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ORIGINAL ARTICLE

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A novel role for proline in plant floral nectars

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Abstract Plants offer metabolically rich floral nectar to attract visiting pollinators. The composition of nectar includes not only sugars, but also amino acids. We have examined the amino acid content of the nectar of ornamental tobacco and found that it is extremely rich (2 mM) in proline. Because insect pollinators preferentially utilize proline during the initial phases of insect flight and can reportedly taste proline, we determined whether honeybees showed a preference for synthetic nectars rich in proline. We therefore established an insect preference test and found that honeybees indeed prefer nectars rich in the amino acid proline. To determine whether this was a general phenomenon, we also examined the nectars of two insect-pollinated wild perennial species of soybean. These species also showed high levels of proline in their nectars demonstrating that plants often produce proline-rich floral nectar. Because insects such as honeybees prefer proline-rich nectars, we hypothesize that some plants offer proline-rich nectars as a mechanism to attract visiting pollinators.

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Introduction

Many plants require pollinator visitation to obtain efficient seed set. Dicotyledonous plants often attract these pollinators with offerings of floral nectar that is secreted into the floral tube at the base of the ovary. Nectar is a rich source of sugars and amino acids and provides a reward to pollinators, thereby increasing the fecundity of those plants that provide nectar. The secretion of nectar is usually under developmental control beginning just before anthesis. After pollination, nectar production ceases, and remaining nectar is frequently resorbed (Burquez and Corbet 1991). While many organisms can function in floral pollination, the class Insecta has excelled at the exploitation of this ecological niche.

The composition of nectar has been widely studied. Nectar is an aqueous combination of substances. Most important among these are sucrose, glucose, and fructose; however, other carbohydrates have also been identified in nectars of some flowers (Baker and Baker 1981; Jackson and Nicolson 2002). Other substances reported in nectar include organic acids (Baker and Baker 1975), terpenes (Ecroyd et al. 1995), alkaloids (Deinzer et al. 1977), flavonoids (Rodriguez-Arce and Diaz 1992), glycosides (Roshchina and Roshchina 1993), vitamins (Carter and Thornburg 2004a; Griebel and Hess 1940), phenolics (Ferreres et al. 1996), and oils (Vogel 1969). Nectars also contain specific plant-defense proteins that appear to function in the protection of the gynoecium from microbial invasion by either windblown or pollinator-transferred microbes to the metabolite-rich floral nectar (Carter and Thornburg 2000, 2004a,b,c; Carter et al. 1999; Naqvi et al. 2005; Peumans et al. 1997; Thornburg et al. 2003).

Most nectars also contain amino acids. The accumulation of amino acids in nectar has been known since the mid-20th century (Lüttge 1961, 1962; Mostowska 1964; Nair et al. 1964; Ziegler 1956). However, it was not until the first major survey that the presence of amino acids in nectar was determined to be common (Baker and Baker 1971). All 20 of the normal amino acids found in protein have been identified in various plant nectars, with alanine, arginine,

serine, proline, glycine, isoleucine, threonine, and valine being the most prevalent (Baker and Baker 1973; Gottsberger et al. 1984; Rusterholz and Erhardt 1998). Tryptophan is typically rare in nectars. The concentration of various amino acids in nectar is generally quite variable; however, the overall composition of nectar is less variable (Gardener and Gillman 2001a). Further, the amino acid composition is known to be affected by the conditions under which plants are grown (Gardener and Gillman 2001b). Among the amino acids found in nectar, proline is unique because insects have the ability to taste this unique amino acid (Gardener and Gillman 2002; Hansen et al. 1998; Shiraishi and Kuwabara 1970; Wacht et al. 2000).

The accumulation of proline in nonfloral plant tissues is generally taken as a sign of stress (Yamada et al. 2005). For example, drought (Kishor et al. 1995), salinity (Jouve et al. 2004), and freezing (Parvanova et al. 2004) all result in activation of the proline biosynthetic pathway (Verbruggen et al. 1993; Yoshiba et al. 1995) leading to proline accumulation. It is thought that during recovery from these stresses, the accumulated proline is rapidly oxidized to glutamate thereby providing the plant with energy, carbon, nitrogen, and reducing power, that permits the plant to restore a stable physiology (Verbruggen et al. 1996).

The accumulation of proline in flowers has received much less attention. It is well-known that high levels of free proline are found in pollen where it can represent up to 70% of total free amino acids (Zhang et al. 1982). Expression of a specific proline transporter in maturing pollen is responsible for this phenotype (Schwacke et al. 1999). While the functional mechanism of such high levels of proline in pollen is not clear, it is generally accepted that pollen serves as a general source of essential amino acids for those insects that consume it (O'Brien et al. 2003). Proline has also been identified at high levels in plant nectars (Gardener and Gillman 2001a; Kaczorowski et al. 2005). This study was therefore undertaken to evaluate the role of proline in floral nectar. To assess the generality of high proline concentrations in nectar of insect-pollinated plants, we analyzed the nectar of ornamental tobacco and of two wild soybean species. To assess the preference of insects to proline in nectar, we tested the preference of the generalist pollinators, honeybees, to various concentrations of proline-enriched sugar solutions.

Materials and methods

Chemicals

Sucrose was obtained from Frutarom, Haifa, Israel, and the other chemicals were from Sigma Chemical Corporation, St. Louis, USA.

Organisms

The ornamental tobacco plants (LxS8) used in this study were derived from an interspecific cross of *Nicotiana langsdorffii* and *N. sanderae* (Kornaga 1993; Kornaga et al. 1997). This line is both male and female fertile, but self-incompatible. It does, however, have high seed set when insect-pollinated. It was propagated clonally to produce large numbers of identical plants.

The soybean plants, *Glycine tomentella* and *G. canescens*, used for nectar collection are Australian perennial species and were grown in a glasshouse at CSIRO, Canberra, Australia. Soybean nectar (approximately 0.50 μl nectar per flower) was collected in microliter capillaries.

Honeybees (*Apis mellifera*) of the New World Carniolan strain were kept in five-frame nucleus hives inside a 7×12×2 m screened enclosure and regularly fed pollen patties as a protein source. The bees had ad libitum access to a water source. Honey store levels were intentionally kept low to maintain foraging motivation.

Feeding experiments

The sugar composition of ornamental tobacco nectar was found to contain 34% (w/v) carbohydrate, and the composition was determined to be 53% sucrose, 23% glucose, and 24% fructose. An artificial nectar solution was prepared with this carbohydrate composition and was supplemented with varying concentrations of proline.

Apparatus

Transparent plastic containers, used to provide water to caged birds, were used for honeybee feeders. When inverted, these containers continually fed a shallow pool (4×5 cm) with liquid for hive consumption. As bees imbibed solution from the feeder, the liquid level gradually declined. A ruler placed adjacent to the container measured the remaining liquid.

In experiment 1, we tested seven proline concentrations: 20, 6, 2, 0.2, 0.06, 0.02, and 0 (artificial nectar with no proline) mM. Seven identical containers were filled with 200 mL of an individual artificial nectar/proline solution. Feeders, in increasing order of proline concentration, were placed on a carousel that turned continuously at 2 rpm to prevent positional bias from affecting choice. To choose a particular concentration, bees had to taste the solutions during every visit to the carousel.

A second experiment was designed to test whether bees would have stronger preferences if the position of the feeders were constant, so that they could learn the positions of the different feeders. We tested four proline concentrations: 100, 20, 4, and 0 mM. We placed four feeders in a row, with 50 cm between them. To facilitate learning their positions, we placed a yellow card (15×15 cm) between the first and second feeders, and a blue card between the third and fourth feeders. The position of each feeder remained constant throughout each day, but was rotated according to a pseudorandom sequence across days.

Procedure

Experiments were conducted in an outdoor enclosure. Experiment 1 (18 replicates) was conducted between December 2002 and February 2003, on days in which the weather permitted, and experiment 2 (10 replicates) between May and June 2003. Mean ambient temperatures ranged between 21 and 28°C for experiment 1 and between 28 and 36°C for experiment 2. Three replicates of experiment 1 were conducted in an indoor flight room (2×3×2 m). Before beginning each experiment, the entrance to all the enclosed hives, except for one, were closed with a net that permitted ventilation but prevented bees from leaving the hive. Thus, each replicate of the experiment was conducted with a single hive. A total of 11 different hives participated in experiment 1 and eight hives in experiment 2. Some hives were tested more than once, but with at least 3 weeks between replicates to insure that the forager population differed between replicates and each replicate was treated as independent.

Every 10 min, we counted the number of bees feeding at each feeder, and every 20 min we recorded the solution level in each feeder. The experiment ended when one of the feeders reached a predetermined low mark (50 mL remaining), or when 5 h had elapsed.

Analysis

To evaluate data from the two experiments, we compared the best fits for data of the two experiments separately and when pooled using an F-test, and also using the corrected Akaike's Information Criterion (AICc), which quantifies how much more likely was one model to be correct than the other (Motulsky and Christopoulos 2003). For both measures of preference, solution level and numbers of bees at each feeder, AICc analysis revealed pooling the data was 2.8 and 10.7 times, respectively, more likely to be correct than using separate data sets. Also, the null hypothesis that pooling the data was better than using separate data sets could not be rejected ($F_{3,160}$=1.43, P=0.24, and $F_{3,160}$=0.55, P=0.65, respectively). Thus, all proline concentration data sets (28 total data sets) were combined for interpretation. Each preference evaluation was evaluated using a nonlinear best fit using a second-degree polynomial (Motulsky and Christopoulos 2003).

Table 1 Amino acid profile of floral nectar from ornamental tobacco

Amino acid	*LxS8* run 1		*LxS8* run 2	
	Mol%	Concentration (μM)	Mol%	Concentration (μM)
Aspartic acid	3.14	109	3.33	114
Serine	8.95	311	9.32	319
Glycine	1.11	40	0.02	0.8
Histidine	1.63	56	1.76	60
Threonine	3.56	124	3.82	131
Proline	56.02	1,937	58.25	2,020
Tyrosine	19.35	670	16.03	547
Valine	1.97	68	1.88	64
Isoleucine	1.39	49	1.57	54
Leucine	1.15	40	1.19	41
Phenylalanine	1.72	60	1.82	62

Amino acid analysis

Three microliters of nectar was processed for routine amino acid analysis by an automated PITC derivatization and separation on a Perkin-Elmer Applied Biosystems Model 130A PCT Amino Acid Analyzer at the ISU protein facility.

Results

Proline in the nectar of ornamental tobacco

We initially evaluated the amino acid composition of tobacco nectar (Table 1). Eleven of the 20 normal protein amino acids were identified in the tobacco nectar. How-

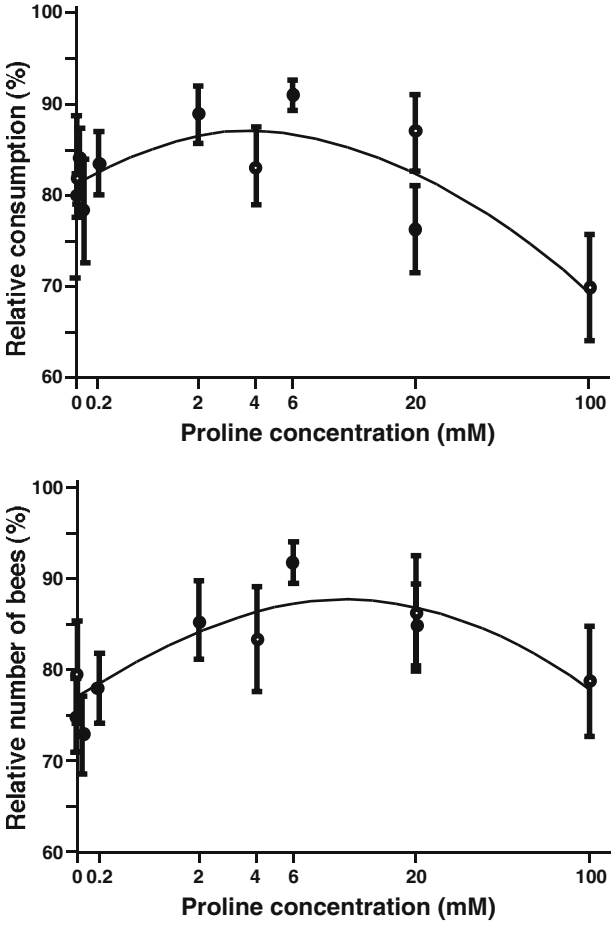

Fig. 1 Mean (+SE) relative consumption **a** and relative number of bees visiting feeders **b** of the various proline concentrations in experiments 1 (*full circles*) and 2 (*open circles*). The *x-axis* is drawn on a logarithmic scale. The *lines* are of a quadratic fit on the log-transformed concentrations

Table 2 Amino acid profile of floral nectar from glasshouse-grown *G. tomentella*

Accession	*G. tomentella* G1133 Sample 2682		*G. tomentella* G1929 Sample 2681		*G. tomentella* G2437 Sample 2676	
Amino acid	Mol%	Concentration (μM)	Mol%	Concentration (μM)	Mol%	Concentration (μM)
Aspartic acid	2.0	58.0	9.5	78.4	10.9	444.1
Glutamic acid	1.4	39.3	5.7	47.2	0.8	32.5
Serine	0.4	12.8	nd	nd	nd	nd
Glycine	0.5	14.9	2.3	18.6	0.6	23.6
Histidine	0.7	20.1	2.2	18.1	0.7	28.9
Arginine	0.5	14.1	0.9	7.1	0.5	20.1
Threonine	1.5	42.8	3.8	31.8	1.5	65.3
Alanine	4.2	122.5	5.7	46.8	5.8	237.0
Proline	57.4	1675.5	44.2	365.1	46.4	1894.5
Tyrosine	9.5	277.2	7.7	63.4	15.0	612.5
Valine	2.4	71.2	7.6	63.1	3.7	152.0
Methionine	0.2	6.5	nd	nd	0.3	11.0
Isoleucine	1.2	35.5	2.2	18.4	0.8	34.4
Leucine	1.2	35.6	2.8	22.8	1.3	53.2
Phenylalanine	16.1	471.0	3.7	30.5	10.6	432.1
Lysine	0.7	21.1	1.8	15.2	1.0	39.3

G numbers represent accessions in the CSIRO germplasm collection (CSIRO, Canberra, ACT, Australia). The PI numbers represent accessions in the USDA germplasm collection (USDA-ARS Urbana, Illinois). G1133=PI 441.001, G1929=PI 591.605, G2437=PI 159.823
nd Not detected

ever, the concentration of proline was inordinately high. In duplicate runs, nearly 60 mol% of the amino acids present in ornamental tobacco nectar was proline. The concentration of proline in the nectar of these plants was approximately 2 mM. Other amino acids that were present in high concentrations included tyrosine and serine. Absent amino acids included the sulfur-containing amino acids cysteine and methionine, the aminated amino acids glutamine and asparagine, the strongly basic amino acids lysine and arginine, as well as tryptophan, glutamate, and alanine.

Honeybees prefer moderate levels of proline in artificial nectar

Because insects have the ability to taste proline (Gardener and Gillman 2002; Hansen et al. 1998; Shiraishi and

Table 3 Amino acid profile of floral nectar from glasshouse-grown *G. canescens*

Accession	*G. canescens* G1232 Sample 2675		*G. canescens* G1232 Sample 2684		*G. canescens* G1232 Sample 2680	
Amino acid	Mol%	Concentration (μM)	Mol%	Concentration (μM)	Mol%	Concentration (μM)
Aspartic acid	25.8	457.3	15.4	524.7	8.5	106.4
Glutamic acid	2.6	46.8	1.3	44.5	3.0	37.1
Serine	1.9	34.4	1.8	60.2	1.7	20.9
Glycine	nd	nd	nd	nd	nd	nd
Histidine	1.6	29.2	1.8	61.6	2.5	32.0
Arginine	nd	nd	1.0	34.19	0.6	7.5
Threonine	1.5	26.5	1.6	55.06	1.3	15.9
Alanine	4.4	77.5	6.5	220.5	4.8	59.9
Proline	47.0	834.3	60.9	2076.0	64.4	806.6
Tyrosine	4.8	84.5	2.7	92.3	2.2	27.3
Valine	8.2	146.0	4.2	143.5	8.3	103.6
Methionine	nd	nd	0.2	5.8	nd	nd
Isoleucine	0.9	16.2	1.0	32.5	0.9	10.9
Leucine	0.4	6.4	0.6	20.2	0.6	7.5
Phenylalanine	0.6	11.3	0.7	23.0	0.8	10.5
Lysine	0.4	6.3	0.5	17.0	0.6	7.2

G numbers represent accessions in the CSIRO germplasm collection (CSIRO, Canberra, ACT, Australia). The PI numbers represent accessions in the USDA germplasm collection (USDA-ARS Urbana, Illinois). G1232=PI 446.934
nd Not detected

Kuwabara 1970; Wacht et al. 2000), we then evaluated the effect of proline on honeybee feeding. Various proline-supplemented artificial nectars were prepared having the same carbohydrate composition as ornamental tobacco. These were evaluated using two separate measures of solution preference. First, we scored the amount of each solution relative to the solution that showed the greatest consumption. Second, we determined the mean number of bees feeding on each solution relative to the feeder showing the greatest consumption.

As shown in Fig. 1a, bees consumed some of each of the artificial nectar/proline solutions. However, those with intermediate proline concentrations were consumed first, with 2 to 6 mM being the most preferred proline concentrations tested. The relative number of bees feeding on the various feeders showed a similar pattern to the relative consumption data, with the greatest number of bees visiting the intermediate proline concentrations (Fig. 1b). The polynomial fits for both the relative consumption and the preferred visitation data were significant (P=0.005), indicating that honeybees both preferred and consumed artificial nectar containing moderate (2 to 6 mM) levels of proline. This level is similar to the physiological level found in ornamental tobacco nectar.

Proline in the nectar of other species

Because honeybees prefer nectars containing moderate levels of proline, we evaluated next whether the nectar of other plant species also contained high levels of proline. For these analyses, we examined the nectar of two species of insect-pollinated wild perennial soybean.

Table 2 shows the nectar amino acid profiles found in *G. tomentella*. As with tobacco, proline had the highest concentration for any amino acid, varying from 0.4 to 1.9 mM, but even when low, proline represented the largest component amino acid in nectar, ranging from 44 to 57 mol%. Overall, we have performed many dozens of amino acid analyses on nectars. In every case, we have found that proline is always the highest amino acid present in the nectars presented in this manuscript. However, as previously noticed (Gardener and Gillman 2001a), the concentration of amino acids in nectar is variable, while the overall composition of the nectar consistently showed high proline levels. Similar observations were made with *G. canescens* nectar (Table 3). Proline concentrations were from 0.8 to 2.1 mM, and proline was the most prevalent amino acid in nectar ranging from 47 to 64 mol%.

Discussion

We have evaluated the amino acid composition of the nectars from several plant species. Each of the species tested showed a number of amino acids accumulating in the nectar of these plants. In every case examined, proline was the most abundant amino acid present in the nectar. Similar observations that proline is common (Baker 1978) or high (Gardener and Gillman 2001a; Kaczorowski et al. 2005) in nectar have been made previously for a number of other species.

Proline is an especially important amino acid for insects. It is the most abundant amino acid in the honeybee hemolymph. In studies using tethered honeybees, proline is selectively degraded in the hemolymph, and it has been proposed that proline is used by foragers in the initial stages or lift phase of flight (Auerswald et al. 1998; Micheu et al. 2000). Proline is also required for egg-laying by the queen (Hrassnigg et al. 2003). Thus, proline is specifically required for insect development and flight.

It is also not surprising that pollinator attraction to nectar could be influenced by chemoperception or taste of nectar containing specific amino acids (Gardener and Gillman 2002; Shiraishi and Kuwabara 1970). In choice tests, both cabbage white butterflies and honeybees preferred a solution of a mixture of sugars enriched by several amino acids, including proline, to an amino acid-deficient solution (Alm et al. 1990). Inouye and Waller (1984) tested the preference of honeybees to many amino acids, including proline. They tested a relatively large concentration range (up to 258 mM), and found a general preference for 1.85 mM, the lowest concentration tested. This level is similar to the physiological level found in ornamental tobacco (Table 1) and several wild soybean species (Tables 2 and 3). The amino acid-enriched sugar solution preferred by honeybees in the study of Alm et al. (1990) contained a proline concentration of 2.22 mM, but also several other amino acids. We found a preference for

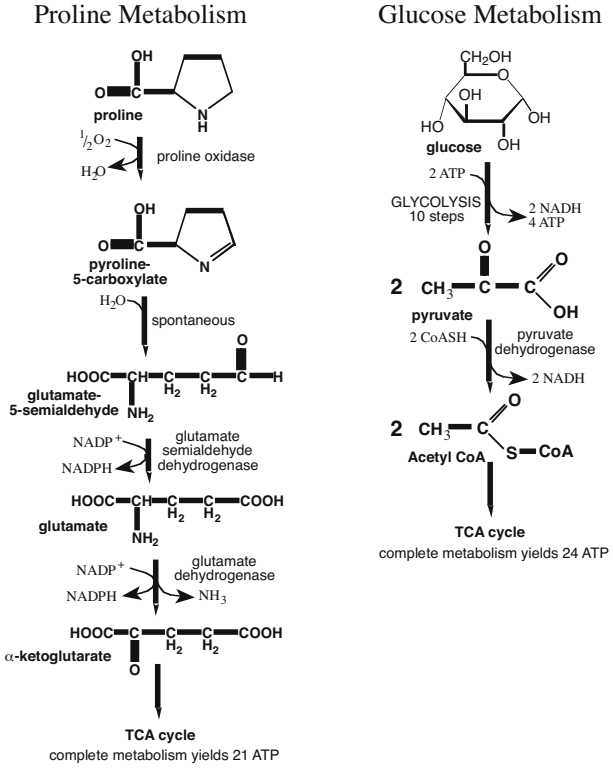

Fig. 2 Comparison of the metabolism of proline with glucose. Intermediary steps of glycolysis and the TCA cycle are not presented

proline concentrations in the order of magnitude of 1 to 10 mM, with a peak at about 6 mM. Preference decreased for both lower and higher proline concentrations. Interestingly, the concentration range preferred by honeybees is that found in several nectars, for example *Lantana camara* Alm et al. (1990), ornamental tobacco, and wild soybean species (this study).

The nonlinear preference function for amino acid concentrations in nectar found by honeybees in this study and also for glycine (Kim and Smith 2000) is probably widespread in insect nutrition choice (Raubenheimer and Simpson 1999; Simpson and Raubenheimer 1993). Our data included many independent replicates at the colony choice level, allowing an accurate assessment of preferences. Still, we found large variability between replicates, which was likely due to slight differences in colony nutritional condition and demography, as suggested for the observed colony variability in the nonlinear preference for glycine (Kim and Smith 2000).

It is not clear whether other amino acids might also prove as attractive as proline for visiting pollinators. Further studies will be required to evaluate this possibility. However, proline (and hydroxyproline) are unique among the naturally occurring amino acids in that they can stimulate the salt cell of labellar chemosensory cells on the proboscis of some insects (Hansen et al. 1998; Wacht et al. 2000). No other naturally occurring amino acids stimulate this cell. This link between the high levels of proline that accumulate in many plant nectars with the ability of insects to 'taste' proline suggests an evolutionary relationship that may function to enhance pollination between plants producing proline-rich nectar and insects that prefer the taste of these nectars.

So, what is the consequence of high levels of proline in nectar? A number of investigations have found that proline is specifically oxidized in insect flight muscle, especially during the first 30 s of insect flight (Balboni 1978; Brosemer and Veerabhadrappa 1965; Crabtree and Newsholme 1970; Njagi et al. 1992). The oxidative proline degradation pathway utilizes proline as a source of energy, specifically during the initial 'lift' phase of insect flight. Reasons for the utilization of proline over glucose become apparent if we examine the metabolic pathways (Fig. 2). Proline is rapidly metabolized and results in the production of multiple nicotinamide adenine dinucleotide phosphate (reduced form) equivalents and high levels of adenosine triphosphate (ATP). No other amino acid can be metabolized as rapidly as proline and release as much ATP without complete metabolism. While glucose ultimately yields more ATP on a molar basis, the initial steps of glucose metabolism require the consumption of ATP. Thus, proline is a more efficient fuel in the short run, while glucose is a far superior fuel in the long run. However, proline is metabolically more expensive than glucose for the plant to produce. Glucose can be produced from atmospheric gases by photosynthesis whereas proline cannot. Proline also contains nitrogen, which is a limiting nutrient. Thus, the accumulation of both glucose and proline in nectar presents insects with a dual action fuel: proline for rapid, short-term bursts of energy production and a large amount of glucose for extended flight. Further, with the ability to taste proline and a preference for nectars that contain proline, insects likely participate in selecting and maintaining plant species containing proline-rich nectars.

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References

Alm J, Ohnmeiss TE, Lanza J, Vriesenga L (1990) Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. Oecologia 84:53–57

Auerswald L, Schneider P, Gade G (1998) Utilisation of substrates during tethered flight with and without lift generation in the African fruit beetle *Pachnoda sinuata* (Cetoniinae). J Exp Biol 201:2333–2342

Baker HG (1978) Chemical aspects of the pollination biology of woody plants in the tropics. In: Tomlinson P, Zimmerman MH (eds) Tropical trees as living systems: the proceedings of the fourth Cabot Symposium, Harvard Forest, Petersham, Massachusetts, 26–30 April, 1976. Cambridge University Press, New York, pp 57–82

Baker HG, Baker I (1971) Amino acids in nectar and their evolutionary significance. Nature 241:543–545

Baker HG, Baker I (1973) Some anthecological aspects of the evolution of nectar-producing flowers, particularly amino acid production in the nectar. In: Heywood VH (ed) Taxonomy and ecology: proceedings of an international symposium, Department of Botany, University of Reading, vol 5. Academic, London, pp 243–264

Baker HG, Baker I (1975) Studies of nectar-constitution and pollinator-plant coevolution. In: Gilbert LE, Raven PH (eds) Coevolution of animals and plants: symposium V, first international congress of systematic and evolutionary biology, Boulder, Colorado, August 1973. University of Texas Press, Austin, pp 100–140

Baker HG, Baker I (1981) Chemical constituents of nectar in relation to pollination mechanisms and phylogeny. In: Nitecki MH (ed) Biochemical aspects of evolutionary biology. University of Chicago Press, Chicago, pp 131–171

Balboni E (1978) A proline shuttle in insect flight muscle. Biochem Biophys Res Commun 85:1090–1096

Brosemer RW, Veerabhadrappa PS (1965) Pathway of proline oxidation in insect flight muscle. Biochem Biophys Acta 110:102–112

Burquez A, Corbet SA (1991) Do flowers reabsorb nectar? Funct Ecol 5:369–379

Carter C, Thornburg RW (2000) Tobacco nectarin I: purification and characterization as a germin-like, manganese superoxide dismutase implicated in the defense of floral reproductive tissues. J Biol Chem 275:36726–36733

Carter C, Thornburg R (2004a) Tobacco nectarin V is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. Plant Physiol 134:460–469

Carter C, Thornburg RW (2004b) Is the nectar redox cycle a floral defense against microbial attack? Trends Plant Sci 9:320–324

Carter C, Thornburg RW (2004c) Tobacco nectarin III is a bifunctional enzyme with monodehydroascorbate reductase and carbonic anhydrase activities. Plant Mol Biol 54:415–425

Carter C, Graham R, Thornburg RW (1999) Nectarin I is a novel, soluble germin-like protein expressed in the nectar of *Nicotiana* sp. Plant Mol Biol 41:207–216

Crabtree B, Newsholme EA (1970) The activities of proline dehydrogenase, glutamate dehydrogenase, aspartate-oxoglutarate aminotransferase and alanine-oxoglutarate aminotransferase in some insect flight muscles. Biochem J 117:1019–1021

Deinzer ML, Thompson PA, Burgett DM, Isaacson DL (1977) Pyrrolizidine alkaloids: their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). Science 195:497–499

Ecroyd CE, Franich RA, Kroese HW, Steward D (1995) Volatile constituents of *Dactylanthus taylorii* flower nectar in relation to flower pollination and browsing by animals. Phytochemistry 40:1387–1389

Ferreres F, Andrade P, Gil MI, Tomas Barberan FA (1996) Floral nectar phenolics as biochemical markers for the botanical origin of heather honey. Zeit Lebensmitt Untersuch Forsch 202:40–44

Gardener MC, Gillman MP (2001a) Analyzing variability in nectar amino acids: composition is less variable than concentration. J Chem Ecol 27:2545–2558

Gardener MC, Gillman MP (2001b) The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* L. (Caryolhyllaceae). Oikos 92:101–106

Gardener MC, Gillman MP (2002) The taste of nectar—a neglected area of pollination. Oikos 98:552–557

Gottsberger G, Schrauwen J, Linskens HF (1984) Amino acids and sugars in nectar and their putative evolutionary significance. Plant Syst Evol 145:55–77

Griebel C, Hess G (1940) The vitamin C content of flower nectar of certain Labiatae. Zeit Untersuch Lebensmitt 79:168–171

Hansen K, Wacht S, Seebauer H, Schnuch M (1998) New aspects of chemoreception in flies. Ann NY Acad Sci 855:143–147

Hrassnigg N, Leonhard B, Crailsheim K (2003) Free amino acids in the haemolymph of honey bee queens (*Apis mellifera* L.). Amino Acids 24:205–212

Inouye D, Waller G (1984) Responses of honey bees (*Apis mellifera*) to amino acid solutions mimicking nectars. Ecology 65:618–625

Jackson S, Nicolson SW (2002) Xylose as a nectar sugar: from biochemistry to ecology. Comp Biochem Physiol B 131B:613–620

Jouve L, Hoffmann L, Hausman JF (2004) Polyamine, carbohydrate, and proline content changes during salt stress exposure of aspen (*Populus tremula* L.): involvement of oxidation and osmoregulation metabolism. Plant Biol (Stuttg) 6:74–80

Kaczorowski RL, Gardener MC, Holtsford TP (2005) Nectar traits in Nicotiana section Alatae (Solanaceae) in relation to floral traits, pollinators and mating system. Am J Bot 92:1270–1283

Kim Y, Smith B (2000) Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*. J Insect Physiol 46:793–801

Kishor P, Hong Z, Miao GH, Hu C, Verma D (1995) Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108:1387–1394

Kornaga T (1993) Genetic and biochemical characterization of a "lost" unstable flower color phenotype in interspecific crosses of *Nicotiana* sp. MS. Iowa State University, Ames

Kornaga T, Zyzak DV, Kintinar A, Baynes J, Thornburg R (1997) Genetic and biochemical characterization of a "lost" unstable flower color phenotype in interspecific crosses of *Nicotiana* sp. WWW J Biol 2:8

Lüttge U (1961) Über die Zusammensetzung des Nektars und den Mechanismus seiner Sekretion. I. Planta 56:189–212

Lüttge U (1962) Über die Zusammensetzung des Nektars und den Mechanismus seiner Sekretion. II. Planta 59:108–114

Micheu S, Crailsheim K, Leonhard B (2000) Importance of proline and other amino acids during honeybee flight (*Apis mellifera carnica* Pollmann). Amino Acids 18:157–175

Mostowska I (1964) Amino acids of nectars and honeys. Zeszyty Kauk Wyzszej Szkoly Rolniczej Olsztynie 20:417–432 (Chem. Abstr. 464: No. 20529, 21966)

Motulsky H, Christopoulos A (2003) Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting. GraphPad Software Inc., San Diego

Nair A, Nagarajan S, Subramanian S (1964) Chemical compositions of nectar in *Thunbergia grandiflora*. Current Sci (Bangalore) 33:401

Naqvi SMS, Harper A, Carter CJ, Ren G, Guirgis A, York WS, Thornburg RW (2005) Nectarin IV, a potent endoglucanase inhibitor secreted into the nectar of ornamental tobacco plants. Isolation, cloning and characterization. Plant Physiol 139: 1389–1400

Njagi EN, Olembo NK, Pearson DJ (1992) Proline transport by tsetse fly *Glossina morsitans* flight muscle mitochondria. Comp Biochem Physiol B 102:579–584

O'Brien D, Boggs CL, Fogel Ml (2003) Pollen feeding in the butterfly *Heliconius charitonia*: isotopic evidence for essential amino acid transfer from pollen to eggs. Proc R Soc Lond B 270:2631–2636

Parvanova D, Ivanov S, Konstantinova T, Karanov E, Atanassov A, Tsvetkov T, Alexieva V, Djilianov D (2004) Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. Plant Physiol Biochem 42:57–63

Peumans WJ, Smeets K, Van Nerum K, Van Leuven F, Van Damme EJM (1997) Lectin and alliinase are the predominant proteins in nectar from leek (*Allium porrum* L.) flowers. Planta 201:298–302

Raubenheimer D, Simpson S (1999) Integrating nutrition: a geometrical approach. Entomol Exp Appl 91:67–82

Rodriguez-Arce AL, Diaz N (1992) The stability of beta-carotene in mango nectar. J Agric Univ PR 76:101–102

Roshchina VV, Roshchina VD (1993) The excretory function of higher plants. Springer, Berlin Heidelberg New York

Rusterholz HP, Erhardt A (1998) Effects of elevated CO_2 on flowering phenology and nectar production of nectar plants important for butterflies of calcareous grasslands. Oecologia 113:341–349

Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB, Rentsch D (1999) LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen. Plant Cell 11:377–392

Shiraishi A, Kuwabara M (1970) The effects of amino acids on the labellar hair chemosensory cells of the fly. J Gen Physiol 56:768–782

Simpson S, Raubenheimer D (1993) A multi-level analysis of feeding behaviour: the geometry of nutritional decision. Philos Trans Roy Soc B 342:381–402

Thornburg RW, Carter C, Powell A, Rizhsky L, Mittler R, Horner HT (2003) A major function of the tobacco floral nectary is defense against microbial attack. Plant Syst Evol 238:211–218

Verbruggen N, Villarroel R, Van Montagu M (1993) Osmoregulation of a pyrroline-5-carboxylate reductase gene in Arabidopsis thaliana. Plant Physiol 103:771–781

Verbruggen N, Hua XJ, May M, Van Montagu M (1996) Environmental and developmental signals modulate proline homeostasis: evidence for a negative transcriptional regulator. Proc Natl Acad Sci USA 93:8787–8791

Vogel S (1969) Flowers offering fatty oil instead of nectar (Abstract No. 229). In: Abstracts of the papers presented at the XI International Botanical Congress, August 24–September 2, 1969 and the International Wood Chemistry Symposium, September 2–4, 1969 Seattle, WA (USA), pp 260

Wacht S, Lunau K, Hansen K (2000) Chemosensory control of pollen ingestion in the hoverfly *Eristalis tenax* by labellar taste hairs. J Comp Physiol A 186:193–203

Yamada M, Morishita H, Urano K, Shiozaki N, Yamaguchi-Shinozaki K, Shinozaki K, Yoshiba Y (2005) Effects of free proline accumulation in petunias under drought stress. J Exp Bot 56:1975–1981

Yoshiba Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, Yamaguchi-Shinozaki K, Wada K, Harada Y, Shinozaki K (1995) Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J 7:751–760

Zhang H, Croes A, Linskens H (1982) Protein synthesis in germinating pollen of petunia: Role of proline. Planta 154:199–203

Ziegler H (1956) Untersuchungen über die Leitung und Sekretion der Assimilate. Planta 47:447–500