuki et al. 2004; Ueda et al. 2008; Katayama and Suzuki 2010).

3 In this context, several authors have found that ants shift their foraging pattern from
4 the use of extrafloral nectar to that of honeydew as aphid (hemipteran insect) colony size
5 increases on plants with EFNs (Sakata and Hashimoto 2000; Katayama and Suzuki 2003a;
6 Suzuki et al. 2004). The shift in ant foraging pattern has different consequences for plants and
7 aphids due to asymmetrical ant-mediated interactions (Offenberg 2001; Suzuki et al. 2004;
8 Katayama and Suzuki 2010). When an aphid colony is small, ants frequently use EFNs and
9 hardly use aphid honeydew (Katayama and Suzuki 2003a; Suzuki et al. 2004). In this case, the
10 small aphid colony may gain indirect benefits from EFNs since ants attracted by EFNs protect
11 the colony which is too small to sufficiently attract ants by itself (Katayama and Suzuki 2010).
12 On the other hand, when an aphid colony grows larger, ants frequently use aphid honeydew but
13 hardly use EFNs (Katayama and Suzuki 2003a; Suzuki et al. 2004). In this case, although plants
14 are directly damaged by sap feeding of aphids, the plants gain “indirect benefits from aphids”
15 because ants attracted by aphids exclude other herbivores from the plants (Suzuki et al. 2004).
16 Thus, the shift in ant foraging behavior is important for determining the dynamics of the
17 ant-plant-aphid interaction.

18 In ant-plant-aphid interactions, an unsolved question has remained: Why does the ant
19 foraging behavior shift from use of EFNs to that of honeydew? Here, we propose two scenarios
20 to explain the ant foraging pattern. The first scenario is that extrafloral nectar secretion may
21 change in response to aphid herbivory, as it is known to do in response to other kinds of
22 herbivory damage (Heil et al. 2001; Ness 2003; Wäckers and Bonifay 2004). If plants gain some
23 benefits by distracting ants away from the aphid colony (ant-distraction hypothesis: Becerra and
24 Venable 1989), plants may increase the quality or quantity of extrafloral nectar. Such “ant
25 distraction” may be more effective against small aphid colonies. However, when an aphid
26 colony grows big enough to attract ants away from EFNs, the plants may stop extrafloral nectar

1 secretion to reduce the costs of nectar secretion. In contrast, the second scenario supposes that
2 plants do not change the quantity or quality of the extrafloral nectar in response to aphid
3 herbivory, but that the ants may preferentially use aphid honeydew because the reward for ants
4 from aphids may exceed that from EFNs at a given aphid colony size. Both scenarios assume
5 that the quality of extrafloral nectar and its attractiveness to ants are superior to those of aphid
6 honeydew; however, it remains unclear if this is indeed the case.

7 In this study, to judge which scenario is more likely, we examined the secretion pattern
8 of extrafloral nectar in response to aphid herbivory, and compared the quality and attractiveness
9 to ants of extrafloral nectar and aphid honeydew, using *Lasius japonicus* ants, *Aphis craccivora*
10 aphids and *Vicia faba* plants with EFNs. We especially focused on the following three points:
11 (1) Does quantity or quality of extrafloral nectar differ among plants attacked by different
12 numbers of aphids? (2) Is extrafloral nectar or honeydew the more sugar-rich resource? and (3)
13 Does extrafloral nectar or honeydew attract a greater number of ants? Our results show there is
14 no inducible secretion of extrafloral nectar in response to aphid herbivory, but find that the sugar
15 concentration in extrafloral nectar is higher than that in honeydew, and that the attractiveness to
16 ants of extrafloral nectar is superior to that of aphid honeydew. Based on these results, we
17 discuss the shift in ant foraging pattern depending on aphid colony size.

18

19 **Materials and Methods**

20 *Organisms*

21 *Vicia faba* (broad bean) is a widely cultivated annual legume herb bearing EFNs. In Japan, this
22 plant germinates in late winter, and begins to grow vigorously in early spring. It bears EFNs in
23 late March and mid-May. Several ant species are often observed visiting the plants in spring to
24 collect extrafloral nectar. To examine whether secretion rate of extrafloral nectar depends on the
25 frequency of its removal, we carried out a preliminary experiment in which we collected
26 extrafloral nectar of *V. faba* every 2 hours (12 times per day), every 4 hours (6 times per day),

1 and every 24 hours (1 time per day), and compared the total volumes of extrafloral nectar
2 secreted over a 24-hour period. In a preliminary experiment, we had previously confirmed that
3 total volume of extrafloral nectar during 24 h was not affected by the method used to collect it
4 (ANOVA, $F_{2,11} = 0.161$, $P = 0.852$, N. Katayama, unpublished data). Although we understand
5 that this collection method does not mimic the foraging pattern of ants, we consider it likely that
6 the removal of extrafloral nectar does not influence its secretion by *V. faba*. *Vicia faba* used for
7 this study was cultured in a temperature-controlled experimental chamber at 22-25°C under
8 natural light conditions.

9 *Aphis craccivora* (cowpea aphid) is the most common ant-tended aphid on *V. faba* in
10 Japan (Takizawa and Yasuda 2006). In the field, ants such as *Lasius japonicus*, *Tetramorium*
11 *tsushimae* and *Formica japonica* (Hymenoptera: Formicidae) visit colonies of this aphid and
12 collect honeydew (Katayama and Suzuki 2003a). We collected one colony of *A. craccivora*
13 from *Vicia angustifolia* L. (Leguminosae) growing on the campus of Saga University (Saga
14 Prefecture: 33° 17' N, 130° 06' E). We reared the aphids on seedlings of *V. faba* grown in
15 polyethylene pots (9 cm in diameter, 8 cm deep) in the laboratory at 20-25°C under a 24L
16 photoperiod.

17 Workers of *L. japonicus* are medium-sized ants (about 4 mm in body length). They
18 prefer sugars and frequently feed on honeydew. A colony of *L. japonicus* (more than 2000
19 workers) was collected on the campus of Saga University, and used in the below experiment.

20

21 *Collection of extrafloral nectar*

22 We examined extrafloral nectar secretion in response to aphid herbivory, using 24
23 seedlings of *V. faba* cultured for a month in a temperature-controlled experimental chamber. The
24 seedlings of *V. faba* were about 20 cm in height, and bore 8 to 10 EFNs each. Before the
25 experiment, all extrafloral nectar was removed from the EFNs by using 0.5- μ L microcapillary
26 tubes (Drummond Scientific Company, Broomall, Pennsylvania, USA). We then assigned these

1 seedlings to each of 3 treatments. Treatment 1 was that no aphids were released on the plant
2 (control), and treatments 2 and 3 were that 5 and 50 adult aphids were released, respectively.
3 Each seedling was put into a clear plastic cylinder with an iron lid in the bottom. The top of the
4 cylinder was covered with fine mesh to prevent aphids from escaping. We placed each cylinder
5 in a temperature-controlled chamber (23°C under a 16L8D condition). After 24 h (Day 1), we
6 used 0.5- μ L microcapillary tubes to collect extrafloral nectar secreted by the seedlings and
7 measured the extrafloral nectar secretion per 24 h. To maintain constant aphid densities, we
8 removed all newborn aphids from the plants every day. After 48 h (Day 2) and 72 h (Day 3)
9 from when we first released aphids on the seedlings, we collected extrafloral nectar following
10 the same procedure. All collected samples of extrafloral nectar were kept in a freezer at -30°C
11 until their sugar content could be analyzed.

12 In natural (and experimental) conditions, the evaporation of extrafloral nectar usually
13 occurs. We consider that the evaporation is not a flaw of the experiment because ants use
14 extrafloral nectar that has condensed after its water content has evaporated. To estimate sugar
15 production from EFNs, we calculated the excretion rates of “sugars” in extrafloral nectar as
16 described below.

17

18 *Collection of honeydew*

19 To compare what kinds of sugars were present in extrafloral nectar and honeydew, we
20 collected honeydew from the aphids. We released 5 adult aphids each onto seven *Vicia faba*
21 seedlings which had been cultured for about three weeks in the outdoors experimental chamber.
22 Each seedling was put into a similar clear plastic cylinder as mentioned. We then reared these
23 aphids under temperature-controlled conditions (23°C under a 16L8D photoregime). After 7
24 days, we cut several leaves with aphids off each plant, and placed them in wet cotton on a Petri
25 dish. While observing the aphids through a microscope, we collected honeydew excreted by the
26 aphids, using 0.5- μ L microcapillary tubes. Honeydew was collected until one microcapillary

1 tube had been completely filled for each plant, for a total of seven tubes. All collected samples
2 of honeydew were kept in a freezer at -30°C until they were analyzed. While collecting the
3 samples, we also measured the volume of one droplet of honeydew excreted by an aphid, using
4 a 0.5- μ L microcapillary tube.

6 *Sugar analysis*

7 The sugar concentrations of extrafloral nectar from Days 1, 2 and 3, and aphid
8 honeydew were analyzed by high-pressure liquid chromatography (HPLC), using a Cosmosil
9 5NH₂-MS packed column (4.6 \times 150 mm; Nacalai Tesque, Kyoto, Japan) and an 80%
10 acetonitrile mobile phase at room temperature. The flow rate was 1 mL min⁻¹. Peak sizes for the
11 various sugars present in the samples were calculated directly by a refractive index detector
12 (RID; Shimadzu Corp., Kyoto, Japan) and used to calculate the concentrations of the sugars in
13 samples. Samples were optimized using eleven sugar standards (fructose, glucose, sucrose,
14 maltose, trehalose, melezitose, xylose, galactose, lactose, melebiose, raffinose), and the
15 composition of each sample was determined by comparison of retention times with those from a
16 standard sample measured on the same day.

18 *Secretion rates of sugars from EFNs and individual aphid*

19 Secretion rates of sugars from EFNs and individual aphid per day were calculated
20 using following formula:

$$22 \text{ [secretion rate (}\mu\text{g d}^{-1}\text{)]} = \text{[conc (}\mu\text{g }\mu\text{L}^{-1}\text{)]} \times \text{[volume (}\mu\text{L d}^{-1}\text{)]}$$

23
24 [conc (μ g μ L⁻¹)] and [volume (μ L d⁻¹)] represent the concentration and the amount secreted per
25 day of extrafloral nectar or honeydew, respectively. Average volume of extrafloral nectar per
26 day collected from the above experiment was used as [volume (μ L d⁻¹)] of extrafloral nectar.

1 Katayama and Suzuki (2002) reported that individual *A. craccivora* aphid excreted a droplet of
2 honeydew approximately once per hour (0.94 h^{-1}). Hence, in this study, [volume ($\mu\text{L d}^{-1}$)] of
3 honeydew excreted by individual aphids was estimated as follows:

4

5 [volume ($\mu\text{L d}^{-1}$)] of honeydew = average honeydew droplet volume ($0.047 \mu\text{L}$, see Results) x
6 number of honeydew droplets excreted per h (0.94 h^{-1}) x 24 h ”.

7

8 *Ant preference between artificial extrafloral nectar and honeydew*

9 We compared the attractiveness to ants of extrafloral nectar and honeydew, using
10 artificial sugar solutions made to mimic extrafloral nectar and honeydew. A colony of *L.*
11 *japonicus* was collected and transferred among 12 test tubes (1.2 cm in diameter and 18 cm
12 length) with 100 workers per tube (ant nest), to produce 12 experimental nests. The bottom of
13 each tube was packed with wet cotton wool about 3 cm deep to maintain a suitable humidity.
14 The tube was covered with aluminum foil to maintain darkness like that of a natural ant nest.
15 Each test tube was connected to a vinyl chloride tube 6 mm in inner diameter and 10 cm long to
16 form an entrance. The ants were fed 10% sucrose solution from a test tube (1.2 cm in diameter,
17 12 cm long) plugged with cotton wool. Before the experiment, ants were starved for 4 d to
18 increase the sensitivity of their reactions to sugar resources. We made two artificial sugar
19 solutions, which were designed to mimic *V. faba* extrafloral nectar (sugar concentrations: 100
20 $\mu\text{g } \mu\text{l}^{-1}$ fructose, $125 \mu\text{g } \mu\text{l}^{-1}$ glucose, $25 \mu\text{g } \mu\text{l}^{-1}$ sucrose) and aphid honeydew (sugar
21 concentrations: $5 \mu\text{g } \mu\text{l}^{-1}$ fructose, $5 \mu\text{g } \mu\text{l}^{-1}$ glucose, $2.5 \mu\text{g } \mu\text{l}^{-1}$ sucrose, $35 \mu\text{g } \mu\text{l}^{-1}$ melezitose).
22 We put 1 mg of cotton wool in each vial (1.2 cm in diameter, 12 cm long), and added 2 ml of
23 either artificial sugar solution into each vials.

24 The experiments were carried out at 25°C under light conditions in a laboratory. We put
25 an ant nest in the center of a plastic tray (length: 30 cm, width: 20 cm, height: 5 cm). The inner
26 side of the tray wall was plastered with fluon (Asahi Glass Company, Tokyo, Japan) to prevent

1 ants from escaping. One hour after putting the ant nest on the tray, we placed one vial
2 containing artificial extrafloral nectar solution on one side in the tray, and placed one vial
3 containing artificial honeydew solution on the other side. After 1 h had passed, the numbers of
4 ants in the two artificial solution vials were counted 10 times at 10 min intervals. The average
5 numbers of ants across the 10 counts of each trial were used for analysis. We replicated this trial
6 12 times using different ant nests.

7

8 *Statistical analysis*

9 To test for inducible extrafloral nectar secretion in response to aphid herbivory,
10 repeated-measures ANOVA was used to examine the effects of time (during Day 1 to 3) and
11 aphid colony size on both extrafloral nectar secretion per 24 h and total sugar concentration in
12 extrafloral nectar. We compared the concentration of each sugar between extrafloral nectar and
13 honeydew using a *t*-test. When comparing sugar concentrations between extrafloral nectar and
14 honeydew, we used the same samples of extrafloral nectar that were collected to test for
15 inducible extrafloral secretion. Because there were no significant differences in the sugar
16 concentrations in extrafloral nectar among treatments (i.e., aphid colony size or time), the
17 average concentration of sugars in the extrafloral nectar produced by each plant was used when
18 comparing with the sugar concentrations in honeydew. A Wilcoxon test was used to compare
19 the number of ants in the vials with artificial extrafloral nectar and in the vials with artificial
20 honeydew in the sugar preference experiment.

21

22 **Results**

23 *Effect of aphids on extrafloral nectar secretion*

24 After we released aphids on the seedlings, we found no significant changes in
25 extrafloral nectar secretion over the three day period, and no significant effect of aphids (aphid:
26 $F_{2,21} = 1.194$, $P = 0.323$; time: $F_{2,20} = 0.917$, $P = 0.416$; aphid \times time: $F_{4,40} = 0.805$, $P = 0.529$;

1 Fig. 1a). Three sugars, fructose, glucose and sucrose, were detected in the extrafloral nectar of *V.*
2 *faba* (Table 1). However, we found no significant effects of time or aphid numbers (i.e., among
3 0, 5, and 50 aphids) on the total sugar concentration in extrafloral nectar (aphid: $F_{2,21} = 0.598$, P
4 $= 0.559$; time: $F_{2,20} = 0.537$, $P = 0.593$; aphid \times time: $F_{4,40} = 1.411$, $P = 0.248$; Fig. 1b).

6 *Comparison of sugar concentrations and amounts between EFN and honeydew*

7 The average volume of a honeydew droplet was 0.047 ± 0.003 μL (mean \pm SE, $n = 26$).

8 Four sugars (fructose, glucose, sucrose and melezitose) were detected in honeydew (Table 1).
9 Concentrations of fructose, glucose, sucrose and total sugars were 14-, 27-, 13- and 4.6-fold
10 greater than those in extrafloral nectar, respectively (fructose: $t_{29} = 5.41$, $P < 0.001$; glucose: $t_{29} = 6.21$,
11 $P < 0.001$; sucrose: $t_{29} = 2.21$, $P = 0.017$; total sugars: $t_{29} = 4.65$, $P < 0.001$; Table 1).

12 The estimated total sugar amount secreted from extrafloral nectar was 99.9 $\mu\text{g d}^{-1}$,
13 which was 1.9-fold greater than that excreted by individual aphids (Table 2).

15 *Ant preference between artificial extrafloral nectar and artificial honeydew*

16 When ants were given the opportunity to chose between the artificial extrafloral nectar
17 solution and artificial honeydew solution, more workers visited the vial with the artificial
18 extrafloral nectar solution than that with the artificial honeydew solution ($z = -2.673$, $P < 0.001$,
19 Fig. 2).

21 **Discussion**

22 The present study demonstrates that the secretion of extrafloral nectar does not change
23 in response to aphid herbivory. This indicates that the shift in ant foraging pattern from
24 extrafloral nectar to honeydew at higher aphid densities (Sakata and Hashimoto 2000; Katayama
25 and Suzuki 2003a; Suzuki et al. 2004) is not caused by the reduction of extrafloral nectar
26 secretion, nor does the plant compensate for the costs of producing useless extrafloral nectar by

1 reducing extrafloral nectar secretion. On the other hand, we found that the sugar concentration
2 in extrafloral nectar is higher than that in honeydew. The ant preference experiment suggests
3 that the attractiveness to ants of extrafloral nectar was superior to that of honeydew of aphids
4 per unit volume. These results indicate that the shift in ant foraging behavior from the use of
5 EFNs to that of honeydew may be explained by our second scenario; i.e., ants would selectively
6 use aphid honeydew because the total reward for ants from aphids exceeded that from EFNs at a
7 given aphid colony size.

8 Our experimental design (i.e., densities of 5-50 aphids and an experimental duration of
9 3 days) is based on previous research (Sakata and Hashimoto 2000; Katayama and Suzuki
10 2003a; Suzuki et al. 2004), which examined how ant foraging pattern changed depending on
11 aphid colony size in field and laboratory experiments. These studies demonstrated that ants
12 shifted their foraging pattern from the use of extrafloral nectar to that of honeydew one to a few
13 days after 50-100 aphids were transferred to *Vicia* plants with extrafloral nectaries (EFNs).
14 Based on previous studies and our present results, we consider that the shift in ant foraging
15 pattern at higher aphid densities is not caused by the reduction of extrafloral nectar secretion. It
16 is likely that more time and higher herbivory pressure by aphids results in the reduction of
17 extrafloral nectar secretion because the plant is weakened by the aphid herbivory. However,
18 investigating this possibility was not our aim in this study.

19 Under natural conditions, aphids gradually increase from a single or a few adult
20 aphid(s) on a plant, and the herbivory pressure by the aphids gradually increases as the aphid
21 colony grows. There is a possibility that the induced change of extrafloral nectar secretion may
22 be elicited by such gradual growth of aphid colony size. It is not possible with existing data to
23 evaluate this possibility at the present time; additional research to test this mechanism.

24
25 *Why are aphids more attractive to ants?*

26 At present, there are few studies that directly compare sugar quality between

1 extrafloral nectar and honeydew (but see Engel et al. 2001), however, it is likely that the sugar
2 concentration is generally higher in extrafloral nectar than in honeydew (extrafloral nectar:
3 25-35%, honeydew: 3-20%) (Koptur 1979; Ruffner and Clark 1986; Helden et al. 1994;
4 Kawano et al. 1999; Völkl et al. 1999; Engel et al. 2001; Yao and Akimoto 2001; Fischer et al.
5 2002). If so, why do ants shift their foraging pattern to use of honeydew? The reason, we
6 suspect, is that at a given colony size, the total volume of honeydew output (and quantity of
7 sugars therein) could be much higher than that of extrafloral nectar produced by the colony's
8 host plant. This study shows that the average honeydew droplet volume of *A. craccivora* aphids
9 was 0.047 μ L. In addition, Katayama and Suzuki (2002) examined the frequency of honeydew
10 excretion by the same aphid species, and found that the aphids excreted about one droplet of
11 honeydew per hour. From these results, we estimated the total volume of honeydew droplets
12 produced per day by a single aphid (Table 2), and compared the sugar secretion rate between
13 EFNs and aphids. The sugar secretion rate of one aphid appears inferior to that of EFNs,
14 however, if the aphid population increases to two aphids, the order is reversed. Although not all
15 droplets of honeydew produced by aphids may be provided to ants, aphids in a colony can still
16 provide a greater quantity of sugar to ants than do EFNs.

17 Engel et al. (2001) reported that the quality of extrafloral nectar of *V. faba* was higher
18 than that of honeydew of *Aphis fabae* (Scop.), and concluded that the presence of the aphids did
19 not influence the attractiveness of EFNs due to the high quality of extrafloral nectar. However,
20 this study did not compare total sugar quantity quantitatively. Our study is the first report which
21 shows that the sugar-productive capacity of aphids in a colony outweighs that of EFNs.

22 Several studies have reported that the honeydew of ant-tended aphids contained
23 melezitose, but that of non ant-tended aphids did not (Stadler and Dixon 1998; Völkl et al. 1999;
24 Engel et al. 2001; Fischer and Shingleton 2001; Fischer et al. 2001; Yao and Akimoto 2001).
25 From these findings, melezitose tends to be considered a key substance for maintaining
26 mutualisms with ants. In this study, we confirmed that the honeydew of *A. craccivora* contained

1 melezitose but the extrafloral nectar of *V. faba* did not (Table 1). However, we found that ants
2 preferred an artificial sugar solution mimicking extrafloral nectar (which did not contain
3 melezitose) to one mimicking honeydew (which contained melezitose) when solution volume
4 was held constant (Fig. 2). This result indicates that melezitose may be not so important in
5 determining ant resource preference, or that the high sugar concentration in extrafloral nectar
6 might outweigh the effect of melezitose in honeydew on ant resource preference.

8 *Multi-species interactions among plants, aphids and ants*

9 Becerra and Venable (1989) proposed the interesting hypothesis that EFNs may
10 function to defend plants from ant-hemipteran mutualisms (ant-distraction hypothesis).
11 According to the ant-distraction hypothesis, EFNs provide an additional sugar resource for ants,
12 so that the ants stop attending the hemipteran insects. Offenberg (2001) reported that *Lasius*
13 *niger* (L.) ants stopped attending and started to prey on *Aphis fabae* aphids in the presence of
14 acacia honey solution, supporting the ant-distraction hypothesis. We suspect this is because
15 honey solution was provided in excess to the ants in that experiment. It is known that ants will
16 prey on aphids in the presence of additional sugar resources (Sakata 1994, 1995), but we think
17 that EFNs of *Vicia* cannot distract ants from aphids because aphids in a colony can provide a
18 much greater amount of sugar to ants than can EFNs. In fact, ants hardly use EFNs at high aphid
19 densities (Sakata and Hashimoto 2000; Katayama and Suzuki 2003a; Suzuki et al. 2004).

20 Furthermore, if plants have evolved the ability to distract ants as a counter-adaptation
21 to ant-tended aphids, they would be expected to secrete more extrafloral nectar in response to
22 sap feeding by aphids, especially since extrafloral nectar secretion is known to be inducible in
23 many plants (Heil et al. 2001; Ness 2003; Wäckers and Bonifay 2004). This reaction may be
24 more effective for the plants, especially at lower aphid density at which the aphids cannot attract
25 ants adequately. However, *V. faba* did not react to sap feeding by aphids (Fig. 1). Thus, our
26 results do not support the ant-distraction hypothesis. Suzuki et al. (2004) reported that when A.

1 *craccivora* parasitized the EFN-bearing vetch *V. angustifolia*, the efficiency of herbivore
2 exclusion by ants increased. This effect was a result of the fact that the aphids attracted ants
3 effectively. That study also did not support the ant distraction hypothesis, but rather indicated
4 that there are indirect benefits to plants with EFNs from the presence of aphids. In addition,
5 when an aphid colony is small, the colony may gain indirect benefits from EFNs since ants
6 attracted by EFNs also protect the colony (Katayama and Suzuki 2010). Therefore, we expect
7 that an indirect mutualistic relationship can potentially occur between plants with EFNs and
8 ant-tended aphids by synergistically attracting ant protection which benefits both parties. This
9 shift in the foraging pattern of ants may be important for the stability and dynamics of
10 ant-plant-aphid interactions, as well as for that of the larger arthropod community structure on
11 the plants.

12

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19

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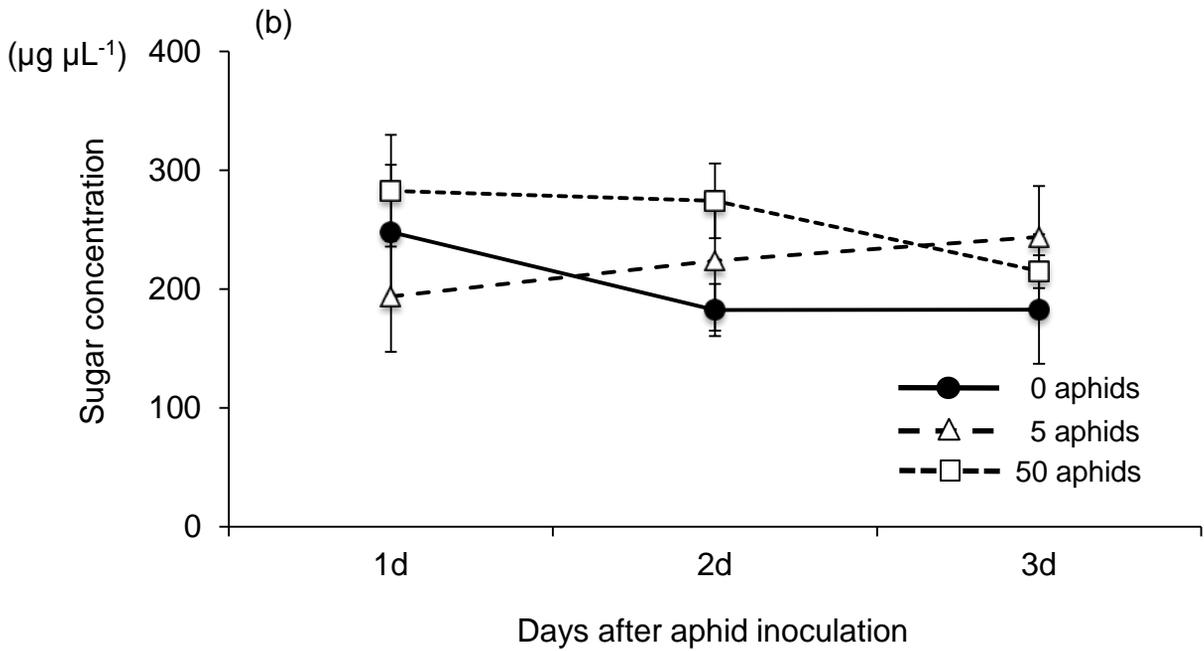
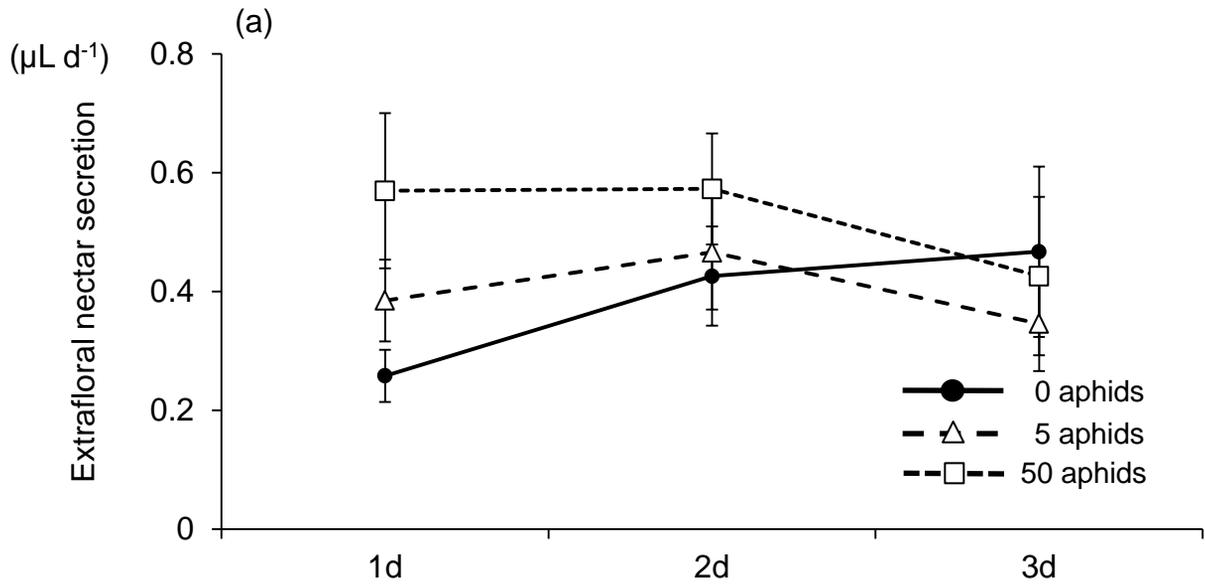
1 **Figure legends**

2 **Fig. 1.** (a) Extrafloral nectar secretion per 24 h and (b) total sugar concentration in extrafloral
3 nectar. Solid circles indicate values in the absence of aphids on a plant. Open diamonds and
4 open squares indicate values in the presence of 5 and 50 aphids, respectively.

5

6 **Fig. 2.** Sugar preference between artificial extrafloral nectar and artificial honeydew by ants.

7 Error bars show SE.



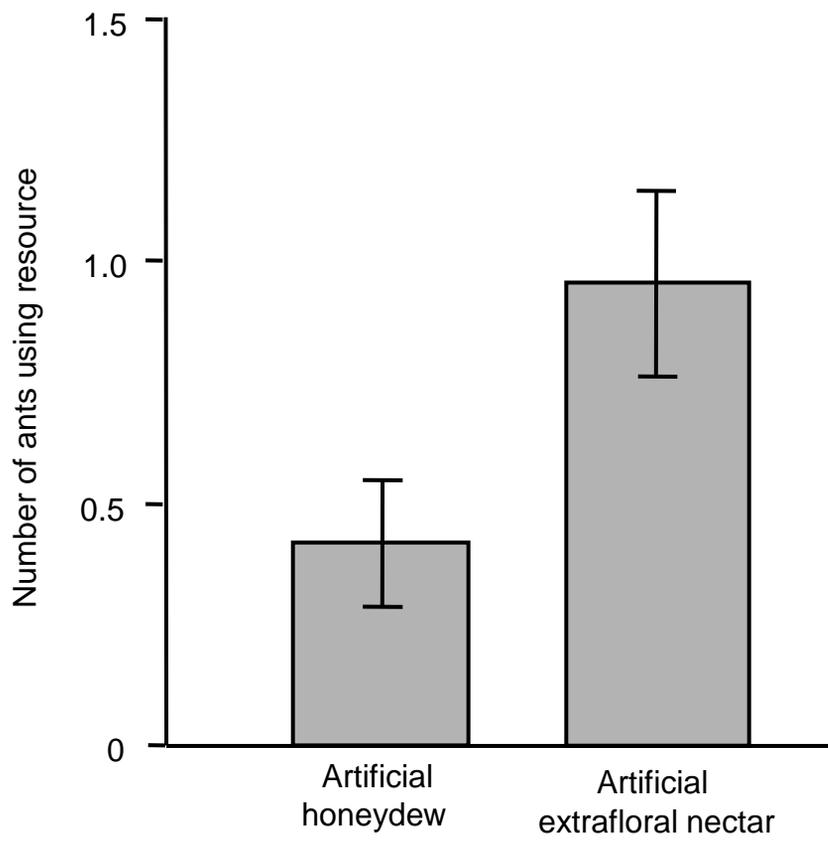


Table 1. Concentration of sugars in extrafloral nectar and honeydew ($\mu\text{g } \mu\text{l}^{-1}$).

	n	Fructose	Glucose	Sucrose	Melezitose	Total sugars
Extrafloral nectar	24	82.4 ± 7.7	113.2 ± 9.3	31.4 ± 7.0		227.3 ± 19.9
Honeydew	7	5.7 ± 1.4	4.2 ± 3.1	2.5 ± 1.0	36.7 ± 14.9	49.2 ± 16.7

Values of extrafloral nectar represented by bold letters indicate significant greater values than that of honeydew (*t*-test test, $P < 0.05$).

Table 2. Excretion rates of sugar in extrafloral nectar and honeydew.

	Volume of secretion ($\mu\text{L d}^{-1}$)	Fructose ($\mu\text{g d}^{-1}$)	Glucose ($\mu\text{g d}^{-1}$)	Sucrose ($\mu\text{g d}^{-1}$)	Melezitose ($\mu\text{g d}^{-1}$)	Total sugars ($\mu\text{g d}^{-1}$)
Extrafloral nectar	0.44	36.3	49.8	13.8	-	99.9
Honeydew	1.06 [†]	6.0	4.5	2.7	38.9	52.1

[†]Volume of honeydew secreted per day was calculated as follows: total volume = average honeydew droplet volume (0.047 μL) x number of honeydew droplets excreted per hour (0.94 h^{-1} : Katayama and Suzuki 2002) x 24 h”.