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Pollen amino acids – an additional diet for a nectar feeding butterfly?

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Abstract: The increase of the amino acid concentration over different time intervals in artificial nectar (i.e., a sucrose solution) due to pollen contamination was investigated in four Californian plant species (*Aesculus californica, Amsinckia lunaris, Brodiaea pulchella, Carduus pycnocephalus*), which are important nectar resources for a Californian colony of the butterfly *Battus philenor* as well as for other insects. The increase of the amino acid concentration in the medium is different in all four species and seems to be determined by a variety of factors including permeability of the pollen grain wall and presence or absence of pores. The results suggest a passive diffusion process of the free pollen amino acids into the medium rather than an active release. Implications from the experiments for *Battus philenor* and for other nectar feeding pollinators are discussed. A possible complementary effect of free pollen and nectar amino acids is proposed for plant species in which pollen is likely to be knocked into nectar by their flower visitors. A possible evolutionary pathway from nectar feeding butterflies such as *Battus philenor* to the complex derived pollen feeding habit in the *Heliconius* butterflies is proposed.

Pollen contamination of nectar has been reported to affect the amino acid concentration and composition of nectar (BAKER & BAKER 1980, 1986; WILLMER 1980; GOTTSBERGER & al. 1984; SCOGIN 1986). Even as early as 1927, BUXBAUM noted in a frequently overlooked paper that the nitrogen content of nectar of *Erythrina crista-galli* L. is increased by contamination with pollen (BUXBAUM 1927). In the context of pollen contaminated nectar, the work of LINSKENS & SCHRAUWEN (1969) is often quoted as a corollary (e.g., GILBERT 1980, SCOGIN 1986). These authors found that 50% of free pollen amino acids are released within only one minute into a sucrose medium from germinating *Petunia hybrida* VILM.-ANDR. pollen. However, beside *Petunia hybrida*, no other plant species have been investigated with respect to their release of free pollen amino acids into a sugar solution over different time intervals. In addition, boron (boric acid) was added to the medium in the experiment of LINSKENS & SCHRAUWEN (1969). Boron has since been recognized as a crucial factor for pollen tube germination (JACKSON & KAMBOJ 1986, SIDHU & MALIK 1986), but has not been found in nectar so far (BAKER & BAKER, unpubl.). Thus pollen does not necessarily germinate if knocked into nectar and

therefore the release of free pollen amino acids might be different in nectar than reported from the germination medium used in the experiment by LINSKENS & SCHRAUWEN (1969).

The present study was carried out to address the following questions:

1. How does pollen contamination influence nectar amino acids quantitatively and qualitatively over different time intervals?

2. Could pollen contamination of nectar be an important source of amino acids for nectar feeding pollinators, in particular for the butterfly *Battus philenor* L.?

To answer these questions, the release of free amino acids from pollen suspended in a plain sugar solution was measured over different time intervals. The experiment was carried out with pollen of four plant species, *Aesculus californica* (SPACH) NUTT., *Amsinckia lunaris* MACBR., *Brodiaea pulchella* (SALISB.) GREENE, *Carduus pycnocephalus* L., the flowers of which are preferably visited by a Californian colony of *Battus philenor* in the field (Hampton Road, Briones reserve, northeast of Berkeley, California; ERHARDT, unpubl.). In addition, pollen of these plant species is likely to be knocked into the nectar by flower visitors. Pollen contaminated nectar samples – not caused by sampling of the investigators, since every precaution was taken – were frequently observed in these species (BAKER & ERHARDT, pers. obs.).

Materials and methods

Pollen of *Amsinckia lunaris, Brodiaea pulchella, Carduus pycnocephalus* and *Aesculus californica* was collected from freshly dehisced anthers from plants growing in the field. Collecting of *Amsinckia lunaris* pollen was very tedious due to the small size of the anthers. Pollen was stored for several days in the refrigerator at 5 °C until sufficient pollen of the four species considered was collected.

For each species, a pollen suspension of 8.8 µg/µl was made, using a sucrose solution of 20% (weight/weight). A sugar concentration of 20% was taken as an average of the nectar sugar concentrations of the four plant species.

The amino acid concentration in the solution was determined after time intervals of 1, 2, 4, 16, 64, 128, 256 min and after 24 h in the following way: the suspension was vortex-mixed each time before aliquots of 3 µl were taken and spotted on strips of chromatography paper (Whatman #1). After the spots were fully dried, the remaining pollen grains were brushed off. The spots were then stained with ninhydrine and their amino acid concentration was read using the histidine scale developed by BAKER & BAKER (1973a, b, 1980). Concentrations were too low for reliable readings of the staining intensity with a spectrophotometer.

To count the number of pollen grains in the different pollen suspensions, aliquots of 2 µl were spotted on slides. Potential viability of the pollen grains was tested by staining them with acid fuchsin in polyvinol lactophenol. Deeply stained grains were assumed to be viable, lightly stained grains as doubtful and unstained grains as non viable. Pollen grains were inspected and counted in an Olympus microscope (BH-2). Measurements of pollen grains were taken at 1000 × (oil immersion). For calculating the volumes of the pollen grains (Table 1), their shapes were approximated by ellipsoids (*Aesculus californica, Amsinckia lunaris, Carduus pycnocephalus*) or by a composition of two ellipsoids (*Brodiaea pulchella*).

The amino acid contribution of a single pollen grain (Table 1) was assessed for each species by taking the maximum amino acid concentration (µg/µl) obtained in the diffusion experiment and dividing it by the number of viable (or viable plus doubtful) pollen grains per µl.

For *Aesculus californica*, amino acid composition and relative proportions of the individual amino acids present after the different time intervals were determined. A pollen sample was taken and again suspended in a 20% (w/w) sucrose solution. Aliquots (3 µl) were taken at the same time intervals as mentioned above, spotted on chromatography paper and dried. The remaining pollen grains were again brushed off. Amino acid composition and the relative proportions of the amino acids were determined with TLC as described in BAKER & BAKER (1977) but taking advantage of the better separation of the amino acids on plates with micropolyamide provided by SCHUELL & SCHLEICHER. To avoid excess carryover of sugar on the plates, the dansylated amino acids were reextracted with a mixture of chloroform (7 parts), methanol (2 parts), and acetic acid (2 parts) before they were spotted onto the plates. Composition and relative proportions of nectar and free pollen amino acids of the investigated species were also determined with TLC including the above mentioned modifications (BAKER & BAKER 1977, YORKS 1979). Scores from 1 – 5 were assigned to the individual amino acids with 1 for the least concentrated amino acid in the sample (BAKER & BAKER 1977).

For determination of arginine, the Sakaguchi test was used, since remaining sugar in the samples often covered the area of arginine on the plates, preventing reliable readings of this amino acid. Phenolics were tested with folin reagent and proteins with brom-phenol blue (BAKER & BAKER 1980, 1982). For arginine, phenolics and protein, scores were given for the different staining intensities in the corresponding tests.

Results

Figure 1 shows the increase of amino acid concentration in the pollen suspension of the four investigated species. Note that the rate of increase is different for each species. In *Carduus pycnocephalus* most of the amino acids had already diffused into the solution after one minute. The other species, however, show a much slower increase. In *Amsinckia lunaris,* the maximum concentration is reached after one hour, in *Aesculus californica* this requires a full day. An even further increase in *Aesculus californica* after one day seems unlikely, however. The increase of *Brodiaea pulchella* during the first 64 minutes is similar to that of *Aesculus californica,* but afterwards a strong increase occurs, which is especially pronounced between 128 and 256 minutes. This sudden increase is most likely due to osmotic bursting of the pollen grains, which then release their contents into the medium. Burst pollen grains of *Brodiaea pulchella* could first be observed microscopically after 64 minutes and more frequently after 128 minutes. In the other three species no burst pollen grains were observed. Both, the relatively slow initial increase in the amino acid

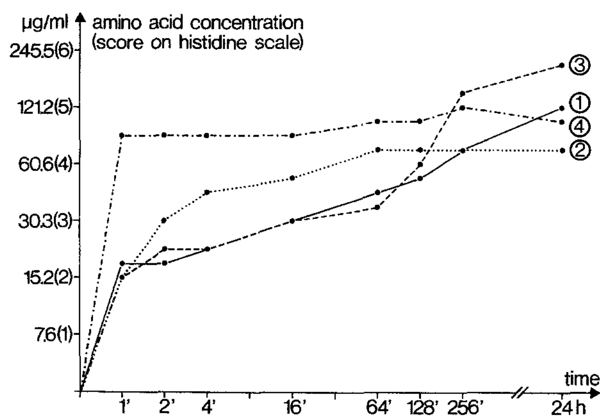

Fig. 1. Increase of amino acid concentration from pollen (8.8 µg/µl) suspended in plain sucrose medium (20% w/w) over different time intervals in *Aesculus californica* (1), *Amsinckia lunaris* (2), *Brodiaea pulchella* (3), and *Carduus pycnocephalus* (4)

Table 1. Parameters of pollen samples and pollen grains of *Aesculus californica, Amsinckia lunaris, Brodiaea pulchella* and *Carduus pycnocephalus*. * Only viable grains considered; ** viable and doubtful grains considered

Parameters	*Aesculus californica*	*Amsinckia lunaris*	*Carduus pycnocephalus*	*Brodiaea pulchella*
No. of pollen grains/µl	1 102	94	137	168
% of viable grains	48	88.8	50.5	89.1
% of doubtful grains	–	–	31.1	3.9
% of nonviable grains	52	11.2	18.3	7.0
Diameter(s) of pollen grain (µm) ± SD (n)	20.1 ± 0.6(21) 23.8 ± 1.0(10)	37.7 ± 1.9(10) 24.1 ± 0.7(10)	39.9 ± 1.2(10)	30.9 ± 2.5(10) 34.5 ± 1.9(10)
Average volume of grain (μm^3)	5 024	7 347	29 732	19 500
No. of pores/grain	3	3	3	–
Diameter(s) of pores (µm) ± SD (n)	4.8 ± 0.3(20)	9.0 ± 0.5(20) 6.8 ± 0.6(20)	8.7 ± 0.9(26)	–
Single pore area (μm^2)	17.8	48.4	59.7	–
Total pore area/vol of grain × 1 000	10.6	19.7	6.0	–
Total pore area/vol of grain × 1 000 × proportion of viable grains	5.1	17.5	3.0* 4.9**	–
Amount of free amino acids per pollen grain × 1 000 (µg)	0.2	0.9	1.7* 1.1**	1.4* 1.36**
Average amino acid concentration in nectar µg/µl (n)	0.15(7)	0.057(5)	0.009(6)	0.038(4)
Score on histine scale	$\sim 5\frac{1}{4}$	$\sim 3\frac{3}{4}$	~ 1	$\sim 3\frac{1}{4}$

concentration and subsequent bursting of the pollen grains could be due to the fact that the pollen grains of *Brodiaea pulchella* do not have pores, in contrast to the other three species.

The different rates of increase of the amino acid concentration in the medium are not directly explained by the different pore sizes of the grains (Table 1, pore area per vol. of grain) nor by the different percentages of viable grains in the pollen samples (which was beyond control for the experiment) nor by a combination of both (Table 1, pore area/vol. of grain × percentage of viable grains). Although these factors certainly influence the release of the free pollen amino acids, other factors, which are difficult to measure and to assess seem to be effective as well. Such factors include thickness, structure and permeability of the pollen wall, proportion of the weight of the pollen wall to the total weight of the pollen grain and especially the structure of the pollen wall at the pores. Note that the numbers of pollen grains in the pollen suspensions differ considerably among the four species due to differences in size and specific weight of the pollen grains. Similarly, the amount of free amino acids of a single pollen grain is quite different in the four species.

Table 2 shows presence and increase of the single free pollen amino acids for *Aesculus californica* over the experimental time. The number of detected amino

Table 2. Release of single amino acids, protein and phenolics from pollen of *Aesculus californica* in plain sucrose solution (20% w/w) after different time intervals. Scores from *1* – *5* indicate increasing levels of concentration with *1* for the least concentrated constituent (BAKER & BAKER 1977)

	50″	2′	4′	16′	64′	128′	256′	490′
Proline	4	4	4	4	4	5	5	5
β-alanine	1	1	1	2	2	2	2	2
Arginine	1	1	1	2	3	3	3	3
Glycine	1	1	1	1	2	2	3	3
Serine	1?	1?	1?	2	2	2	2	2
Threonine	1?	1?	1?	2	2	2	3	3
Valine			1	1	2	2	3	3
Phenylaline			1	1	2	2	3	3
Leucine			1	1	2	2	3	3
Isoleucine			1	1	1	2	3	3
Alanine				2	2	2	2	2
Glutamine				1	1	2	2	2
Asparagine				1	1	2	2	2
Glutamic				1	1	1	2	2
γ-amino buturic				1	1	1	2	2
Tyrosine				1	1	2	2	3
Lysine					2	2	3	3
Tryptophane					1	2	2	3
Methionine							2	2
Hydroxyproline								3?
Phenolics	1	1	1	2	2	3	3	3
Protein				trace	1	2	3	3

acids increases over time and the most easily soluble and/or most strongly represented amino acids could be detected first in the medium. This pattern is strongly suggesting a diffusion process of the free amino acids. The increase of protein and phenolics in the medium is also best explained by diffusion.

Finally, in Table 3, composition and proportions of the free pollen amino acids of the four investigated species are indicated. For comparison, nectar amino acid concentrations and compositions are also listed.

In the free pollen amino acids, both composition and relative proportions are similar in the four species. They all have a fair number of free amino acids with proline dominating. This is consistent with the results of other investigations on free pollen amino acids (STANLEY & LINSKENS 1974). A comparison of nectar and pollen amino acids shows that not surprisingly the number of free amino acids in pollen outnumbers the amino acids found in the nectar of the corresponding species. In addition, the free amino acids found in both nectar and pollen seem to be present in larger proportions in pollen. In only one case an amino acid present in nectar was not detected in pollen (methionine in the nectar of *Amsinckia lunaris*), but this exception does not appear to be very meaningful. Note that also in the nectars of the four investigated species proline is the dominant amino acid.

Table 3. Comparison of nectar and free pollen amino acids in *Aesculus californica, Amsinckia lunaris, Brodiaea pulchella* and *Carduus pycnocephalus*. * From pollen sample suspended in water; ** from pollen sample suspended in 20% sucrose for 8 h. Scores from *1* – *5* indicate increasing levels of concentration with *1* for the least concentrated amino acid (BAKER & BAKER 1977)

	Aesculus californica			*Amsinckia lunaris*		*Carduus pycnocephalus*		*Brodiaea pulchella*	
	Nectar	Pollen		Nectar	Pollen	Nectar	Pollen	Nectar	Pollen
Alanine		2*	2**	1	2		3	1	3
Arginine	+	1	3	+	+	+	3	+	3
Asparagine		2	2	1(?)	1		3		2
Aspartic		1					1		2
Cysteine, etc.					2(?)				
Glutamic			2	1(?)	2		1	1(?)	2
Glutamine	1(?)	2	2	2	1		2	1	3
Glycine		3	3	1	2	1	2	1	3
Histidine	1(?)						2		
Isoleucine	2	2	3	1	2	1	2	1	2
Leucine		2	3		2	1	2	1	2
Lysine	2	1	3		1		2		1
Methionine	1	1	2	1			1		
Phenylalanine	2	1	3	1	2	1	2	2	3
Proline	5	5	5	3	4	2	5	3	4
Serine	1(?)	2	2	1	2		2		3
Threonine	1(?)	2	3	1	2		2	1	2
Tryptophan		1	3		1		2		2
Tyrosine	1	3	3	1	2	2	3	2	2
Valine	2	1	3	1	2	+	2	2	2
β-alanine		3	2	1	2		2	1	
γ-amino buturic			2	1(?)	2		3		2
Ornithine					1				
Unknowns	2, 2			1	3, 2, 2, 2 1, 1, 1, 1, 1		2, 1, 1		3, 3, 1

The amino acid concentration in the nectar is very low in *Carduus pycnocephalus*, it is also surprisingly low in *Brodiaea pulchella*, which is butterfly-pollinated and therefore would be expected to have a higher nectar amino acid concentration (BAKER & BAKER 1986). In *Amsinckia lunaris* fresh nectar has also a relatively low amino acid concentration. In *Aesculus californica*, the nectar amino acid concentration is more in the range of butterfly-pollinated flowers.

Discussion

Release of free pollen amino acids into sucrose medium. The results first show that different plant species differ considerably in their release of free pollen amino acids into a plain sucrose medium. These differences cannot be explained with differences in a single character of the pollen grains (e.g., different ratios of pore size to volume, Table 1) but seem to result from a combination of different properties of these pollen grains (e.g., permeability of the pollen wall, proportion of the weight of the pollen wall to total weight of the pollen grain, presence and structure or absence of pores, etc.). However, the steady increase of the amino acids in the medium in three of the four investigated species suggests that the amino acids diffuse passively out of the pollen grains into the medium and are not actively released as mentioned in GILBERT (1980). A diffusion process is also suggested by the sequence of amino acids released into the sucrose medium as well as by the release of phenolics and proteins from pollen of *Aesculus californica* (Table 2). Diffusion of protein into a plain sucrose medium has also been reported from pollen of *Petunia hybrida* (JACKSON & KAMBOJ 1986).

The first increase of amino acids in the medium after one minute could well be caused by amino acids located in and dissolved from the pollen wall as suggested by LINSKENS & SCHRAUWEN (1969). Except for *Carduus pycnocephalus*, however, the release of amino acids was distinctly slower in the present experiment than reported from pollen of *Petunia hybrida*, where 50% of the free amino acids were released into the medium after only one minute (LINSKENS & SCHRAUWEN 1969). This difference could well be due to the fact that boron was added to the sucrose medium of the *Petunia hybrida* pollen suspension and therefore germinating pollen was investigated in the experiments by LINSKENS & SCHRAUWEN (1969). As mentioned earlier, JACKSON & KAMBOJ (1969) found that *Petunia hybrida* pollen did not germinate in a sucrose medium if boron was absent. Thus the plain sucrose solution used in our experiment and most likely also nectar in nature are not necessarily a germination medium for pollen. The slower amino acid release in our experiment could therefore be explained by the fact that the free pollen amino acids were diffusing out of pollen grains which were not germinating. Although no pollen tubes could be observed in the pollen suspension of *Carduus pycnocephalus*, the rapid release of amino acids in this species could nevertheless result from an initial germination reaction of the pollen grains or alternatively also from an especially high permeability of the pollen wall.

The slower release of amino acids by diffusion [as opposed to the faster (active?) release of amino acids in germinating pollen] would also explain why the pollen feeding *Heliconius* butterflies handle their pollen loads for such extended time periods („several hours“, GILBERT 1972). It remains doubtful, however, if they are able to extract the entire nitrogen content out of the pollen as assumed by GILBERT

(1980). It seems more likely that the free pollen amino acids constitute the bulk of the nutrients these butterflies are getting from pollen, since no gut enzymes were detected in the pollen loads of the butterflies. On the other hand, salivary enzymes might considerably increase the level of amino acids (and proteins?) that the butterflies are able to extract from pollen, since the clear liquid which is exuded from the proboscis of the butterflies is saliva (BOGGS 1987) and not nectar as assumed by GILBERT (1972). It would be useful to test saliva for presence of proteases.

Pollen contamination of nectar: possible consequences for pollinators and plants. The results clearly confirm that the amino acid content of nectar can be considerably increased by pollen contamination as suggested and reported in other investigations (BUXBAUM 1927; BAKER & BAKER 1980, 1986; GOTTSBERGER & al. 1984; SCOGIN 1986). However, a significant increase of the nectar amino acid concentration requires contamination with a fair amount of pollen (Table 1) and the pollen grains have to remain long enough in the nectar to allow the amino acids to diffuse out. Contamination with only a few pollen grains would in most cases not affect the amino acid composition of nectar significantly except for flowers producing very small amounts of nectar as in the small florets in capitula of some *Asteraceae* (e.g., *Achillea millefolium, Leucanthemum vulgare*).

On the other hand, there are plant species where considerable amounts of pollen are inevitably knocked into the nectar by their visitors and pollinators or freely drop into the nectar. In such plants, the free pollen amino acids released into nectar are likely to be an important source of amino acids for the pollinators. This has again been proposed already by BUXBAUM (1927) for the bird-pollinated flowers of *Erythrina crista-galli* (BAKER & BAKER 1982) and more recently by SCOGIN (1986) for flowers pollinated by bats and non-volant mammals. The results of the present study also suggest that in the investigated plant species pollen amino acids released into nectar could be an important additional nutrient for visitors and pollinators. This might be true especially for pollinators which are exclusive nectar feeders such as the butterfly *Battus philenor*.

Since the pollen amino acid composition is similar in the four investigated species, the qualitative contribution of pollen amino acids to nectar is also similar. Proline, the dominant amino acid in pollen, has been suggested to play a role in pollen tube growth (STANLEY & LINSKENS 1974). It is also an important amino acid in nectar (BAKER & BAKER 1986). Accumulation of proline in nectar from pollen contamination could be important to insect pollinators, since proline is an important energy storage compound in insect haemolymph and flight muscle (BURSELL 1963, SACKTOR & CHILDRESS 1967) and also a source of flight energy in honey bees and probably also in other insects (BARKER & LEKNER 1972). There could even be a complementary effect between pollen and nectar amino acids, leading to a reduced secretion of amino acids into nectar in plant species in which the amino acid contribution from contaminating pollen is regularly high. Such an effect could operate to some degree in *Carduus pycnocephalus* and in *Brodiaea pulchella*. In the latter species the pollinating butterflies inevitably knock pollen into the nectar. Interestingly, uncontaminated nectar of *Brodiaea pulchella* has an amino acid concentration well below the average of butterfly pollinated flowers (BAKER & BAKER 1986). In *Carduus pycnocephalus* the nectar amino acid concentration is even lower. Since the wide range of visitors on capitula of *Asteraceae* behave quite "messily",

covering themselves with pollen and moving pollen all over the capitulum, they are also likely to knock fair amounts of pollen into the nectar. In doing so, they might add to the nectar the amino acids which in *Carduus pycnocephalus* are not secreted by the nectary.

However, the degree to which the proposed complementary effect is operating, if at all, is still speculative. Further information on plants regularly presenting pollen contaminated nectar to their visitors is needed.

A possible evolutionary pathway. Pollen-contaminated nectar may have been the starting point for the evolution of the pollen feeding habit of the famous *Heliconius* butterflies (GILBERT 1972, 1980). By imbibing pollen contaminated nectar, butterflies may well experience higher levels of amino acid concentration than they would from feeding on plain nectar. This in turn might enhance their sensitivity to amino acids. Furthermore, pollen might stick to their proboscides, which they would have to clean. In cleaning, the taste buds at the tip of the proboscis might come into close contact with this pollen and the butterflies might experience even higher concentrations of amino acids. This then might result in behavioural changes leading to more active collecting and handling of pollen. Since amino acids in the adult food of butterflies seem to be a clear selective advantage in longevity and egg production of females (GILBERT 1972, DUNLAP-PIANKA & al. 1977, MURPHY & al. 1983), behavioural changes leading to pollen feeding might have been strongly reinforced and followed also by morphological changes at the tip of the proboscis as observed in the pollen feeding *Heliconius* butterflies (GILBERT 1972).

The question might arise why pollen feeding has not evolved more often in butterflies. A possible reason for this restriction could be the very complex behaviour and environment involved with this particular way of feeding (GILBERT 1980). On the other hand, further examples of pollen feeding butterflies may still await detection. A first hint in this direction is the observation of pollen loads sticking to the proboscides of some tropical species of the genus *Parides* and *Battus* in Costa Rica (DE VRIES 1979). These butterflies might indeed feed on pollen in a similar way as do the *Heliconius* butterflies (DE VRIES 1979).

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