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Single amino acids in sucrose rewards modulate feeding and associative learning in the honeybee

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ABSTRACT

Obtaining the correct balance of nutrients requires that the brain integrates information about the body's nutritional state with sensory information from food to guide feeding behaviour. Learning is a mechanism that allows animals to identify cues associated with nutrients so that they can be located quickly when required. Feedback about nutritional state is essential for nutrient balancing and could influence learning. How specific this feedback is to individual nutrients has not often been examined. Here, we tested how the honeybee's nutritional state influenced the likelihood it would feed on and learn sucrose solutions containing single amino acids. Nutritional state was manipulated by pre-feeding bees with either 1 M sucrose or 1 M sucrose containing 100 mM of isoleucine, proline, phenylalanine, or methionine 24 h prior to olfactory conditioning of the proboscis extension response. We found that bees pre-fed sucrose solution consumed less of solutions containing amino acids and were also less likely to learn to associate amino acid solutions with odours. Unexpectedly, bees pre-fed solutions containing an amino acid were also less likely to learn to associate odours with sucrose the next day. Furthermore, they consumed more of and were more likely to learn when rewarded with an amino acid solution if they were pre-fed isoleucine and proline. Our data indicate that single amino acids at relatively high concentrations inhibit feeding on sucrose solutions containing them, and they can act as appetitive reinforces during learning. Our data also suggest that select amino acids interact with mechanisms that signal nutritional sufficiency to reduce hunger. Based on these experiments, we predict that nutrient balancing for essential amino acids during learning requires integration of information about several amino acids experienced simultaneously.

1. Introduction

Learning about food is a mechanism that provides animals with flexibility in food choices that improves chances of survival. The taste of food is an important cue used during learning to identify palatable and nutritious foods. Insects detect nutrients like sugars and amino acids (AAs) using sensory neurons housed in sensilla on the mouthparts, antennae, and feet (Scott et al., 2001; Amrein and Thorne, 2005). During appetitive learning, information from taste sensilla is integrated in the brain with visual, olfactory, and tactile cues to form a learned association (Hammer, 1993). This information can be further modulated by cues that arise after food has been consumed. For example, when nutrients have been consumed in association with taste cues, animals form lasting memories of food (Sclafani and Ackroff, 1994; Burke and Waddell, 2011). The taste of food is also an important cue associated with the post-ingestive consequences of eating toxins or unsuitable foods (Garcia et al., 1955; Behmer et al., 1999; Zhang et al., 2005; Wright et al., 2010; Simoes et al., 2012).

Nutrient balancing is a complex process in which animals integrate taste cues with post-ingestive information about food quality to obtain optimal nutrition (Simpson and Raubenheimer, 1997). Most studies of associative learning use carbohydrates to reward animals, but animals can also learn to associate chemical cues like odours and tastes with the presence of protein or AAs in food (Simpson and White, 1990; Raubenheimer and Tucker, 1997). During nutrient balancing, the body detects deficiencies in nutrition and modulates food intake to identify and consume foods that satisfy this deficiency. When AAs or proteins are deficient in diet, insects such as locusts and Drosophila have greater taste sensitivity towards solutions containing AAs (Abisgold and Simpson, 1987; Simpson et al., 1991; Toshima and Tanimura, 2012). Deficiencies also influence how quickly animals learn cues associated with appropriate nutrients. Locusts are more likely to learn to associate odours and tastes with protein in food if they are protein-deficient (Raubenheimer and Tucker, 1997).

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It remains unclear whether nutritional oversufficiency can also influence learning. Just as appetitive learning is enhanced by deficiencies, it is possible that aversion learning could be driven by too much of specific nutrients as over consumption of nutrients can be metabolically costly to animals (Zanotto et al., 1994, 1997). Too much protein or essential AAs in diet, for example, has high costs for social insect workers, as it decreases lifespan (Pirk et al., 2010; Dussutour and Simpson, 2012; Paoli et al., 2014). If animals are forced to consume foods that are nutritionally deficient in specific essential AAs but sufficient in others, they learn to avoid these foods (Simpson et al., 1991; Koehnle et al., 2003; Toshima and Tanimura, 2012). However, few studies have identified whether nutritional oversufficiency produces learned aversions towards specific nutrients such as individual AAs when they are overabundant.

Honeybees learn to associate visual and olfactory cues with food during foraging for nectar and pollen on flowers, and for this reason, have become an important model system for the study of learning and memory (Menzel, 1983; Hammer and Menzel, 1995). Foraging worker honeybees collect floral nectar but also use it for their own nutritional needs. Floral nectar contains the sugars, sucrose, glucose and fructose, but also contains essential and non-essential AAs (Baker and Baker, 1973; Nicolson et al., 2007). High concentrations of AAs are toxic to honeybee foragers but low concentrations are nutritionally important (Paoli et al., in review). Few studies of appetitive learning in bees have used AAs in rewards during associative learning, and most of these have concluded that the honeybee's responses towards AAs are often indistinguishable from a sugar solution (Inouye and Waller, 1984; Kim and Smith, 2000). One study showed that the forager honeybee's intake of proline, a non-essential AA used by bees as fuel for flight commonly found in nectar (Micheu et al., 2000; Suarez et al., 2005) is modulated by its concentration in solution (Carter et al., 2006), such that bees preferred concentrations around 6 mM and consumed significantly less of sucrose solution containing 100 mM proline. It remains unclear whether the bees in this study were learning to avoid the 100 mM solution or whether they simply avoided drinking it because it tasted repellent.

Here, we tested how nutritional state affected the taste of specific AAs (isoleucine, proline, phenylalanine, and methionine) and whether or not bees learned to avoid relatively high (100 mM) concentrations of these AAs in sucrose solutions. We first tested how feeding with sucrose solution containing a specific AA influenced whether or not bees would consume the solution the next day. We also tested how being fed with sucrose solutions containing individual AAs influenced the honeybee's rate of learning when these solutions were used as the reward during an olfactory appetitive conditioning task.

2. Methods

2.1. Subjects

Returning forager honeybees (*Apis mellifera Buckfast*) were collected from a population developed at the National Bee Unit (York, UK). They were captured at a hive entrance between the months of March–July 2011 at Newcastle University and restrained as described in Wright and Smith (2004). They were anesthetized on ice for ~3 min in glass vials, and then placed in a restraining harness. Bees were restrained within 30 min of catching them at the colony entrance. Restrained bees were used in the feeding assay and in the learning assay. In these assays, bees were commonly fed to satiety (i.e. until they would no longer consume solution or lift the proboscis to feed when stimulated on the antennae). Feeding was accomplished using a 0.2 ml Gilmont micrometer syringe (Gilmont Instruments). To feed the bees, each was tapped on the antennae briefly with 1 M sucrose solution to elicit proboscis extension and then fed 0.4 μl droplets on the proboscis until each bee would no longer consume the solution (i.e. satiety).

2.2. Solutions

Solutions of 1 M sucrose and 1 M sucrose with 100 mM of a single amino acid (isoleucine, methionine, phenylalanine or proline, powdered forms, Sigma–Aldrich) were made using distilled water.

2.3. Influence of AA solutions on feeding

Bees were restrained as above. A one hour after they were restrained, they were fed to satiety with one of the following solutions: 1 M sucrose or a 1 M sucrose solution containing 100 mM of the following AAs: isoleucine, proline, phenylalanine, or methionine. The volume required to produce satiety was measured; bees generally achieved this within 1–2 min of the start of feeding. After 24 h, each bee was fed to satiety again using the same solution they had been fed the day before. The volume required to produce satiety was measured.

We also tested whether the AAs elicited PER on their own when applied to the mouthparts. Bees were restrained as above and fed 1 M sucrose to satiety. After 24 h, each bee was tapped on the antenna with 1 M sucrose and then fed to satiety using one of the compounds in solution: sucrose, isoleucine, phenylalanine, proline, or methionine. The following concentrations were tested: 100 μM, 1 mM, 10 mM, 100 mM. Separate groups of bees ($N = 30$ each) were tested with each concentration.

2.4. Olfactory conditioning protocol

Subjects for the olfactory PER conditioning assay were restrained as above and fed to satiety with either 1 M sucrose or with 1 M sucrose containing 100 mM of isoleucine, methionine, proline, or phenylalanine and left for ~18–24 h. Immediately prior to conditioning, bees were tested for responsiveness to feed by touching both antennae with 1 M sucrose to elicit PER; those that failed to respond were not used in the conditioning assay. Subsequently, each subject underwent a 12 trial olfactory conditioning paradigm of the PER (Bitterman et al., 1983; Wright et al., 2010) with a 5 min inter-trial interval (ITI). The conditioned stimulus (CS) was a 4 s odour pulse controlled by a programmable logic controller; the odour was delivered via a 5 mm × 75 mm glass tube containing filter paper covered with a 3 μl aliquot of pure odour solution (1-hexanol, Sigma–Aldrich). The unconditioned stimulus (US) was presented approximately 3 s after the start of the CS. The US was a 0.4 μl droplet of experimental solution (1 M sucrose or 100 mM AA in 1 M sucrose) delivered via a 0.2 ml Gilmont syringe. On each trial of conditioning, following CS delivery, a 1 M sucrose solution was presented to both antennae to provoke PER, then to the US was presented to the proboscis for consumption. If a subject refused to eat the US, they were force-fed the solution. Subjects responding spontaneously to the conditioned stimulus on the first trial were excluded from analysis. The conditioned response was recorded as a binary variable (PER or no PER) following CS delivery just prior to US presentation.

2.5. Statistical analysis

All data analyses were performed using SPSS 21. Pearson's correlation and a linear regression (lreg) were carried out for the 2-day gustatory mouthparts assay data. The amount consumed gustatory assay was compared using 1-way ANOVA. PER and conditioning data were analysed using logistic regression or a

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