RESEARCH ARTICLE

Taste preference for amino acids is dependent on internal nutritional state in Drosophila melanogaster

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SUMMARY

Like mammals, insects need to ingest proteins from foods because they cannot synthesise several amino acids. Amino acids are also essential nutrients for Drosophila melanogaster, especially for female egg production, but how flies detect amino acids and how the feeding response to amino acids is regulated are unknown. In this study, the two-choice preference test, the proboscis extension reflex test and a CAFE assay were performed to explore the ability of D. melanogaster to detect and discriminate amino acids. To determine whether D. melanogaster change their feeding preference to amino acids after being deprived of them, as previously reported in the locust, two groups of flies raised on the usual medium or on glucose medium were compared. Amino-acid-deprived flies demonstrated enhanced preference to an amino acid mixture and to several amino acids. These flies ingested amino acids even when they were replete with glucose. The proboscis extension reflex to particular amino acids was induced only in amino-acid-deprived flies. Our findings indicate that the sensitivity of labellar taste cells to amino acids may change when flies are deficient in amino acid supply, and also reveal that the detection pathways for individual amino acids may differ. We suggest the existence of an amino acid receptor and a monitoring system regulating the feeding responses to amino acids.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/16/2827/DC1

Key words: Drosophila, taste, amino acid, feeding, nutrition, oviposition, proboscis extension reflex, specific hunger.

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INTRODUCTION

To survive and reproduce, all organisms must locate and ingest nutrient substances present in the environment. How do they detect substances necessary for survival and reproduction? Gustation is governed not only by peripheral gustatory information but also by internal sensors (e.g. Gutierrez and Simon, 2011). Expression of taste receptors in the mammalian gut suggests that the intestines may also be involved in feeding regulation (Margolskee et al., 2007). In addition, absorbed chemicals circulating in the blood are sensed in the brain (Ren et al., 2009).

The possibility that an unidentified nutrient sensor might regulate feeding behaviour in animals is supported by recent findings. For instance, mutant mice that lack the function of sweet taste sensation can discriminate between an artificial sweetener, sucralose, and a sugar (de Araujo et al., 2008); Drosophila can learn the nutritional value of non-sweet sugar (Fujita and Tanimura, 2011; Burke and Waddell, 2011); and mutant flies with no taste reception can also detect a nutritional sugar (Dus et al., 2011).

It is not clear whether animals have an ability to discriminate and ingest particular nutrient chemicals in which they are deficient. An animal’s taste sensitivity to such a chemical might change upon being deficient in it. This response has been observed in the locust and in the cotton leafworm Spodoptera littoralis (Abisgold and Simpson, 1987; Simpson et al., 1991; Simmonds et al., 1992). Two recent studies revealed that mated females increase their preference for yeasty food and that yeast deprivation enhances the preference for yeast (Vargas et al., 2010; Ribeiro and Dickson, 2010). These studies also revealed that the TOR/S6K signalling pathway is involved in regulating the feeding behaviour. Furthermore, Vargas et al. (Vargas et al., 2010) proposed that serotonin is a key neurotransmitter regulating the behavioural switch. Thus, it is intriguing to ask whether Drosophila can sense amino acids by gustation. Sophisticated genetic tools have been developed in Drosophila to investigate and identify specific neuronal pathways and molecules (Venken et al., 2011). Drosophila was also recently recognised as an excellent model with which to study metabolism and homeostasis (Rajan and Perrimon, 2011). Here, we investigated how the feeding response to amino acids is regulated by nutritional state in Drosophila melanogaster.

Sugars are important energy sources, and amino acids are essential for protein synthesis. Although adult flies can survive with only sugar, proteins present in various cells of adult flies are continuously renewed at a slow rate through metabolic turnover (Maynard Smith et al., 1970). Female flies in particular require proteinaceous foods for egg production to propagate offspring. Females do not lay eggs without a protein supply (Bouletreau-Merle, 2011). Female flies in particular require proteinaceous foods for egg production to propagate offspring. Females do not lay eggs without a protein supply (Bouletreau-Merle, 1971). The essential amino acids for Drosophila are arginine plus the nine human essential amino acids. Egg production decreases remarkably if flies are given medium lacking one of these essential amino acids (Sang and King, 1961). A previous study reported that mated females show enhanced preference for yeasty food (Ribeiro and Dickson, 2010; Vargas et al., 2010), suggesting that they require amino acids contained in yeast.

Studies in the fleshfly and the blowfly have indicated that the labellar sensilla respond to amino acids (Shiraishi and Kuwabara, 1970; Shimada and Tanimura, 1981). Other studies have
demonstrated that taste cells in the mosquito and tsetse fly also respond to amino acids (Dimond et al., 1956; Van der Goes van Naters and Den Otter, 1998). By contrast, it is unknown whether the gustatory receptor neurons of *Drosophila* can respond to amino acids. Recent studies have revealed the molecular nature of sugar and bitter receptors (e.g. Montell, 2009), but the gustatory receptors for amino acids have not yet been identified.

Here, we first obtained basic data on the amino acid preferences of *D. melanogaster*. Using a two-choice preference test, we found that flies prefer an amino acid mixture to a low concentration of sugar, and that preference for each amino acid differs. We compared a group of flies raised in standard medium containing amino acid sources with another group raised in medium containing only glucose. The amino-acid-deficient flies demonstrated an enhanced preference for amino acids. This enhanced intake may be regulated at the level of peripheral sensitivity or by unknown internal mechanisms depending on amino acid levels in the haemolymph. Proboscis extension reflex test confirmed that the sensitivity to amino acids was elevated in amino-acid-deprived flies. These results raise questions concerning the neural mechanism modulating feeding behaviour depending on internal nutritional state.

**MATERIALS AND METHODS**

**Fly strains**

*Drosophila melanogaster* Meigen 1830 were reared on standard cornmeal–yeast–agar–glucose medium under a 12 h:12 h light:dark cycle (lights on at 06:00 h and off at 18:00 h) at 25°C. Canton-Special (CS) was used as a wild-type strain. Female and male flies were mixed and used for behavioural tests. Therefore, all the females were mated.

**Amino acid deprivation**

Flies were placed under two different conditions. Flies eclosed within 12 h were placed in vials containing either standard medium (SM) or 500 mmol l\(^{-1}\) glucose (1% agar; GM). The vials were replaced with new vials every 2 days and 1 day before the test. Flies were placed in these conditions for 6 days after eclosion. Flies were usually food deprived for 24 h in a vial containing a wet Kimwipe before tests. In other cases, flies were used without food deprivation.

**Chemicals**

As a tastant, D-glucose was obtained from Wako Pure Chemical Industries (Osaka, Japan). Agar (purified powder) was obtained from Nacalai Tesque (Kyoto, Japan). Amino acids of special grade were obtained from Nacalai Tesque, Wako Pure Chemical Industries or Sigma-Aldrich (St Louis, MO, USA). The composition of the amino acid mixture was as follows: 1 mmol l\(^{-1}\) arginine, 1.75 mmol l\(^{-1}\) aspartic acid, 2 mmol l\(^{-1}\) glutamic acid, 0.25 mmol l\(^{-1}\) tyrosine, 2.5 mmol l\(^{-1}\) tryptophan, and 5 mmol l\(^{-1}\) each of alanine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine. This solution corresponds to 82.5 mmol l\(^{-1}\) amino acids and was used without dilution or at 1/5 dilution. Food Blue No. 1 and Food Red No. 106 were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan).

**Proboscis extension reflex test**

Proboscis extension reflex (PER) testing was performed as described previously (Kimura et al., 1986). Flies were food deprived for 22 h and then fixed with myristyl alcohol on a plastic plate. Before testing the response to sugars and amino acids, fixed flies were left for 2 h in a humidified chamber. Experiments were conducted between 14:00 and 17:00 h. Flies were first satiated with water until they stopped responding to it. After testing with amino acid solution, flies were stimulated with water again. Stimulation was performed under a compound stereomicroscope, and one prothoracic leg was carefully touched with a small droplet of solution for 2 s. It was difficult to touch the labellar sensilla with a droplet without allowing flies to drink it. The proboscis was quickly touched by a cone-like wick made from a strip of Kimwipes (Shiraiwa and Carlson, 2007). Proboscis extension was observed within 2 s.

**CAFE assay**

To quantitate the amino acid intake we used a capillary feeder (CAFE) assay. The original method (Ja et al., 2007) was modified as previously reported (Fujita and Tanimura, 2011), and the assay was performed without food deprivation. Vials were placed in a humidified Peltier incubator (CN-25C, Mitsubishi Electric Co., Tokyo, Japan) in darkness. Experiments were conducted from 15:00 to 21:00 h.

**RESULTS**

**Two-choice preference test**

In a previous two-choice preference test, wells on the microtest plates were filled with agar solution (Tanimura et al., 1982), but several amino acids hindered the agar solution from solidifying. Some amino acids also tend to be chemically modified after repeated heating to melt agar. Therefore, the two-choice method using a Petri dish system previously developed in our laboratory was applied. This method does not employ agar; instead, amino acid solution is applied to pieces of filter paper placed on a Petri dish (supplementary material Fig. S1A).

We first compared this assay method with the microtest plate method by conducting the two-choice preference test between 10 and 5 mmol l\(^{-1}\) glucose. The PI values obtained by the microtest plate method were significantly higher than those obtained by the Petri dish method (data not shown). Nevertheless, flies chose the
higher concentration of glucose even in the Petri dish method. Next, we compared 10 mmol l\(^{-1}\) glucose and 5 mmol l\(^{-1}\) glucose alternatively combined with the red and the blue colours (supplementary material Fig. S1B). The PI values were not significantly different between the two combinations. Thus, in the following two-choice tests, we always used an amino acid solution coloured blue and a glucose solution coloured red.

**Amino-acid-deprived flies ingest more amino acids**

Intake of amino acids is necessary for female flies to produce eggs, but we do not know whether flies are able to detect amino acids by gustation and/or internal nutrient sensing. We performed a two-choice preference test between an amino acid mixture and a low concentration of sugar (Fig. 1A). We found that flies lacking an amino acid supply preferred amino acids significantly more than flies raised in SM. The preference for amino acids increased depending on the concentration of the amino acid mixture (data not shown).

Because the two-choice preference test could only evaluate the comparative preference to amino acids against glucose, the CAFE assay was performed to measure the amount of amino acid intake under a no-choice condition (Fig. 1B). We determined the amount of intake of amino acid mixture (diluted 1/5) every 1 h. Significant differences between SM and GM were observed in males at 5 h and females at 4 h. The difference in the amount of intake exceeded sevenfold in females and was approximately threefold in males at 6 h. These results suggest that flies change preference for amino acids and increase their intake depending on their internal amino acid level.

**Taste preference for individual amino acids**

We found that flies preferred the amino acid mixture and their preference and amount of intake increased when flies were raised on GM. We then asked which amino acids are responsible for the attraction and tested the preference for 20 individual amino acids using flies raised under SM and GM conditions. We performed a two-choice preference test between 10 mmol l\(^{-1}\) amino acid and 5 mmol l\(^{-1}\) glucose and calculated PI values (supplementary material Table S1). Glycine showed high PI values in both female and male flies raised on SM. However, the value did not significantly increase when flies were raised on GM. GM flies preferred cysteine, phenylalanine and tyrosine to glycine.

Fig. 2 shows the ratio between the two PI values obtained from the two groups (Ratio=[(PI of GM flies)/(PI of SM flies)]–1). The ratios demonstrated large values in females for cysteine, phenylalanine, threonine and tyrosine, and in males for histidine and leucine. The amino acids that demonstrated a significant ratio increase differed between females and males. Demonstration of such a ratio increase was unrelated to the classification of amino acids as essential or non-essential or their chemical properties. These results indicate that flies can sense at least these several amino acids using gustation and/or internal homeostasis and have the ability to modulate feeding behaviour depending on internal amino acid levels.

**Sugar-satiated flies selectively ingest deficient nutrients**

Two-choice preference tests thus far were performed using flies food deprived for 24 h so as to maximize the proportion of flies that initiate feeding in 2 h. However, the feeding behaviour of food-deprived GM flies reflects the lack not only of amino acids but also sugar. Therefore, we performed two-choice preference tests using flies without food deprivation solely to evaluate the influence of amino acid deficiency on feeding behaviour (Fig. 3). First, we performed a two-choice preference test using a mixture of 20 amino acids and glucose. Approximately 40% of SM flies were coloured in 2 h. By contrast, 70–80% of GM flies were coloured.

When we compared the preference between SM and GM coloured flies, the proportion of flies that preferred the amino acid mixture increased among GM flies, whereas the proportion of flies that preferred glucose was similar between GM and SM flies (Fig. 3A). Remarkably, GM flies ingested amino acids although they were replete with glucose. Fig. 3B,C shows the results of two-choice tests between
phenylalanine or cysteine and glucose using flies without food deprivation. Both sexes of flies raised on GM preferred phenylalanine and cysteine. However, a significant increase in the preference for these amino acids was not observed in male flies with food deprivation (Fig. 2). This difference may be caused by the fact that male flies are more readily food deprived (see Discussion). The preference for glycine did not increase by amino acid deprivation (Fig. 3D).

**Amino-acid-deprived flies show PER to amino acids**

We tested both GM and SM flies to determine whether stimulation of the tarsal and labellar chemosensilla induced PER. We used only female flies because their internal need for amino acids is evident. A positive reflex to labellar stimulation with the amino acid mixture or individual amino acids was observed in flies without food deprivation raised on GM, but not in flies raised on SM (data not shown). However, because the percentage of flies that demonstrated a positive reflex was small, we chose to examine food-deprived flies. We tested three amino acids, phenylalanine, cysteine and glycine, that demonstrated high PI values in the two-choice preference test. Tarsal stimulation by the amino acid mixture or each of the three amino acids failed to induce a reflex (Fig. 4A), suggesting that tarsal gustatory receptor cells show low sensitivity to amino acids. Next, we stimulated the labellar sensilla with a wick wetted with amino acid solution. Robust reflex responses were induced by stimulation.

![Graph](image-url)

**Fig. 2.** Effect of amino acid deprivation on preferences of flies for individual amino acids. Two-choice preference tests were performed between 20 amino acids (10 mmol l⁻¹, except tyrosine at 1.8 mmol l⁻¹) and 5 mmol l⁻¹ glucose. Preference ratios were calculated based on the data shown in supplementary material Table S1 (N=5); Ratio=[(PI of GM flies)/(PI of SM flies)]−1. Significant changes were observed in females for cysteine, phenylalanine, threonine and tyrosine, and in males for histidine and leucine (Wilcoxon–Mann–Whitney test, *P<0.05, **P<0.01, N=5).

**Fig. 3.** Two-choice preference test between amino acids and glucose using flies without food deprivation. Tests were conducted between 10 mmol l⁻¹ glucose and amino acid mixture (A) and between 5 mmol l⁻¹ glucose and phenylalanine (B), cysteine (C) and glycine (D). The height of each column represents the ratio of coloured flies that ingested either amino acid or glucose. In each column, the grey portion indicates the ratio of flies that preferred amino acid, and the black portion indicates the ratio of flies that preferred glucose. These values were calculated by (B+M/2)/(B+R+M+O) and (R+M/2)/(B+R+M+O), where B, R and M represent blue, red and purple, respectively, and O represents the number of uncoloured flies. Significant differences in the proportion of flies that preferred amino acids were observed in both sexes between the SM and GM groups, except for glycine (Wilcoxon–Mann–Whitney test, *P<0.05, **P<0.01, N=5). No significant differences in the proportion of flies that preferred glucose were observed in both sexes between the SM and GM groups for amino acid mixture, phenylalanine or cysteine (Wilcoxon–Mann–Whitney test, P>0.10, N=5).
with amino acid solutions in GM flies, but no response was observed in SM flies (Fig. 4B). Nearly 40% of GM flies responded to the amino acid mixture. These results indicate that the sensitivity of labellar chemosensilla to amino acids increased after internal amino acid deprivation. As a positive control, the labellar chemosensilla were stimulated with 20 and 100 mmol l⁻¹ glucose (supplementary material Fig. S2). PER was observed when the tarsus was stimulated with 100 mmol l⁻¹ glucose and when the labellum was stimulated with 20 and 100 mmol l⁻¹ glucose. SM and GM flies demonstrated no significant differences in PER ratios to 100 mmol l⁻¹ glucose with labellar stimulation (Wilcoxon–Mann–Whitney test, $P=0.72, N=40$), whereas GM flies demonstrated a lower response to tarsal stimulation with 100 mmol l⁻¹ glucose than SM flies (Wilcoxon–Mann–Whitney test, $P=0.001, N=40$). At present, we have no explanation to address why GM flies demonstrate a lower response to glucose. Nonetheless, GM flies respond to amino acids, and this behaviour is not due to hyperactivity of GM flies. The stimulating effectiveness of 20 mmol l⁻¹ phenylalanine on GM flies was between that of 20 and 100 mmol l⁻¹ glucose. Glycine induced no PER either by tarsal or labellar stimulation, although glycine is rather preferred by flies. These results suggest that sensitivity to particular amino acids is elevated in GM flies, but that feeding responses to amino acids are not totally regulated by external taste organs.

**DISCUSSION**

The present study showed that *D. melanogaster* sense and demonstrate preferences to amino acids. First, we performed a two-choice preference test between glucose and an amino acid mixture. To test whether the preference for amino acids was affected by the nutritional state of flies, we compared flies raised on SM with those raised on GM. We found that flies raised on GM preferred amino acids and ingested relatively larger amounts of amino acid solution. These results indicate that *D. melanogaster* have a gustatory sense to detect amino acids and are able to change preference for amino acids depending on their internal nutritional state.

One unresolved question addressed in this study was whether *D. melanogaster* sense the taste of individual amino acids. In larger flies, some amino acids taste sweet and others are salty or tasteless (Shiraishi and Kuwabara, 1970). The results of our two-choice test indicate that *D. melanogaster* prefer several amino acids. Preference to an amino acid was unrelated to its chemical properties or whether it was essential or non-essential.

We found that amino-acid-deprived flies ingest amino acids even when they are replete with glucose. *Drosophila melanogaster* demonstrate an ability to monitor internal haemolymph amino acid levels to control the feeding response to amino acids. We found that taste preference for phenylalanine and cysteine was elevated by amino acid deprivation in females, whereas in males, preference for these two amino acids did not increase after food deprivation. By contrast, the taste preference for phenylalanine and cysteine was enhanced in male GM flies without food deprivation. If flies are food deprived, they are hungry for sugar. Food-deprived GM flies are hungry both for sugar and amino acids. Then under a two-choice test between amino acids and sugar, flies are attracted by both substances. When GM flies are tested without food deprivation, they should be attracted by amino acids. Thus, we propose that amino acid requirements are better evaluated using flies without food deprivation.

Amino-acid-deprived flies demonstrated the PER to the amino acid mixture as well as to phenylalanine and cysteine, whereas flies raised on normal medium did not. These results are striking in their indication that the taste sensitivity of labellar taste cells is increased by internal nutritional condition. When locust nymphs were placed under protein-deficient conditions for just 4 h, electrophysiological studies showed that the taste cells responded more vigorously to amino acid mixtures (Simpson et al., 1991). Locust nymphs are therefore likely to require a continuous amino acid supply. In *D. melanogaster*, amino acid deprivation for several days is necessary to induce the specific preference to amino acids. Thus, change in the peripheral sensory system may account for enhanced feeding responses to amino acids upon amino acid deprivation. However, although glycine was the most potent attractant for flies, no PER was observed with glycine stimulation of the tarsus or labellum, even at a high concentration. This result suggests that glycine may be sensed by an internal taste receptor. Pharyngeal taste cells or interpseudotracheal papillae taste cells are possible glycine sensors, and in addition, a sensor may be present in the alimentary canal.

How does deprivation lead to taste sensitivity to amino acids? The neural transmission of the gustatory receptor neurons that sense amino acids might be enhanced (Inagaki et al., 2012). Because the taste sensitivity to amino acids specifically increases, an alternative possibility is that deprivation induces expression of a gene or genes encoding a putative taste receptor for amino acids. In mammals, the heterodimer T1R1+T1R3 functions as an amino acid receptor (Nelson et al., 2002). In *Drosophila*, 68 gustatory receptor genes are known, but the function of only a few of these genes has been elucidated (Montell, 2009). Genetic tools available in *Drosophila* should help to identify amino acid receptors.

Typically, chemosensilla in *Drosophila* contain four gustatory receptor cells that respond to sugar (S), water (W), salt at low concentration (L1) and salt at high concentration (L2), respectively (Tanimura et al., 2009). Among them, S, W and L1 cells receive signals from phagostimulants; therefore, S and/or L1 cells may sense...
amino acids. We are now conducting electrophysiological recordings from three types of labellar chemosensilla in amino-acid-deprived flies to identify amino-acid-responsive gustatory cell types.

Mating is known to modify the feeding behaviour of female *Drosophila*, and the sex peptide is a key molecule involved in this change (Yapici et al., 2008). Two reports have demonstrated an enhanced preference for yeast in mated females (Ribeiro and Dickson, 2010; Vargas et al., 2010). It will therefore be interesting to determine whether taste sensitivity to amino acids is influenced by mating, and to investigate the molecular and neural mechanisms involved in the behavioural plasticity. Intake of amino acids is also linked to longevity (Lee et al., 2008; Grandison et al., 2009), and elucidation of the amino acid detection system may help to understand these relationships.

Specific hunger for proteins was first reported in the blowfly and locust many years ago (Dethier, 1976; Simpson et al., 1991). Our novel finding that the taste sensitivity to amino acids is regulated by the internal nutritional state in *D. melanogaster* indicates that this species provides an excellent system in which to investigate the neuronal and molecular mechanisms involved in such changes by employing electrophysiological and genetic approaches.

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**FUNDING**

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**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>GM</th>
<th>glucose medium</th>
<th>PER</th>
<th>proboscis extension reflex</th>
<th>PI</th>
<th>preference index</th>
<th>SM</th>
<th>standard medium</th>
</tr>
</thead>
</table>

**REFERENCES**


Fig. S1

A

B

10 mmol l⁻¹ glucose (Red) vs. 5 mmol l⁻¹ glucose (Blue)

5 mmol l⁻¹ glucose (Red) vs. 10 mmol l⁻¹ glucose (Blue)
Fig. S2

A

PER ratio

<table>
<thead>
<tr>
<th></th>
<th>20 mmol l⁻¹ glucose</th>
<th>100 mmol l⁻¹ glucose</th>
<th>water</th>
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B

<table>
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<tr>
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<th>20 mmol l⁻¹ glucose</th>
<th>100 mmol l⁻¹ glucose</th>
<th>water</th>
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Table S1. Two-choice preference test between 20 amino acids and glucose: effect of amino acid deprivation on *Drosophila melanogaster*.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Female</th>
<th>Male</th>
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<tbody>
<tr>
<td></td>
<td>SM</td>
<td>GM</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.61±0.14</td>
<td>0.54±0.19</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.51±0.13</td>
<td>0.65±0.10</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.41±0.18</td>
<td>0.38±0.16</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.58±0.13</td>
<td>0.42±0.16</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.40±0.13</td>
<td>0.81±0.07**</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.40±0.06</td>
<td>0.33±0.24</td>
</tr>
<tr>
<td>Glutamine</td>
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<td>0.58±0.04</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.63±0.08</td>
<td>0.73±0.24</td>
</tr>
<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Isoleucine</td>
<td>0.39±0.15</td>
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<tr>
<td>Leucine</td>
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<tr>
<td>Lysine</td>
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<td>0.43±0.14</td>
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<td>Phenylalanine</td>
<td>0.33±0.14</td>
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<tr>
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<tr>
<td>Threonine</td>
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<td>0.69±0.09**</td>
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<tr>
<td>Tyrosine</td>
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<td>0.77±0.13*</td>
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<tr>
<td>Valine</td>
<td>0.40±0.10</td>
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</table>

Mean ± s.d. values of the preference index are shown. Values between standard medium (SM) and glucose medium (GM) were statistically analysed using the Wilcoxon–Mann–Whitney test (*P*<0.05; **P*<0.01).