Original article

The effect of avocado (*Persea americana*) nectar composition on its attractiveness to honey bees (*Apis mellifera*)¹

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Abstract – Honey bees are important avocado pollinators, but due to low attractiveness of avocado flowers pollination is often inadequate. Since honey is of nectar origin, we used it to test the effect of nectar composition on the preference of honey bees and we identified avocado honey due to its perseitol concentration. Bees preferred feeders containing non-avocado honey to avocado honey even when total sugar concentration in the avocado honey was higher. Crop loads were smaller for bees feeding on avocado than non-avocado honey. Finally, the learning performance of bees in a proboscis extension conditioning experiment was lower when they were rewarded with avocado honey than with non-avocado honey or sucrose solution. Moreover, only for avocado honey did the percentage of bees refusing to consume reward increase during the experiment. Our results indicate that honey bees prefer honey whose floral origin is of a competing flora over that of avocado. We conclude that avocado nectar composition may contribute to the low attractiveness of avocado flowers.

Persea americana / crop load / proboscis extension conditioning / repellency / pollination / perseitol / citrus

1. INTRODUCTION

Bees often show preference for some flowers over their competitors. This phenomenon is an important process in the evolution of flowers (Barth, 1991). The preference of honey bees can greatly impact agricultural systems when foragers abandon crops that require pollination to set fruit or seeds in favor of the surrounding flora (Delaplane and Mayer, 2000). Nectar volume (Fulop and Menzel, 2000), sugar concentration (von Frisch, 1967), and sugar quantity per flower (Rabinowitch et al., 1993) may affect honey bees choice behavior, but the effect of nectar composition, especially of trace elements, is not so clear (Gardener and Gillman, 2002).

Avocado (Persea americana Mill.) is native to the neotropics, but it has become an important crop in many tropical and subtropical regions around the world (Knight, 2002). In its native environment, avocado is visited by a diversity of insect species, many of which perform efficient pollination (Ish-Am et al., 1999). In areas where avocado is grown commercially and native pollinators are absent, honey bees are used successfully and almost exclusively for pollination (Davenport, 1986; Vithanage, 1990; Ish-Am and Eisikowitch, 1993; Gazit and Degani, 2002). The importance of honey bees in avocado pollination is evident from the strong positive correlation between bee activity in an orchard and fruit set and yield (Vithanage, 1990; Ish-Am and Eisikowitch, 1998; Gazit

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and Degani, 2002). However, inadequate pollination is still common in avocado, even when honey bee colonies are placed in an orchard. This phenomenon has been attributed to the honey bee preference for flower species other than avocado (Bergh, 1967; Vithanage, 1990; Ish-Am and Eisikowitch, 1998; Gazit and Degani, 2002).

Several explanations have been proposed for the low attractiveness of avocado flowers and one of them is nectar composition (Ish-Am, 1994). Nectar usually does not repel bees, but a particular nectar may be less attractive than nectar of competing flowers (London-Shafir et al., 2003). Therefore, the attractiveness of nectar of a particular floral origin should be compared with that of nectar from the different sources that are available to the bees at the same time.

Limited nectar amounts make it difficult to conduct preference experiments based on manually collected nectar. Honey bees, on the other hand, gather large amounts of nectar and store it in the hive as honey. In this work, we used avocado honey, and established its floral origin by detecting the concentration of perseitol, a unique avocado carbohydrate, which comprises up to 6% of the nectar sugars (Ish-Am, 1994; Liu et al., 1995). We compared the response of honey bees to avocado honey with their response to honey from competing flowers on three levels: (1) Preference of the colony between feeders containing honey from the different sources. (2) Crop loading of single bees when honey solutions were supplied ad lib. (3) Learning performance of harnessed bees in response to honey rewards of only a few microliters.

2. MATERIALS AND METHODS

2.1. Honey sources

Avocado honey is characterized by its dark color, high minerals content and high pH value (Dag et al., 2005), yet the most accurate method to identify this honey is due to perseitol concentration. The honey samples that were used in the present work were extracted from colonies that were placed in a few avocado orchards in Israel during the avocado blooming season. Even though colonies were placed inside the orchard, avocado nectar was not the only nectar source. To identify nectar source, honey was

extracted from each colony separately and its perseitol concentration measured by HPLC (Dag et al., 2003). Perseitol concentrations ranged between 0.2 and 2.4% of total sugars. The honey from each colony was classified as avocado honey if the perseitol concentration was higher than 1.2%, or as non-avocado honey if the perseitol concentration was lower than 0.5%. In the preference experiment and the crop loading experiment each tested colony received avocado honey from one extracted colony and non-avocado honey from another extracted colony. For the learning experiments, which were conducted a year later, new honey sources were used. Only one source of avocado honey (2.5% perseitol) and one source (a citrus grove) of non-avocado honey (0% perseitol) were used.

2.2. Preference between honey sources

Twelve colonies of honey bees were kept in five frames nucleus hives, inside a $6 \times 12 \times 3$ m enclosure (15 mesh), in Rehovot, Israel, between September and October 2002. The bees had ad lib access to a water source and were provided with a pollen patty once a week. Only one colony was tested at a time and the entrances to the rest of the hives were closed the evening before each test.

Six different honey solutions were prepared each day by diluting honey with distilled water. One sample of avocado honey and one sample of non-avocado honey were each diluted to prepare three honey dilutions with total dissolved solids (TDS) of 60%, 30% and 10% w/w measured by a hand-refractometer (brix units). Even though the tested honeys contain mainly glucose and fructose (Dag et al., 2005), their refractive index is similar to that of sucrose (Kearns and Inouye, 1993). The solutions were presented to the bees in 200 mL bird feeders.

The six feeders were placed in a circle 10 cm apart, on a carousel that rotated at a velocity of 2 rpm, to prevent a potential location bias. The experiment ended when 130 mL were consumed from one of the feeders, or after 4 h had elapsed. The volume of solution consumed from each feeder was measured at the end of the experiment. The honey solution from which bees consumed the highest volume was considered as 100% consumption and the decreased volumes from the rest of the feeders were compared with the volume representing the 100%. The results are presented as the relative percent of consumption from the different solutions.

2.3. Crop loading

One month after the end of the previous experiment, 10 of the 12 colonies that survived were also tested in a crop loading experiment. In this experiment, we weighed the crop load that bees imbibed from the six diluted honey solutions used in the preference experiment. During each day of experiment, bees from one colony were tested for the six different honey solutions and a total of 558 foragers were tested. The crop loading of 9–10 bees from each colony was measured for each honey solution. We attempted to test the bees from every colony with the six different honey solutions during each day of experiment. We always started with the 30% solutions, to ensure at least one common concentration to compare between colonies. For most colonies we were able to complete the six tests, but for two colonies we were unable to conduct the last two tests, with the 10% solutions. In all cases, the two solutions with the same TDS were tested one after the other.

Honey bees were trained to visit a 5 mL feeder filled with 30% sucrose solution, placed on an electric balance (Precisa 505M, accuracy of ± 1 mg). When the feeder was visited by many bees it was removed and the experiment began. A new, identical feeder, filled with one of the six honey solutions, was placed in the same location and was covered by a plexiglass cage. Only one bee at a time was allowed to enter the cage and to imbibe from the feeder. When the bee stopped imbibing and flew to the top of the cage, she was collected to prevent repeated measures of the same bee. The reduction in the solution weight indicated the amount she had collected. The consumed volume was calculated by multiplying the consumed weight by the specific density of each solution using the parameters from Weast (1988) for sucrose.

2.4. Learning performance

The learning experiment was conducted during December 2003 and January 2004, by conditioning of the proboscis extension response (Bitterman et al., 1983). Bees from a total of eight hives were tested. These colonies were not related to the colonies used for the previous experiments. Each morning, 30 bees from one colony were caught into glass vials as they flew out of the hive. The vials were placed in ice for 1-2 min until the bees were motionless, and then the bees were strapped into a sectioned hollow plastic tube 6 mm in diameter, by a 3 mm wide strip of duct tape that wrapped around the tube and (dorsal) thorax of the bee (Shafir et al., 1999). When they awoke, the bees were fed 1 µL of a 30% sucrose solution. Typically, only a few bees did not feed and they were removed from the experiment. Twenty-four bees were chosen for the experiment, and were allowed one hour to adapt to the harness.

During the experiment, odor of eugenol (conditioned stimulus) was delivered to each bee for 3 s, followed by a reward of 1 μ L of sugar or honey solution (unconditioned stimulus) delivered with a Gilmont microsyringe, simultaneously with additional 2 s of odor. The odor was supplied using a 1 mL syringe tube in which a strip of filter paper held 0.7 μ L pure eugenol (MERCK-Schuchardt). An air pump delivered air through valves that were controlled by a computer, and through the tube toward the bee antennae. Each bee experienced six conditioning trials of odor followed by reward and one test trial, in which odor was delivered for 3 s, but was not followed by reward. The intertrial interval was 13 minutes. Learning curves were plotted from the proportion of bees that extended the proboscis to the odor (prior to delivery of reward) during each of the seven trials. We also calculated the proportion of bees that consumed the entire reward droplet in each of the six conditioning trials.

The 24 bees were separated at random into three groups of eight bees each. Each group received a different unconditioned stimulus during the conditioning trials. The three unconditioned stimuli were: 60% (w/w) sucrose solution, avocado honey diluted in distilled water to a 60% TDS, and non-avocado honey diluted to a 60% TDS, all measured with a hand refractometer. A total of 192 bees were tested. Only two bees extended their proboscis to the odor during the first trial, prior to receiving the unconditioned stimulus. Since we were interested in comparing the reinforcement ability of the different unconditioned stimuli in the conditioning process, we excluded these two bees from the analysis (Hoban et al., 1996).

2.5. Statistical analyses

The significance of the results for the preference experiment was tested by two-way ANOVA including the honey source and the TDS as fixed factors, and their interaction. Differences between pairs of treatments were tested by least square means contrast. The arcsin square root transformation was employed on the honey consumption percent data in this experiment prior to analysis (Sokal and Rohlf, 1995). The analysis of the crop loading results was tested using a similar model, but we also added the colony as a random factor. We were also interested in the colony x treatments interactions, but this could not be done on the full model due to missing data for two colonies with the 10% solution. We therefore conducted separate two-way ANOVA for each TDS. Learning curves in the learning experiment were compared using logistic growth curve analysis in SAS (Hartz et al., 2001), comparing two treatments each time.

3. RESULTS

3.1. Preference between honey sources

For 11 of the 12 colonies tested, the feeder that was emptied most at the end of the experiment

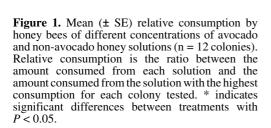
*

60%

*

30%

Sugar (TDS) concentration (w/w)



was one containing non-avocado honey solution. The interaction between honey source and TDS was significant ($F_{2.66} = 14.8, P < 0.0001$; Fig. 1). Bees consumed the non-avocado honey significantly faster than the avocado honey when the TDS was 60% ($F_{1.66} = 60.2$, P <0.0001), and 30% ($F_{1,66} = 10.3$, P = 0.002). They even consumed the 30% non-avocado honey faster than the 60% avocado honey $(F_{1.66} = 7.6, P = 0.008)$. No significant differences were found between the 60% and 30% avocado honey solutions ($F_{1.66} = 0.2$, NS). It is possible that increased attractiveness due to higher sugars content was reduced by higher concentration of any repelling component in the avocado honey. The consumption of the two 10% solutions was negligible, and no significant difference was found between them $(F_{1.66} = 0.014, \text{NS}).$

3.2. Crop loading

Crop load weight was affected by both honey source ($F_{1,543} = 8.5$, P = 0.004; Fig. 2) and TDS ($F_{2,543} = 66.6$, P < 0.0001), and the interaction between them was not significant ($F_{2,543} = 0.58$, NS). Crop load weight increased

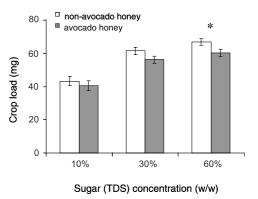


Figure 2. Mean (\pm SE) crop load weight (mg) of honey bees feeding on different concentrations of avocado and non-avocado honey solutions (n = 558 bees). * indicates significant differences between treatments with *P* < 0.05. At 30% TDS, there was a significant interaction between colony and honey source.

with increasing TDS, while the calculated consumed volume, which was also affected by the TDS ($F_{2,543} = 27.3, P < 0.0001$), was highest when bees received the 30% solution. The differences between crop load weight and volume results are explained by differences in the specific density of solutions that varied in TDS. Resembling the pattern in the preference experiment, bees imbibed a greater weight (and volume) of non-avocado honey than avocado honey when the TDS was 60% ($F_{1,180} = 7.7$, P = 0.021). For TDS of 30%, the interaction between honey source and colony was significant ($F_{9,180} = 2.4, P = 0.015$), indicating a difference between colonies in their discrimination between the honey sources. For TDS of 10%, there was no difference in imbibing of the nonavocado and avocado honey ($F_{1.142} = 0.43$, NS).

3.3. Learning performance

The statistical method we used to compare learning curves compares acquisition rates between two treatments (Hartz et al., 2001). The lowest acquisition rate was achieved using avocado honey as a reward (Fig. 3a), and it was significantly lower than when using non-avocado honey as reward ($Z_{7,126} = 2.02$, P =0.043) or sucrose solution ($Z_{7,128} = 3.33$, P = 0.0009). The best acquisition rate was

Relative Consumption (%)

100

80

60

40

20

0

non-avocado honey

avocado honev

10%

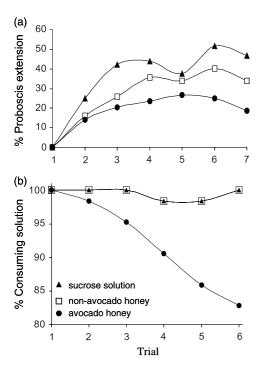


Figure 3. (a) Learning curves showing the percentage of honey bees that extended their proboscis in response to the conditioned odor, using different solutions as reward. (b) The percentage of honey bees that consumed the different solutions used as rewards. There were six rewarded conditioning trials and one unrewarded test trial (trial 7).

achieved with sucrose solution, but it was not significantly different than with non-avocado honey ($Z_{7,126} = 1.37$, NS).

Comparing the feeding behavior of the bees, it was found that bees almost never avoided feeding on sucrose solution or non-avocado honey (Fig. 3b). In contrast, the percentage of bees that avoided the avocado honey increased consistently, and reached 17% in the last feeding trial.

4. DISCUSSION

Our results revealed that honey bees are able to discriminate between honey from different botanical sources and that they prefer non-avocado honey to avocado honey. While the crop load and learning experiments reflect only the effect of avocado honey on the behavior of the tested individuals, the preference experiment may have also been influenced by the recruitment activity of the colony to the more preferred honey source. This was also the only experiment in which honey bees could choose between the alternatives. Attractiveness of honey bees to repellent nectars, honey or specific components is usually evaluated in comparison to sucrose solution (Waller et al., 1972; Majak et al., 1980; Hagler and Buchmann, 1993; London-Shafir et al., 2003), or glucose solution (Allsopp et al., 1998). Foraging honey bees do not have the alternative of pure sugar solutions, but rather, different nectar sources that may be more or less attractive than sugar solutions. Even though citrus nectar, the main competitor of the avocado during the Israeli spring, is known to be highly attractive to honey bees (Ish-Am and Eisikowitch, 1998), the results of the learning experiment suggest that the difference in attractiveness between avocado and non-avocado (putatively of citrus origin in this experiment) honey, is smaller than between avocado and sucrose solution. Therefore, it is important to compare between nectars from competing floral sources, rather than only with sugar solution.

The crop load of honey bees is hypothesized to indicate the perceived quality of the food source. It increases with increasing sugar concentration (von Frisch, 1950) or flow rate (Moffatt, 2000). Our results of crop load weight support this hypothesis; crop load weight increased with sugar concentration, but it was also greater for non-avocado honey than for avocado honey, especially when the honey solution was more concentrated (Fig. 2). Interestingly, calculations of crop load volume revealed that bees tended to collect higher volumes of the 30% solution than of the more concentrated solution, possibly due to weight limits reached by the imbibed volume of the 60% solution, or due to difficulty in imbibing highly viscous solutions (Waddington, 1990). Differences in the effect of sugar concentration between imbibed volume and weight may depend on the species studied and on the range of concentrations tested (Wells and Giacchino, 1968; Roubik and Buchmann, 1984). However, crop loads of non-avocado honey were greater than of avocado honey, regardless of whether they were expressed as weight or volume.

Bees avoided imbibing a full crop load of avocado honey, as they do with sugar solutions containing salt (von Frisch, 1950), or high ethanol concentrations (Abramson et al., 2000). These results may indicate the presence of some repellent component in avocado honey. Dag et al. (2003) measured crop loads of honey bees foraging on avocado flowers and found smaller loads compared with bees that foraged on the competing flowers. The suggested explanation was that the avocado foragers flew shorter distances than those foraging on alternative blooms, and therefore had smaller crop loads. The crop load results of this work provide an additional explanation for the previous findings.

The increasing percentage of honey bees that refused to imbibe avocado honey in the learning experiment also supports the hypothesis of a repellent component in avocado nectar. We have recently characterized avocado honey (Dag et al., 2005). The chemical composition of avocado nectar remains to be analyzed and the components that render it unpalatable to bees remain to be identified. Such components are likely to be relatively nonvolatile, because it was found that honey bees could hardly distinguish between odors of honey from different sources (Bonod et al., 2003). The presence of the unique carbohydrate perseitol in avocado nectar and honey, calls for testing how honey bees respond to it, but our preliminary results (unpublished data) indicate that bees cannot perceive it.

Foraging on flowers, bees and other insects are usually rewarded by very small volume of nectar for a single visit and many visits are needed to achieve a full crop load. Trying to understand the importance of repelling components in the nectar, we have to identify not only their (post-ingestion) effect on the fully loaded bee, but also how they affect the foraging process as the bee is collecting small amounts (possibly due to pre-ingestion effects, such as taste). For example, sucrose solution containing ethanol, reduced the learning performance of bees after consumption of only a few microliters (Abramson et al., 2000) possibly due to a preingestion effect, while feeding on nectar containing miserotoxin resulted in postponed death in the hive (Majak et al., 1980). Most of the studies in which repellency and toxicity of nectars were examined compared preference (Waller et al., 1972; Hagler and Buchmann, 1993; London-Shafir et al., 2003) or longevity (Majak et al., 1980; Allsopp et al., 1998) of honey bees that were fed ad lib with the tested solutions. Only a few studies tested how the learning process of bees is affected by limited nectar amounts enriched with components like glycine (Kim and Smith, 2000) or ethanol (Abramson et al., 2000). The results of our learning experiment reveal that the repelling components in the avocado honey and possibly in avocado nectar have an immediate negative effect on foraging behavior of honey bees.

Using honey as an indicator for nectar quality is a useful method when the amount of nectar secreted per flower is limited (Hagler and Buchmann, 1993), but it requires that the botanical origin of honey can be unequivocally identified. Also honey composition includes both components that originate directly from the nectar and components that change their composition during the process of honey ripening (White, 1992). Therefore, it would be more accurate to test the behavior of honey bees in response to nectar instead of honey. Though it is difficult to collect large amounts of nectar that are required for choice experiments with free-flying bees, the proboscis extension conditioning assay requires much smaller volumes. The differences that we observed in learning performance between bees rewarded with minute volumes of different honeys, suggests that this could be a powerful technique to compare between nectars. Avocado and citrus nectar, in particular, can be readily collected, and the results of comparing learning behavior in response to these nectars could complement our understanding of the competition between avocado flowers and their neighbors.

Floral nectars consist mainly of sugars and water (Shuel, 1992), but trace elements in nectar may also influence the foraging behavior of pollinators (Adler, 2000). Here we used several methods to establish the importance of nectar composition compared with nectars of competing flora. The preference experiment is important to determine the effect of nectar composition in the presence of competing blooms, isolated from other parameters like nectar concentration or flower structure. However, this method may not be applied when nectar amount or other specific components are limited. The crop loading method tests whether a potential pollinator would agree to consume the nectar and how much of it, in the absence of other alternatives. The learning experiment examined the effect of limited nectar amounts, which is more likely to replicate flower visits in the field. This method may also be useful for limited nectar amounts. This work revealed that avocado honey deters honey bees and we assume that the deterring component originates from the avocado nectar. Honey bees are important pollinators of avocado trees, mainly in cultivated plots, but they are not its natural pollinators. In trying to understand the possible role of the composition of avocado nectar we should apply similar methods using the natural pollinators of the avocado. The response of stingless bees, which are considered to be the main avocado pollinators (Ish-Am et al., 1999), has to be compared with the response of other flower visitors like diptera and hemiptera. The results of such experiments would tell us whether the avocado nectar composition has a role in attracting specific pollinators (Rhoades and Bergdahl, 1981; Adler, 2000).

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Résumé – Influence de la composition du nectar d'avocat (*Persea americana*) sur son attractivité vis-à-vis des abeilles domestiques (*Apis mellifera*). Les abeilles domestiques sont des pollinisateurs importants de l'avocat mais, même lorsque les ruches sont placées dans les vergers d'avocatiers, la pollinisation est souvent inadéquate. La principale raison en est la plus forte attractivité des plantes fleurissant en même temps. Nous avons utilisé la composition chimique du nectar pour tester la préférence des abeilles et avons identifié le miel d'avocatier par sa teneur en perseitol, sucre caractéristique de l'avocatier, qui peut représenter jusqu'à 6 % des sucres du nectar.

Dans la première expérience, les nourrisseurs contenaient soit du miel d'avocatier (miel A), soit un autre miel (miel non-A). Les abeilles ont préféré les nourrisseurs avec le miel non-A, même lorsque sa teneur totale en sucres était plus faible (Fig. 1). La seconde expérience visait à mesurer la charge du jabot. Pour cela une abeille à la fois venait boire dans des nourrisseurs placés sur une balance électrique et renfermant une quantité donnée de solution. La différence de poids de la solution donnait la quantité prélevée par l'abeille. La charge du jabot des abeilles visitant les nourrisseurs de miel A était inférieure à celle des abeilles visitant les nourrisseurs de miel non-A (Fig. 2). Ceci suggère que le miel d'avocatier renferme un composé répulsif. La troisième expérience a testé les performances d'apprentissage des abeilles à l'aide d'un test de conditionnement de l'extension du proboscis. Elles ont été plus faibles lorsque les abeilles étaient récompensées avec du miel A plutôt qu'avec du miel non-A ou avec une solution de saccharose (Fig. 3a). En outre le pourcentage d'abeilles refusant de consommer la récompense n'a augmenté au cours de l'expérience que pour le miel A (Fig. 3b).

pollinisation / charge du jabot / conditionnement / extension du proboscis / répulsif / perseitol / *Persea americana* / citrus

Zusammenfassung - Einfluss der Zusammensetzung des Avocado-Nektars (Persea americana) auf seine Attraktivität für Honigbienen (Apis mellifera). Honigbienen sind wichtige Bestäuber bei Avocado, aber auch wenn Völker in die Avocado-Plantagen gestellt werden, ist die Bestäubung oft unzureichend. Als ein Hauptgrund für die nicht ausreichende Bestäubung wurde die höhere Attraktivität von Blüten anderer Pflanzen beschrieben. Da Honig auf Nektar basiert, nutzten wir ihn, um den Einfluss der Zusammensetzung des Nektars auf eine Bevorzugung durch die Bienen zu prüfen. Die Herkunft der Honige wurde durch Bestimmung der Perseitolkonzentration festgestellt, einem einzigartigen Avocado-Karbohydrat, dessen Anteil bis zu 6 % der Zucker im Avocado-Nektar betragen kann. Während des ersten Versuchs wurden den Bienen an der Futterstelle Avocado- und Nichtavocado-Honig geboten. Die Bienen bevorzugten die Futterstelle mit Nichtavocado-Honig gegenüber der mit Avocado-Honig, auch dann, wenn die Zuckerkonzentration im Nichtavocado-Honig geringer war (Abb. 1). Im 2. Versuch durfte jeweils nur eine Biene an Futterstellen trinken, die auf einer elektrischen Waage standen. Die Reduktion im Gewicht der Lösung zeigte an, wie viel die Biene aufgenommen hatte. Die von Bienen aufgenommene Menge war bei Avocado-Honig geringer als bei Nichtavocado-Honigen (Abb. 2). Das ließ vermuten, dass Avocado- Honig eine abstoßende Komponente enthält. Im 3. Test wurde die Lernfähigkeit von Bienen mit der Konditionierung des Rüsselreflexes überprüft. Die Lernfähigkeit war schlechter bei einer Belohnung mit Avocado-Honig als bei Nichtavocado-Honig

oder einer Zuckerlösung (Abb. 3a). Überdies nahm der Prozentsatz an Bienen, während des Versuchs die Belohnung nicht anzunehmen, nur bei Avocado-Honig zu. (Abb. 3b).

Unsere Ergebnisse zeigen, dass Honigbienen Honig bevorzugen, der von Blüten der Konkurrenzpflanzen des Avocado stammt. Das lässt vermuten, dass auch Avocadonektar abstoßende Substanzen enthält, die zu der geringen Attraktivität von Avocadoblüten beitragen.

Aufnahmemenge / Konditionierung des Rüsselreflexes / Repellenteffekt / Bestäubung / Perseitol / *Persea americana* / Citrus

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