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RESEARCH ARTICLE

Sucrose acceptance threshold: a way to measure sugar perception in ants

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Abstract Variation in the perception of sweet taste is a well-known phenomenon in the animal kingdom. Well-established protocols for measuring sucrose responsiveness in non-social insects and honeybees have made it possible to understand many aspects of their biology and behaviour. Ants are also advanced social insects that present a plethora of life histories with diverse strategies and behaviours; however, a universal paradigm possible to measure this response in different ant species has not yet been developed. Here, we present a protocol for measuring the *sucrose acceptance threshold (SAT)* under controlled conditions in harnessed ants with different feeding habits. By testing the response to antennal and palp sucrose stimulation and using the *occurrence of licking as the response,* we developed a non-ambiguous evaluation that allowed easy detection of threshold changes. The results showed that the response to both antennal and palp stimulation varied widely among species. Some species licked in response to antennal stimulation while others did so in response to palp stimulation. Using the appropriate kind of stimulation, we tested the SAT protocol in ants of different genera and ants of the same species with different levels of sugar reserve. The differences detected in both cases imply that the protocol is appropriate for measuring and detecting variations in sugar perception in ants.

Keywords Ants · Sucrose responsiveness · Taste · Response threshold · Licking behaviour

Abbreviations
SAT Sucrose acceptance threshold

Introduction

Variation in the perception of taste of different chemical substances is a well-known phenomenon in both vertebrates and invertebrates. Depending on the animal model, different protocols for measuring the sugar response threshold are available and represent a useful tool in the understanding of sweet taste. An individually standardized sucrose response threshold (SRT) protocol is available for non-social insects such as flies (Sudlow et al., 1987; Edgecomb et al., 1987; Scheiner et al., 2004), and social insects such as honeybees (Page et al., 1998). The social condition allows taste perception to be related not only to different aspects of individual biology, but also to those of social biology, as colony organization, genotype, role in the colony, current feeding status, and concentration of solution circulated within the colony, among other factors (Page et al., 1998; Pankiw and Page, 1999, 2000; Pankiw et al., 2001, 2004; Martinez and Farina, 2008). Ants are advanced social insects like honeybees, and, in addition, present a plethora of life histories and commonly show polymorphism in the worker caste. Numerous studies in ants have analysed the modulation of complex behavioural

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outputs—both at social and individual level—triggered by different sugar concentrations. However, very few dealt with the individual threshold in sugar perception (Wada et al., 2001).

SRT in honeybees is based on proboscis extension as a reflex response to antennal stimulation with sugar solution (Page et al., 1998). In ants, the extension of the glossa (the distal end of the labium) has been used instead as the response to sugar under controlled conditions. However, even for species of the same genus, different paradigms were used in harnessed workers: palp stimulation in *Camponotus japonicus* (Wada et al., 2001) while antennal stimulation in *Camponotus aethiops* (Guerrieri and d′Ettorre, 2010). The former study focuses on the feeding response while in the latter the authors developed a novelty paradigm for olfactory conditioning in which the named *Maxilla-labium extension response* (MaLER) was quantified (Guerrieri and d′Ettorre, 2010). This response is not always provoked by mere antennal stimulation in ants and often occurs after touching the mouthparts with sugar solution (Falibene, pers. obs.; Guerrieri, pers. com.), which hinders the use of the protocol used in bees for measuring SRT in ants. In addition, we observed that harnessed *Camponotus mus* workers sometimes present the Maxilla–labium complex extended with the still glossa protracted even before the stimulation and sometimes they only protract it partially afterwards, making the quantification of this response ambiguous in this species.

Ants of different genera drink fluids by sucking or licking (Paul and Roces, 2003). Some species always lick while others either suck or lick depending on the amount of solution available (Josens and Roces, 2000; Paul and Roces, 2003). Licking is prompted by sugar stimulation and involves *a movement* in which the glossa is protracted and retracted repeatedly to drive the solution into the mouth (Josens and Roces, 2000; Paul and Roces, 2003); four to five of these cycles are performed per second (Paul and Roces, 2003). This behaviour is very conspicuous under a stereomicroscope and therefore, can be easily quantified as a response to sugar stimulation in harnessed ants.

In the present work, we use licking behaviour as the response to sucrose to establish a protocol for measuring sugar sensitivity in harnessed ants of different species. As a first step, we compared licking behaviour in response to antennal and palp stimulation with a sucrose solution. Then, using the appropriate kind of stimulation for each species, we measured the sucrose acceptance threshold (SAT) by stimulating the ants with an increasing concentration of sugar solution and quantifying the lowest concentration that caused licking behaviour. Finally, we evaluated the sensitivity of the protocol to differences between treatments by testing ants of the same colony with different sugar reserve levels.

Materials and methods

Ants of different species were captured within the campus of the University of Buenos Aires and the surrounding area (34° 32′ S, 58°26′ W), Buenos Aires, Argentina: *Acromyrmex lundi, Cephalotes jheringi, Crematogaster* sp., *Solenopsis richteri* (Myrmicinae), *Camponotus mus, Camponotus punctulatus* (Formicinae), *Linepithema humile* (Dolichoderinae) and *Pseudomyrmex* sp. (Pseudomyrmecinae). They were maintained in the laboratory for 1 or 2 days to allow acclimatization, with free access to water. Then, they were individually placed in eppendorf tubes and anaesthetized on ice for about 2–4 min until the first signs of immobility. Afterwards, they were harnessed into a micropipette tip (10–100 µl for large species and 0.1–10 µl for small ones), the end of which had been cut off. Only the head was exposed through the resulting hole, allowing the ants to move their antennae and mouthparts freely.

A group of 20-30 ants of the same species were harnessed on each occasion. The mounting procedure lasted around 40 min and measuring began 1 h after the last ant was mounted in the harness. Prior to measurement, all ants were offered water and allowed to drink until satiety.

Response to antennal or palp stimulation

We tested the response to sucrose stimulation on both the antennae and palps of different ant species. Individuals were harnessed and separated into two equal groups. Stimulation of each individual ant was carried out by touching the antennae or the palps (maxillary and labial) with a sharpen toothpick imbibed with 50% w/w sucrose solution. One group received first antennal stimulation and then palp stimulation (antenna/palp group) while the other was first stimulated on their palps and then on their antennae (palp/antenna group). The interstimulus interval varied between 4 and 5 min. The occurrence or lack of licking after stimulation (*positive* or *negative response,* respectively) was computed for each individual.

For each ant species, the *proportion of response* was calculated as the proportion of ants that licked after either one or both stimulations with respect to the total number of harnessed individuals. Then, and considering only those ants that showed licking behaviour to any stimulation, we calculated the *proportion of responses to antennal stimulation* by dividing the number of positive responses after antennal stimulation by the number of ants that responded positively to any stimulation. In the same way, we calculated the *proportion of responses to palp stimulation.* Both proportions were obtained for each group (antenna/palp and palp/antenna). Note that if some ants lick in response to both stimuli, the sum of the proportions of responses after antennal and palp stimulation for each group will be greater

than 1. We compared the proportion of positive responses to the first stimulus between the two groups (*antenna* in antenna/palp group *vs. palp* in palp/antenna group) using a two-proportion Z-test (significance level: 5%).

Sucrose Acceptance Threshold

SAT for different species

SAT was evaluated in four ant species (*C. mus*, *L. humile*, *C. jheringi* and *A. lundi).* For those species in which the proportion of response to antennal stimulation prevailed over the response to palp stimulation, SAT was measured by means of antennal stimulation. On the other hand, for those species that responded more to palp stimulation, the latter was used instead.

Depending on the species, the antennae or palps of harnessed ants were touched with a toothpick imbibed with 0.3, 1, 3, 10, 30 or 50% (w/w) sucrose solution. These concentrations were presented to the ants in ascending order. Before the first sugar stimulation trial and between each subsequent trial, ants were tested in the same way for their response to water. The interstimulus interval varied between 4 and 5 min.

In all cases, the response was considered *positive* only when the ant showed *licking behaviour after contacting the solution*, and *negative* when *there was no licking behaviour after stimulation*, independently of whether the glossa remained exposed without movement either before or after the stimulation (see in the Supplementary Online Material, negative and positive responses after palp stimulation in *C. mus* ants).

All the ants were tested until the first positive response to sucrose solution and then they were eliminated. The concentration at which an ant showed licking behaviour represents its SAT, which is an indicator of the individual responsiveness to sucrose.

SAT for the same species; effect of sugar reserve level

The feeding behaviour of the nectivorous ant *C. mus* has been widely studied in our laboratory. Given that the level of colony starvation modifies the acceptance of a dilute solution in free-moving ants (Josens and Roces, 2000), we used this species to test whether the SAT varies with the sugar reserve level and to evaluate whether the protocol is sensitive enough to detect this variation.

Previously starved *C. mus* ants from a single laboratory colony were separated into two groups of identical size. Both groups were fed at the same time with 20% w/w sucrose solution, one group with an amount of solution equivalent to 0.1 µl per ant (*low volume group*) and the other with ten times more, i.e. 1.0 µl per ant (*high volume group*). Thus, the time elapsed since the last meal and the quality of this food was the same for both groups and only the level of reserve varied. Ants were maintained with free access to water until the following day when they were harnessed and tested for sucrose responsiveness as described above.

Statistical analysis

In order to compare the responses of different groups (among species or treatments) statistically, we ascribed a score value to each ant. Those ants that responded positively to the first sucrose solution (0.3% w/w) received a score of 1. If the first positive response was shown with the second concentration (1% w/w), they received a score of 2, and so on. Thus, the SAT score of an individual ant ranged between 1 and 6 (as six concentrations were evaluated). Then, score values of different groups were compared by Kruskal–Wallis ANOVA test because the data did not meet the assumption of homogeneity of variance (Sokal and Rohlf, 2000), followed by Dunn comparisons between groups when corresponded. Ants that responded to water stimulation immediately before the first positive response and ants that did not respond to any of the sucrose stimuli were eliminated from the analysis. The percentage of no responses was also measured.

Results and discussions

Response to antennal or palp stimulation

The response to antennal or palp stimulation varied widely among species (Table 1). While *A. lundi* and *Crematogaster* sp. responded more to antennal stimulation than palp stimulation, in *C. mus*, *L. humile* and *Pseudomyrmex* sp. the proportion of responses to palp stimulation was higher. On the other hand, *C. punctulatus* and *C. jheringi* showed no significant differences in their response to antennal and palp stimulation.

Some species showed an increase in the response to antennal stimulation when they had previously been stimulated on their palps (Table 1). *C. mus* and *Pseudomyrmex* sp. showed an initial response to antennal stimulation of 0.04 and 0.2, respectively (antenna/palp group). However, when these ants were stimulated on their antennae after being previously stimulated on their palps (palp/antenna group) the response increased to 0.35 for *C. mus* and 0.56 for *Pseudomyrmex* sp. This increase may be due to sensitization caused by palp stimulation. In honeybees, a single presentation of sucrose aroused the animal for a short period of time, increasing the proboscis extension response (Menzel, 1999).

Table 1 Evaluation of licking response after antennal or palp stimulation in different ant species

Species	Proportion of response	Antenna/palp		Palp/antenna		Antenna *vs.* palp (p)[a]
		Antenna	Palp	Palp	Antenna	
Myrmicinae						
Acromyrmex lundi	0.37 (105)	0.95	0.05	0.11	0.89	<0.001
Cephalotes jheringi	0.31 (117)	0.75	0.69	0.70	0.65	0.26
Crematogaster sp.	0.49 (81)	0.76	0.43	0.35	0.95	0.004
Solenopsis richteri[b]	0.39 (36)	1	–	–	–	–
Formicinae						
Camponotus mus	0.88 (58)	0.04	1	0.96	0.35	<0.001
Camponotus punctulatus	0.95 (20)	1	0.9	1	0.78	1
Dolichoderinae						
Linepithema humile	0.36 (109)	0.32	0.86	0.76	0.35	0.003
Pseudomyrmecinae						
Pseudomyrmex sp.	0.74 (38)	0.2	1	0.72	0.56	0.004

For each species, two groups were stimulated with 50% w/w sucrose solution: one group on their antennae first and then on their palps and the other group in the opposite order. The *proportion of response* indicates the proportion of ants that licked (positive response) either after one or both stimulations with respect to the total number of harnessed individuals (N). Considering only the ants that licked, the proportion of responses to antennal or palp stimulation is shown for each group. P indicates comparison of antenna *versus* palp (two-proportion Z-test)

[a] Comparison between the first stimulation of each group

[b] Because of their size, behaviour and morphometry of their mouthparts, this species was evaluated only by antennal stimulation

In some individuals, the mere protraction of the glossa—without the subsequent movements—was also observed instead of licking and only occurred in those species that responded more to palp stimulation, while in the others it did not occur at all or occurred in a proportion less than 0.1. Antennal stimulation promoted the glossa protraction in the antenna/palp group in the following proportions: 0.30 for *C. mus*, 0.39 for *L. humile* and 0.37 for *Pseudomyrmex* sp., with respect to the total number of harnessed ants.

In several of the species tested here some individuals had their maxilla–labium complex protracted with the still glossa exposed before the start of stimulation. On the other hand, some individuals that did not lick partially exposed their maxilla–labium complex after stimulation, making the evaluation of this response difficult. Therefore, quantifying the occurrence of licking after stimulation as the response eliminated ambiguity in the evaluation. Licking is a conspicuous behaviour that is easily recognisable in both ants that are specialized for collecting nectar and ants that rarely feed on it. Licking is even clear in ants that mainly suck sugar solutions, such as *C. mus* (Josens and Roces, 2000).

The genus *Camponotus* seems to be variable in the responses to sucrose stimulation. While *C. mus* responded mainly to palp stimulation, *C. punctulatus* licked after any stimulation. In addition, *C. aethiops* has been reported to perform MaLER consistently during associative learning conditioning after antennal stimulation (Guerrieri and d′Ettorre, 2010) and *C. japonicus* to extend and move the glossa after palp stimulation (Wada et al., 2001).

Sucrose Acceptance Threshold

SAT for different species

SAT was evaluated in ants of different genera and feeding habits. Based on the results of the previous experiment, we used palp stimulation for *C. mus* and *L. humile* and antennal stimulation for *A. lundi* (Fig. 1). As *C. jheringi* showed no difference between the two types of stimulation, we decided to perform the SAT test using antennal stimulation. During the SAT evaluation, the percentage of responses varied among species: *A. lundi* 69% ($N = 29$), *C. jheringi* 39% ($N = 47$), *C. mus* 75% ($N = 24$) and *L. humile* 69% ($N = 32$). All ants showed a unimodal distribution of responses (Fig. 2) and medians coincided with modes in all cases (*C. mus*: 10%; *L. humile*: 30%; *C. jheringi*: 3%; *A. lundi*: 30% w/w). SAT scores varied among species ($H_{3,N=78} = 14.2$, $p = 0.003$, Kruskal–Wallis test). The point of stimulation (antennal or palp) seemed to bear no relation to the score obtained, as *L. humile* received palp stimulation and *A. lundi* antennal stimulation but they had the same SAT ($p > 0.5$, Dunn Comparison); on the other hand, *C. jheringi* and *A. lundi* received both antennal stimulation and they showed different SATs ($p < 0.02$, Dunn Comparison). *L. humile* and *C. jheringi* also differed in their SATs ($p < 0.05$, Dunn Comparison) while the rest of the comparisons did not differ. In conclusion, the proposed protocol can be used to compare different species with different feeding habits under conditions or treatments

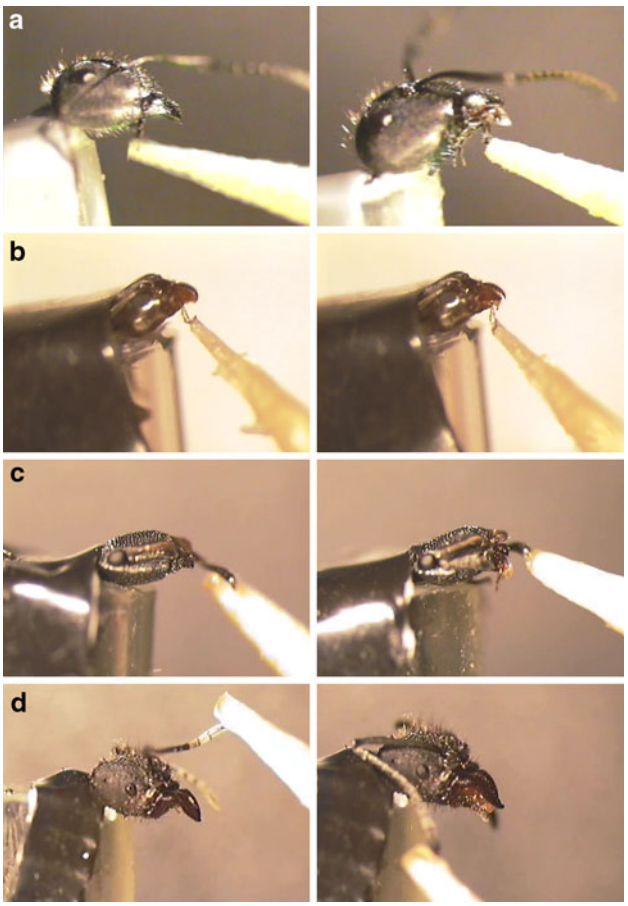

Fig. 1 SAT recorded in different ant species. **a** *Camponotus mus* and **b** *Linepithema humile* were stimulated on their palps while **c** *Cephalotes jheringi* and **d** *Acromyrmex lundi* were stimulated on their antennae. Harnessed ants were stimulated with sucrose solution in increasing concentrations. *Left hand column* ants with mouthparts retracted while stimulated with sucrose solution of a sub-threshold concentration. The response was considered negative when no licking behaviour occurred. *Right hand column* ants licking while stimulated with sucrose solution at their threshold concentration. The response was considered positive when licking behaviour was triggered by stimulation

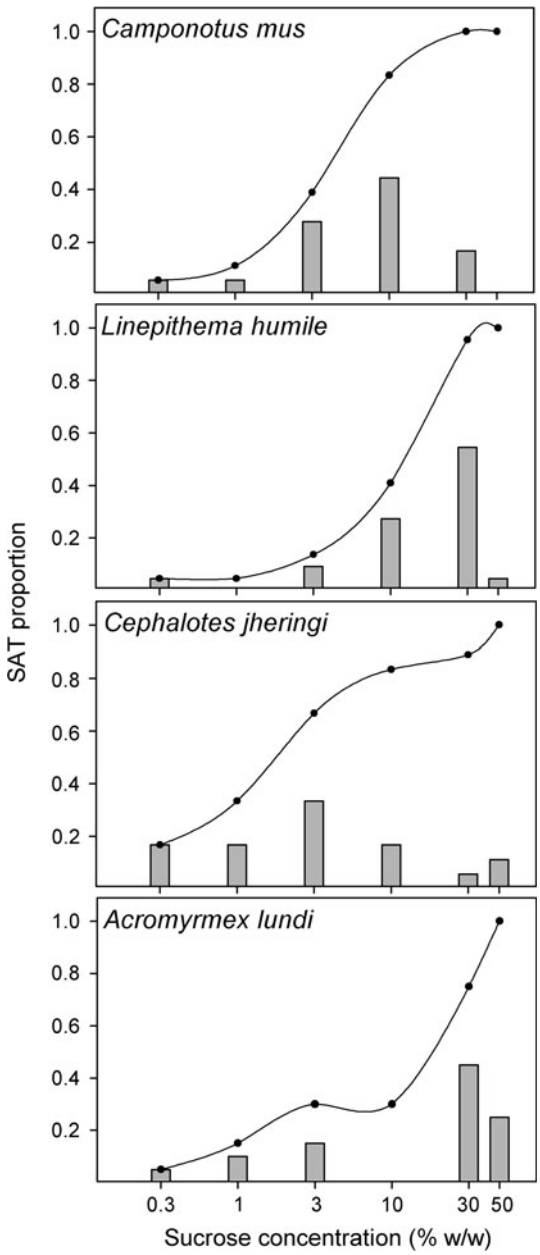

Fig. 2 Distribution of SAT in harnessed ants of four different species captured in the field. The SAT was recorded as the lowest concentration of sucrose solution that elicited licking behaviour. *Bars* indicate the proportion of ants that showed a positive response for different sucrose concentrations (logarithmically scaled). *Line* indicates the cumulative proportion of response

to be evaluated. However, in our results we evaluated individuals without any particular treatment, and we did not control the previous feeding status, a fact that could affect the SAT among other factors. Field experiments performed with free moving ants have shown that sucrose threshold differ among species in the same habitat as well as with the nutrient availability (Kay, 2002, 2004).

SAT for the same species; effect of sugar reserve level

In order to evaluate the protocol's sensitivity to differences between treatments, we compared the SATs of *C. mus* ants with different sugar reserve levels. Ants that had drunk a high volume of solution prior to the test (high volume group) had a significantly higher SAT score than those that had drunk the low volume (low volume group) ($H_{1,N=96} = 22.38$, $p < 0.0001$, Kruskal–Wallis, Fig. 3). While the SAT of ants from the low volume group was 3% w/w (mode and median value), the ants in the high volume group mostly responded to 10% w/w (Fig. 3). These results showed that the protocol allowed differences between ants under different treatments to be detected and compared.

In nectar-feeding insects, sucrose responsiveness could be a good indicator of the internal state or motivation to feed

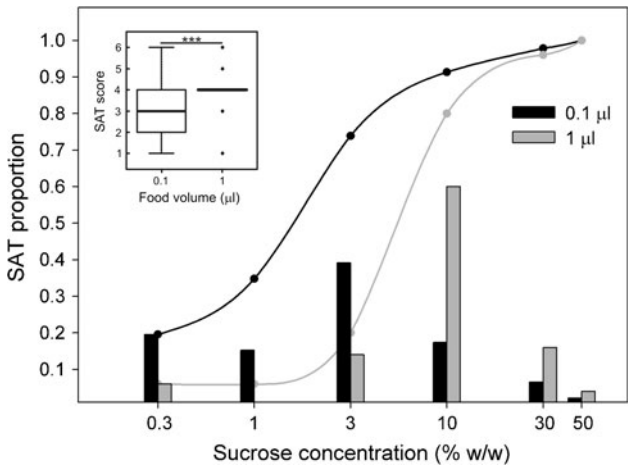

Fig. 3 Distribution of SAT for two different groups of previously starved *C. mus* ants that were fed at the same time with different quantities of a 20% w/w sucrose solution 1 day before the SAT evaluation: one group received 0.1 µl per ant ($N = 50$, low volume, *black bars* and *line*), while the other received 1 µl per ant ($N = 46$, high volume, *grey bars* and *line*). *Bars* indicate the proportion of ants that showed the first positive response for different sucrose concentrations (logarithmically scaled). *Lines* indicate the cumulative proportion of response. Insert: Box plot of SAT scores. *Thick horizontal lines* within each box represent medians, *boxes* show quartiles, *whiskers* show the extreme values and *circles* indicate outliers. (***$P < 0.001$)

as it changes according to the time since the last meal and the quality of the food previously ingested (Sudlow et al., 1987; Page et al., 1998; Kay, 2002). Our results showed that the level of carbohydrate reserve (i.e. food volume ingested during the last meal) modified the SAT: the lower the reserve, the lower the SAT. As the time since the last meal and the quality of this food were the same for the high volume and low volume groups, changes in SAT must have been a consequence of a modification in sugar sensitivity according to the level of sugar reserve.

In this study, we developed a method of measuring and comparing sugar perception in ants under controlled conditions. Our results show the importance of identifying the appropriate stimulation site for each particular species. The SAT can then be evaluated by quantifying the occurrence of licking behaviour after stimulation without ambiguity. This protocol was successful for ants of different taxonomic groups and with different feeding habits. This new protocol is relatively fast and gives a reliable metric of taste in ants. Including ants as a new model in taste sensitivity studies will help to develop a better understanding of many aspects such as individual functions, network structure, group organization, pattern of interactions and relationships among individuals in a social insect society.

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Conflict of interest The authors declare that they have no conflict of interest.

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