Olfactory Detectability of L-Amino Acids in the European Honeybee (*Apis mellifera*)

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Abstract

The honeybee is one of several insect model systems for the study of olfaction, yet our knowledge regarding the spectrum of odorants detectable by *Apis mellifera* is limited. One class of odorants that has never been tested so far are the amino acids, which are important constituents of floral nectar. Using the proboscis extension response paradigm, we assessed whether the odor of amino acids is detectable for honeybees and determined olfactory detection thresholds for those amino acids that were detectable. We found that honeybees are able to detect the odor of 5 of the 20 proteinogenic amino acids when presented at a concentration of 50 or 100 mM. Median olfactory detection thresholds for these 5 amino acids were 12.5 mM with L-tyrosine and L-cysteine, 50 mM with L-tryptophan and L-asparagine, and 100 mM with L-proline. All detection thresholds were much higher than reported concentrations of amino acids in floral nectars. We conclude that in the foraging and feeding context, honeybees are likely to detect amino acids through taste rather than olfaction. Across-species comparisons of the detectability of and sensitivity to amino acids suggest that the number of functional genes coding for olfactory receptors may affect both a species' sensitivity for odorants and the breadth of its spectrum of detectable odorants.

Key words: amino acids, *Apis mellifera*, detection, honeybee, olfaction, sensitivity

Introduction

The honeybee's sense of smell has been well studied, both anatomically (e.g., Schröter and Malun 2000; Haddad et al. 2004) and physiologically (e.g., Stopfer et al. 1997; Galizia et al. 1999; Sachse et al. 1999; Deisig et al. 2006; Fernandez et al. 2009; for review, see Laurent 2002). Several studies demonstrate that honeybees have an excellent ability to quickly and robustly learn the reward value of new odors (e.g., Vareschi 1971, Gil et al. 2007; Wright, Choudhary, et al. 2009; Wright, Smith, et al. 2009; for review, see Menzel 2008) and to discriminate between monomolecular odorants that are components of flower odors (Laska et al. 1999; Laska and Galizia 2001). However, little is known about the spectrum of odorants that honeybees are able to perceive. This is of interest given that honeybees possess only ≈160 functional genes coding for olfactory receptors (Robertson and Wanner 2006), a considerably smaller number of olfactory receptor types compared with mammals, such as mice (≈1060, Nei et al. 2008) and even humans (≈390, Niimura and Nei 2006). Several authors have argued that the number of functional olfactory receptor genes should be predictive of a species' sensitivity for odorants and of the breadth of its

spectrum of detectable odorants (Rouquier et al. 2000; Gilad et al. 2004).

One class of odorants that has never been tested with honeybees so far are the amino acids. This is surprising given that it is widely known that free amino acids are the second most abundant group of compounds in nectar after carbohydrates (Baker HG and Baker I 1973, 1986). Behavioral observations have shown that honeybees prefer natural and artificial sugar solutions that contain amino acids (Inouye and Waller 1984; Alm et al. 1990; Carter et al. 2006; Petanidou et al. 2006), but the sensory basis of these preferences is unknown. Because amino acids evoke specific smell sensations in vertebrates, such as fish, mice, and primates including humans (Nikonov and Caprio 2007; Laska 2010; Wallén et al. 2012), we hypothesize here that honeybees might also be able to perceive the odor of amino acids.

The aim of the present study therefore was to evaluate whether and at which concentrations the odors of amino acids are detectable for honeybees. We used a classical conditioning paradigm, the proboscis extension response (PER), which takes advantage of the honeybee's ability to quickly

build a robust association between an odor stimulus and a sucrose reward (Bitterman et al. 1983). This method is widely used to study olfactory perception in honeybees (e.g., Deisig et al. 2002; Wright et al. 2002; Guerrieri et al. 2005) and suitable for the determination of behavioral detection thresholds.

Materials and methods

Animals

Honeybees (*Apis mellifera L.*) were kept on the grounds of the University of Exeter. Departing foragers were collected, cooled down, and restrained individually in metal tubes so that only their mouthparts and antennae were free to move. Each subject was fed with 50% sucrose solution until satiety and left for approximately 20 h before conditioning. At least 20 min prior to the start of each experiment, bees were stimulated with a droplet of sucrose solution on their antennae to provoke the PER. Only those animals that extended their proboscis were used in the conditioning experiments. Each bee was fed with water prior to testing until they no longer extended their proboscis when stimulated with water on their antennae. This was done to minimize the risk of bees responding to water vapor.

Odor stimuli

The following amino acids were used as odor stimuli: L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. These substances comprise the 20 proteinogenic amino acids and have all been reported as constituents of floral nectar (Baker HG and Baker I 1973, 1986). All amino acids were obtained from Sigma-Aldrich and had a purity of 99.8%. They were diluted in demineralized water to obtain a specific molar concentration. Room temperature and thus stimulus temperature was kept constant at 21–22 °C.

Experimental set-up

A 60 mL odor saturator bottle containing 20 mL of a given odorant at a desired dilution served as odor source. The bottle was connected with silicone tubing to a pump which provided an airstream of 160 L/h and to a plastic syringe directed at the head of a bee, at a distance of 4 cm, which delivered the odorized airstream. The airstream was gated by a solenoid valve and extracted by an exhaust system located behind the bee. Each stimulus was presented for 4 s and consisted either of odorized air from the headspace inside the odor saturator bottle or clean air.

The bee was placed in front of the syringe 25 s prior to odor delivery. Three seconds after stimulus onset (CS, conditioned stimulus), the bee received 30% sucrose solution as

an unconditioned stimulus (US) to the antennae to initiate proboscis extension, and then the sucrose was delivered to the proboscis. Thus, the interstimulus interval was 3 s, and the overlap between CS and US was 1 s. The bee was then left in front of the syringe for another 25 s before returning to its resting position.

Experiment 1: olfactory detectability of amino acids

Experimental procedure

Bees were collected in the morning around 11.00 AM and in the afternoon around 04.00 PM, fed, and tested the next day at 7.00 AM and 12.00 PM as described above. Eighty bees were tested per amino acid, giving a total sample size of 1600 bees. All amino acids were tested both in morning and afternoon sessions. Half of the bees were conditioned with the odor of a given amino acid as the CS. As a control, the other half of the bees in each session were conditioned with the solvent (demineralized water) only. Each bee received 5 conditioning trials with an intertrial interval (ITI) of 10 min. The sixth trial was conducted as an unrewarded test 10 min after the last conditioning trial to evaluate the detectability of each amino acid and of the solvent alone. An ITI of 10 min was selected based on the results of previous studies, which suggest that this period of time is sufficient in order to minimize the possibility of adaptation effects negatively affecting the outcome of threshold determinations.

Amino acids were presented at 100 mM, except for L-glutamic acid, L-aspartic acid, and L-tyrosine, which were presented at 50 mM due to their limited solubility. L-tryptophan has a hydrophobic side chain and thus has also a limited solubility. A saturated aqueous solution of L-tryptophan corresponding to a concentration of 56 mM was therefore used in lieu of a 100 mM solution.

Data analysis

During conditioning and in the final test trial, proboscis extensions to the CS (prior to the delivery of the US) were measured as binary response variable. Responses were calculated as the percentage of all bees tested that responded to the odor of a certain amino acid. Responses in the unrewarded test trial (sixth trial) were compared using the Fisher's Exact test between bees receiving the odor of an amino acid and control bees receiving only solvent stimulation (demineralized water). An amino acid was considered to be detectable if significantly more bees responded with proboscis extension to that odor compared with the solvent alone. The alpha level was set at 0.05.

Experiment 2: olfactory detection threshold

Experimental procedure

A total of 150 new bees were conditioned in this experiment, but only bees that reliably learned the odor were used.

Olfactory detection thresholds for the 5 amino acids that were detectable in experiment 1 (L-asparagine, L-cysteine, L-proline, L-tryptophan, and L-tyrosine) were determined by testing 8–10 bees per amino acid. All odorants, at different concentrations, were freshly prepared each day, immediately prior to the start of the experiment.

Groups of 5 bees were differentially conditioned. The amino acid odor (CS+) was paired with a reward in form of a droplet of 30% sucrose solution delivered to the antennae and proboscis. The solvent (demineralized water, CS–) was paired with an aversive US, 1.5 M NaCl solution applied to the antennae only (Wright, Smith, et al. 2009). In the initial training phase, the bees were presented with a total of 20 trials (10 CS+ and 10 CS–, delivered alternately). The ITI was 5 min between CS+ and CS– and 10 min between the CS+ trials. Only bees that showed significant discrimination between the solvent and the amino acid odor were used further in the threshold experiment. To reach significance, a bee needed to give 6 correct responses in a row or at least 15 correct responses out of 20 (binomial test, $P < 0.05$). Bees that gave less than 15 correct responses out of 20 were not used further. Using this procedure, we were able to determine thresholds in motivated individuals and to reduce the number of animals used in the experiment.

In the threshold experiments, bees were trained to discriminate between the solvent and a given amino acid presented at different concentrations in descending order. Bees were exposed to variable number of trials until showing significant discrimination between the CS+ of a given concentration (Table 1) and the solvent (CS–). The number of trials was, however, limited to maximally 30 trials (15 CS+ and 15 CS–) per concentration step. A one-tailed binomial test was used to determine significant discrimination. Only the bees that significantly discriminated between these 2 stimuli were tested with the next (lower) concentration step (Table 1). This process continued until a bee failed to reach the significance criterion.

Data analysis

It was recorded whether a bee extended its proboscis, or not, in response to the delivery of the odor CS+ or solvent CS–,

before receiving the appetitive (sucrose) or aversive (NaCl) reinforcer. A correct response for a CS+ trial was proboscis extension, whereas a correct response for a CS– trial was an absence of proboscis extension. Significant detection for every concentration step was determined using a one-tailed binomial test ($P < 0.05$).

Results

Olfactory detectability of amino acids

When presented at a concentration of 50 or 100 mM, the honeybees detected the odors of 5 of the 20 amino acids tested (L-tyrosine, L-proline, L-cysteine, L-tryptophan, and L-asparagine) (Figure 1, left column). Response scores in the sixth trial for these 5 amino acids ranged from 42.5% for L-tyrosine to 27.5% for L-tryptophan and L-asparagine, respectively. These scores were significantly higher than the corresponding detection scores in the sixth trial for the solvent ($P < 0.05$, Fisher's Exact test).

The odors of the 15 remaining amino acids tested (L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-serine, L-threonine, and L-valine) were not detectable for the honeybees (Figure 1B–D). Response scores in the sixth trial for these amino acids did not differ significantly from the corresponding detection scores in the sixth trial for the solvent ($P > 0.05$, Fisher's Exact test).

Olfactory detection threshold

Figure 2 shows the distribution of individual olfactory detection thresholds for the 5 amino acids that were found to be detectable for the honeybees.

L-tyrosine

Of 20 bees tested for their ability to discriminate L-tyrosine presented at 50 mM from the solvent, 9 succeeded. Two of these 9 bees were found to have their olfactory detection threshold at 25 mM, 5 at 12.5 mM, and 2 at 6.25 mM, respectively (Figure 2A). Thus, the median olfactory detection threshold concentration was 12.5 mM for L-tyrosine.

L-proline

In the initial training phase, 10 of 25 bees successfully detected L-proline when presented at 100 mM. Eight of these 10 bees were found to have their olfactory threshold at 100 mM, and one bee each at 75 mM and another at 50 mM (Figure 2B). Thus, the median olfactory detection threshold for L-proline was 100 mM.

L-cysteine

Ten of 30 initially trained bees discriminated between 100 mM L-cysteine and the solvent. Seven of these bees were

Table 1 Concentration steps used in the threshold experiment

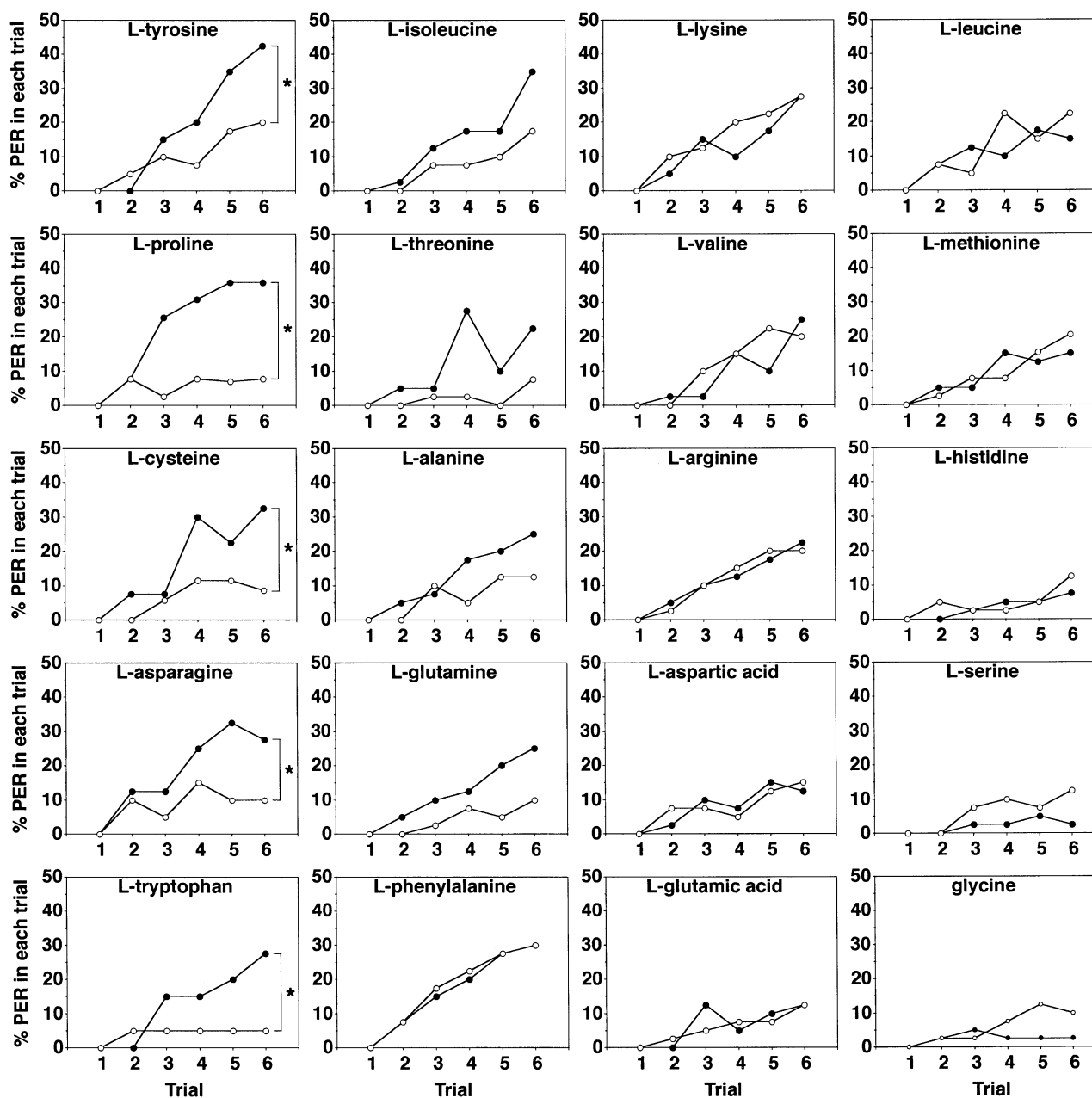| Step	L-tyrosine (mM)	L-proline (mM)	L-cysteine	L-asparagine (mM)	L-tryptophan (mM)
1	50	100	100 mM	100	56
2	25	75	12.5 mM	50	50
3	12.5	50	6.25 mM	25	25
4	6.25	25	3.125 mM	12.5	12.5
5	3.125	12.5	1.625 mM	6.25	6.25
6	1.625	6.25	781 μM	3.125	3.125

Figure 1 Acquisition curves for the 20 amino acids tested (*N* = 40 bees in each group). Each panel shows the percentage of bees that responded with PER to the presentation of either an amino acid (black symbols) or the solvent (white symbols) in each of the 6 trials performed. Asterisks indicate a statistically significant difference between the number of bees that responded to a given amino acid in the sixth trial and the number of bees that responded to the solvent in the sixth trial (*P* < 0.05).

found to have their olfactory detection threshold at 12.5 mM, 2 at 6.25 mM, and 1 at 3.125 mM (Figure 2C). Thus, the median olfactory detection threshold for L-cysteine was 12.5 mM.

L-asparagine

When trained to discriminate between 100 mM L-asparagine and the solvent, 10 of 30 bees succeeded. Nine bees were found to have their olfactory detection threshold at

50 mM and 1 bee at 25 mM (Figure 2D). Thus, the median olfactory detection threshold for L-asparagine was 50 mM.

L-tryptophan

Eight of 30 bees significantly discriminated between 100 mM L-tryptophan and the solvent. Six bees were found to have their olfactory detection threshold at 50 mM and 2 at 25 mM (Figure 2E). Thus, the median olfactory detection threshold for L-tryptophan was 50 mM.
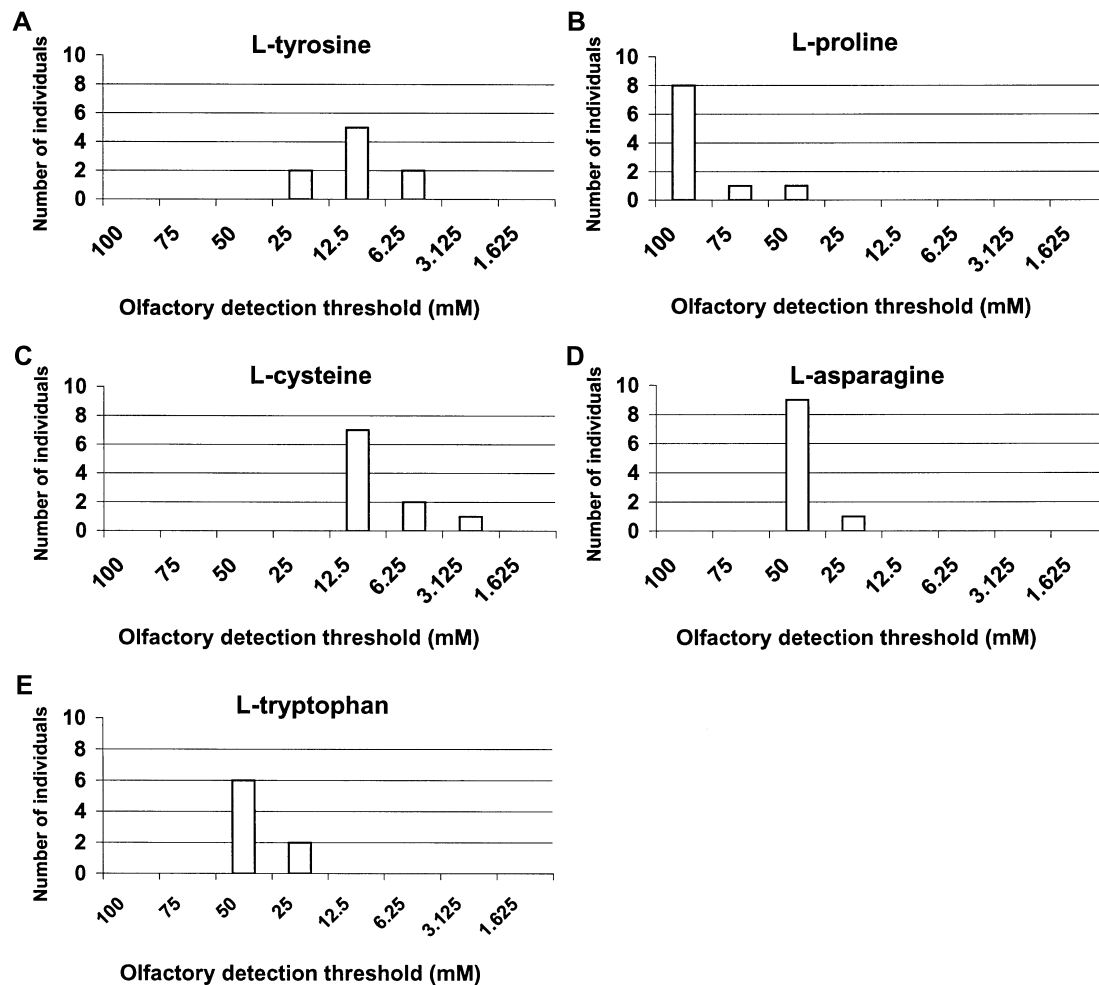
Figure 2   Distribution of olfactory detection thresholds. For each of the 5 amino acids (**A–E**), the bars show the number of animals which reached their olfactory detection threshold at a given concentration.

Interindividual variability was comparatively low, and with 3 of the amino acids (L-proline, L-asparagine, and L-tryptophan), olfactory detection threshold values differed only by a dilution factor of 2 between the highest and the lowest scoring animals. With the remaining 2 amino acids (L-tyrosine and L-cysteine), individual threshold values differed by a factor of 4 (Figure 2).

Discussion

The results of the present study demonstrate that honeybees are able to detect the odor of 5 of the 20 proteinogenic amino acids. Median olfactory detection thresholds for these 5 amino acids were found to be 12.5 mM with L-tyrosine and L-cysteine, 50 mM with L-tryptophan and L-asparagine, and 100 mM with L-proline. Our results do not rule out the possibility that honeybees could be sensitive to more than 5 amino acids. Here, we used the PER-conditioning paradigm as a reliable experimental procedure for investigating olfactory perception in the context of foraging and feeding

behavior. However, it has been observed in *Manduca* moths that certain odor stimuli are not learned in the feeding context despite the fact that they elicit a clear electrophysiological response (Daly et al. 2001). It has been also shown that some odors, such as the bee's alarm pheromone, are less effective as CS in PER conditioning (Smith 1993). Further studies are needed to corroborate these questions and also to assess whether the failure of the bees to respond to certain amino acids was due to a physical property of the stimuli such as their comparatively low volatility or to a perceptual problem resulting from a lack of corresponding olfactory receptors.

Our finding that honeybees failed to detect the odor of 15 of the 20 amino acids tested may at first seem surprising given that free amino acids are known to serve as important nutrients for honeybees (De Groot 1953) and are the second most abundant group of compounds in floral nectar after carbohydrates (Baker HG and Baker I 1973, 1986). Also, bees prefer sucrose solutions containing certain amino acids (Inouye and Waller 1984; Alm et al. 1990; Carter et al. 2006).

Although this evidence points to the potential use of olfactory cues, it is equally likely that the preferences for these amino acid–rich nectars could arise from taste cues and nutritional reward values. In the latter case, bees may not need to have a highly developed sense of smell for amino acids present in floral nectars.

The question that arises is whether the 5 amino acids that were detectable by smell may have a special significance for honeybees. L-tyrosine had the highest detection score in the present study (see Figure 1). Feeding preference tests with free-flying honeybees have shown that artificial nectar containing L-tyrosine is clearly preferred over control solutions without this amino acid (Inouye and Waller 1984). L-tyrosine is also critically involved in the formation of sclerotin, a mixture of proteins that makes up the cuticles of insects including honeybees (Andersen 2004).

L-proline is considered to be one of the most abundant amino acids in a wide variety of floral nectars (Gottsberger et al. 1984; Gardener and Gillman 2001). It is also found in high concentrations in the hemolymph of honeybees and thought to play a crucial role as a source of energy in the takeoff phase of flight (Micheu et al. 2000). Similarly to the findings with L-tyrosine, honeybees have been shown to prefer sucrose solutions containing L-proline over solutions containing only sucrose (Carter et al. 2006) and those containing L-alanine and L-serine (Bertazzini et al. 2010). Furthermore, bees showed a perceptual bias in olfactory PER learning when odors were reinforced differentially with 2 different rewards, pure sucrose solution and sucrose solution containing 10 mM proline (Wright, Choudhary, et al. 2009). That study demonstrated that bees can detect concentrations of this amino acid as a tastant in ingested solution, well below the threshold for olfactory detection that we report here.

L-cysteine was detected by the honeybees down to a concentration of 3.125 mM (see Figure 2). Sulfur-containing odorants such as thiols are detected at very low concentrations by a variety of terrestrial vertebrate species (Laska et al. 2007), which is commonly regarded as a mechanism to avoid consumption of spoiled food due to putrefaction processes (Kamiya and Ose 1984). Because the odorants released via the microbial degradation of proteins are also among the first signs of the death of an animal (Dekeirsschieter et al. 2009), it may be useful for honeybees to detect L-cysteine in order to remove dead conspecifics from the hive.

L-asparagine and L-tryptophan were also detected by the honeybees in the present study (see Figure 1). Both these amino acids have been shown to act as a repellent for bees when present in relatively high concentrations in floral nectar (Petanidou et al. 2006).

Studies reporting either an attractive (L-tyrosine and L-proline) or an aversive (L-asparagine and L-tryptophan) property of a given amino acid for honeybees are all based on measurement of the consumption of solutions mimicking floral nectar (Inouye and Waller 1984; Carter et al. 2006;

Bertazzini et al. 2010). Therefore, they do not allow us to determine whether the animals' choice was based on olfactory and/or gustatory cues. Unfortunately, no information as to the honeybees' taste sensitivity for amino acids is available.

A comparison between the olfactory sensitivity of honeybees as determined in the present study and the concentrations of free amino acids commonly found in floral nectars may be useful in order to elucidate whether the odors of amino acids may indeed have a behavioral significance for these insects. Several studies have analyzed the qualitative and quantitative composition of amino acids in floral nectars from a wide variety of flowering plants (Baker HG and Baker I 1973, 1986; Gottsberger et al. 1984; Carter et al. 2006; Petanidou et al. 2006). Individual amino acid concentrations reported in floral nectar rarely exceed 1 mM and are thus lower than the olfactory detection thresholds found in the present study, although it has been reported that in some plants, they may add up to higher concentrations, for example, to a total concentration of amino acids of 50 mM in the nectar of *Aloe marlothii* (Nicolson and Thornburg 2007). However, the generally observed low concentrations do not exclude the possibility that honeybees may use the odor of amino acids in foraging decisions. Contamination with pollen as a result of flower handling by floral visitors may dramatically increase the concentrations of free amino acids in floral nectar (up to 1900 μg/mL, Gottsberger et al. 1984). Such contamination occurs naturally during the process of pollination, whereas the above-mentioned analytical studies employed uncontaminated nectar.

Although these functional considerations are interesting and important, it is fundamentally important to understand the honeybee's ability to smell amino acids in a phylogenetic context. Whereas several species of aquatic animals have been reported to be able to detect the odor of all 20 proteinogenic amino acids (e.g., hammerhead shark: Tricas et al. 2009; channel catfish: Nikonov and Caprio 2007; zebra fish: Michel and Lubomudrov 1995; and sea bream: Hubbard et al. 2011), human subjects, similar to the honeybees, were found to detect the odor of only 5 amino acids when presented at 100 mM (L-lysine, L-cysteine, L-methionine, L-phenylalanine, and L-proline; Laska M, unpublished data). Interestingly, it appears that bees and humans, as terrestrial species, have a more limited ability to smell amino acids than aquatic animals. This emerging pattern is intriguing because olfactory receptors fall into 2 major phylogenetic classes representing olfactory receptors from aquatic (class I) and terrestrial (class II) animals (Sanz et al. 2005). It supports the hypothetical scenario that the transition from an aquatic to a terrestrial lifestyle may have decreased the selective pressure on genes coding for class I receptors in terrestrial species, such as honeybees and humans (Nei et al. 2008). This idea is supported by the finding that the expansion of the honeybee olfactory receptor family started approximately 100 Ma during the emergence of the earliest bees and

coinciding with the emergence of angiosperm plants (Robertson and Wanner 2006).

A final aspect of the present study is our finding that the olfactory detection thresholds for the 5 amino acids tested here ranged from 3.125 to 12.5 mM with L-cysteine to 50–100 mM with L-proline (see Figure 2). At this point, it should be noted that behavioral detection thresholds as determined in the present study may be significantly higher than electro-physiological response thresholds (e.g., Daly et al. 2007). Nevertheless, behavioral detection thresholds probably give a more accurate reflection of the real-world relevance of the stimuli in question compared with electro-antennogram re-sponse thresholds. With this caveat in mind, a comparison between the behavioral detection threshold values obtained in the present study and those observed in other terrestrial species might be helpful to clarify which mechanisms may underlie a species' olfactory sensitivity for a given odorant or class of odorants. Although such across-species compar-isons need to take into consideration the fact that method-ological differences may lead to widely differing results (Hastings 2003), it seems admissible to state that both spider monkeys (0.3–1.0 mM with L-cysteine and 3–30 mM with L-proline) and mice (0.01–0.03 mM with L-cysteine and 3.3–10 mM with L-proline) are more sensitive than the honeybees to these 2 amino acids (Wallén et al. 2012). Human subjects have recently been shown to detect the odors of L-cysteine and L-proline at concentrations as low as 0.2 and 100 mM, respec-tively, and thus perform better than the honeybees with only one of these 2 amino acids (Laska 2010). It is interesting to note that in line with findings suggesting that sulfur-containing functional groups generally increase olfactory sensitivity within a given chemical class of odorants (Jameson 2005), all 4 species displayed lower olfactory detection thresholds for the sulfur-containing amino acid L-cysteine compared with L-proline which lacks sulfur. One possible explanation for the higher olfactory sensitivity of spider monkeys, mice, and (partially) humans compared with honeybees may be that they all possess a considerably higher number of functional olfactory receptor genes (spider monkeys: ≈900, Rouquier et al. 2000; mice: ≈1060, Nei et al. 2008; and humans ≈390, Niimura and Nei 2006) than the ≈160 reported in honeybees (Robertson and Wanner 2006). Although several studies have failed to find a negative correlation between the size of the olfactory receptor repertoire and olfactory detection thresholds (Laska et al. 2007), the results of the pres-ent study support the view that the number of functional genes coding for olfactory receptors may affect both a species' sensitivity for odorants and the breadth of its spectrum of detectable odorants (Rouquier et al. 2000; Gilad et al. 2004). Whether neuroanatomical factors such as the total number of olfactory sensory neurons or ecological factors such as the behavioral relevance of the odor stimuli in question may provide a better explanation for the observed between-species differences in detectability of and sensitivity to amino acids remains to be tested.

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