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Larval selectivity for yeast species by *Drosophila mojavensis* in natural substrates

(larvae–substrate comparisons/foraging strategy)

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ABSTRACT **The yeast flora found in the major substrates of *Drosophila mojavensis* and in larval guts was studied both qualitatively and quantitatively. Quantitative studies show that, in four of the five substrates tested, the larvae did not contain a random sample of the yeasts present in the substrates. One widely distributed cactus yeast, *Pichia cactophila*, was typically in greater frequency in the larvae than in the substrates. Another cactus yeast, *Candida sonorensis*, typically exhibited the opposite relationship. Laboratory tests support larval preference behavior rather than differential digestion as being primarily responsible. Larvae are capable of distinguishing between patches of different yeast species and spend more time in patches of preferred yeasts. *D. mojavensis* appear to be ecological (host plants) generalists and physiological (yeasts) specialists in contrast to the other cactophilic *Drosophila*. Selective feeding by *D. mojavensis* larvae in natural substrates may represent optimal foraging behavior.**

Interest in the microorganisms upon which *Drosophila* feed in nature dates back about 35 years (1–12). The researchers who participated in these early studies realized the importance of putting the population genetics of *Drosophila* into an ecological context. Food sources are certainly major factors of the ecology of any animal, and yeasts are considered to be a major food source for the majority of species of *Drosophila* in both adult and larval stages (13). The last decade, however, has seen an acceleration of interest in this subject, particularly in the yeasts that inhabit the decaying stems of various species of columnar cacti in the Sonoran Desert.

The Sonoran Desert of the southwestern United States and northwestern Mexico has provided a unique opportunity to study yeasts and their relationship to *Drosophila* species because the breeding and feeding sites of cactophilic *Drosophila* are well known (14, 15). The four species of *Drosophila* that are endemic to the Sonoran Desert are *D. pachea*, *D. nigrospiracula*, *D. mettleri*, and *D. mojavensis*. These desert-adapted flies utilize necrotic sections of cacti (or soil soaked with juices from necrotic tissue) for all stages of their life cycles. Of these four, *D. mojavensis* is the most polyphagous and also the most polytypic species. The major host plants of *D. mojavensis* include agria (*Stenocereus gummosus*) in Baja; organpipe cactus (*Stenocereus thurberi*) in Sonora, Mexico; and barrel cactus (*Ferocactus acanthodes*) in southern California. Cochal cactus (*Myrtillocactus cochal*) is used at times where it is found in Baja California.

Recent advances in the study of yeasts, cactophilic *Drosophila*, and their host plants started with two yeast surveys published in 1976 by Starmer *et al.* (16) and Heed *et al.* (17). These papers reported the kinds and quantities of yeasts present in rotting stems of the giant cacti and isolated from adult *Drosophila*. Most of the yeast isolates were found to be specific for cactus plants (18–20). Unfortunately, both papers suffered from the misclassification of several predominate yeast species. This problem was subsequently rectified by the reclassification of many of the yeast isolates into several new species (21–24). This new information was then used in a more extensive survey by Starmer *et al.* (25). The comprehensive work on these cactophilic yeasts has also provided the selective techniques necessary for more accurate quantification of the yeast flora of both cactus rots and *Drosophila*.

To date, research on the yeasts associated with *Drosophila* in the Sonoran Desert and elsewhere has essentially neglected the study of the yeasts ingested by *Drosophila* larvae. This is regrettable because, as Carson (13) clearly pointed out, adults will feed on a wide variety of fermenting substances, but evidence suggests that larval feeding is a more specialized and delicately adapted behavior. Larval feeding ecology is relevant to all aspects of insect population biology, including environmental effects on genetic variability, gene–environment interactions, and the selection pressures that direct evolution. This paper examines all of the major substrates of *D. mojavensis* and compares the types and frequencies of the yeasts present with the types and frequencies found in the guts of resident larvae.

MATERIALS AND METHODS

Yeast Isolation. The initial approach in the study of the yeast flora of natural substrates of *D. mojavensis* involved streaking samples or dilutions of samples directly on acidified yeast extract/malt extract agar [Difco YM agar plus 0.7% (vol/vol) 1 M HCl, pH 3.7–3.8] in the field. Acidified plates were used to reduce the growth of bacteria. The plates were stored until colonies appeared. Counts of morphologically distinct colony types were then made, and a representative of each type was brought into pure culture by two successive platings on YM agar for identification. Identification was done by standard methods currently used in yeast taxonomy (26).

The isolation of yeasts from larval guts and specific substrates was accomplished as follows: Naturally occurring necroses were examined for the presence of *Drosophila* larvae. If they were present, three or four 1-g samples of the rotting tissue were collected from the area of the rot containing the larvae. Six to eight second- or third-instar larvae were also collected. The larvae were surface sterilized in 70% (vol/vol) ethanol for 1 min, rinsed twice in sterile water, and ground up in pairs in a small glass homogenizer. Dilutions of both the tissue and larval samples were made in sterile water. Appropriate dilutions were plated on selective synthetic media containing carbon sources utilized by only one or two of the species present. A key to frequently recovered cactus yeasts based on carbon source utilization is presented by Starmer *et al.* (25). After a sufficient in-

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Abbreviation: YM agar, yeast extract/malt extract agar.

cubation period, counts of the yeast species present on each selective plate were made. A representative of each species was brought into pure culture by two or three successive platings on YM agar. Each representative was then identified to the species level by contemporary yeast taxonomy (26).

Larval Preference. Larval preference tests for specific yeasts were performed in the laboratory, using Petri plates of YM agar and pairs of yeast species. Yeast cells were obtained from 3-day-old cultures and were transferred to the YM plates with a loop. Four patches of yeast per plate (each about 5 mm in diameter) were established. Patches of one species of yeast were placed at 12 and 6 o'clock and the other species was placed at 3 and 9 o'clock on the plates. Twenty to 50 *D. mojavensis* larvae were then introduced into the center of each plate. The number of larvae in each patch type was recorded at 5, 15, 30, 45, and 60 min after introduction.

Larval Digestion. Digestion experiments utilized first- and second-instar larvae of *D. mojavensis* (strain A761) from axenic cultures. This strain was originally collected from an agria rot in northern Baja California in December of 1979. *Pichia cactophila* (strain 79-267.1) and *Clavispora opuntiae* (strain 79-267.12) were isolated from an agria rot at the same time and in the same area as the *Drosophila*. Lawns of these two yeasts were produced by plating high-concentration suspensions on YM agar plates and allowing growth for 48 hr. Larvae (100–150) were then introduced into these yeast plates. The larvae fed on the yeasts for approximately 12 hr, were removed and rinsed in 0.7% sterile saline, and were placed on nutrient-deficient (for yeast growth) agar plates. The time points in this experiment were 0, 4, 6, 8, and 10 hr after cessation of feeding. At these times, four larvae that had ingested *P. cactophila* were removed, surface sterilized in 70% ethanol for 1 min, rinsed in sterile water, ground up in a glass homogenizer, diluted with sterile water, and plated on YM agar. Four larvae that had fed on *C. opuntiae* were treated identically. After incubation, the numbers of colonies on the YM agar plates were counted and recorded.

RESULTS AND DISCUSSION

Table 1 presents the collection record of yeasts isolated from *D. mojavensis* substrates and includes all of our isolation data as of July 1980. It is evident that some differences exist in the frequency of isolation of certain yeasts from the different substrates. Starmer (27) has shown that the phyletic division of the host plants influences the proportions of the yeasts in their necroses. Because agria, organpipe, and cochal all belong to the same subtribe of cacti (Stenocereinae) and barrel cacti are in an entirely different tribe (Cacteae), differences in yeast communities between substrates should be most noticeable in comparisons of these two groups. The data in Table 1 support this contention. Most of the major yeast species appear to be generalized for agria, organpipe, and cochal. Some yeasts are conspicuously absent from barrel rots—e.g., *Candida* sp. "A," *P. mexicana*, and *C. albidus*. On the other hand, the predominantly isolated variety of *P. amethionina* from barrel rots (*pachycereana*) is not normally found in necroses of cacti belonging to the Stenocereinae subtribe. The most frequently isolated yeasts, *P. cactophila* and *C. sonorensis*, are found in all four substrate types.

Comparisons of the yeast communities of the substrates with the yeasts found in the guts of larvae living in the substrates are shown in Table 2. Only yeasts for which the average relative frequency was greater than 1% are included in this table. The differences between Tables 1 and 2 in the ranking of yeasts reflects the difference between qualitative and quantitative techniques. For example, it is possible that a yeast species may be

Table 1. Collection record of yeasts isolated from *D. mojavensis* substrates

Yeast	No. isolates/no. plants sampled			
	Agria (n = 105)	Organ-pipe (n = 42)	Cochal (n = 15)	Barrel (n = 13)
Pichia cactophila	0.734	0.595	0.733	1.000
Candida sonorensis	0.420	0.476	0.800	0.231
Pichia amethionina v. *amethionina*	0.200	0.072	0.067	0.077
Pichia amethionina v. *pachycereana*	0.010	0	0	0.154
Candida ingens	0.076	0.262	0	0.077
Candida sp. "K"	0.105	0.215	0	0
Candida sp. "A"	0.066	0.048	0.200	0
Pichia mexicana	0.029	0.190	0.067	0
Cryptococcus cereanus	0.029	0.143	0	0
Clavispora opuntiae	0.010	0	0.133	0
Cryptococcus albidus v. *diffluens*	0.029	0.024	0.067	0
Candida mucilagina	0.095	0.020	0	0
Cryptococcus albidus v. *albidus*	0	0.024	0.067	0
Kluyveromyces marxianus	0.010	0	0.067	0
Candida boidinii	0	0	0.067	0
Candida guilliermondii	0.010	0.024	0	0
Trichosporon cutaneum	0	0.024	0	0
Rhodotorula minuta v. *texensis*	0	0.024	0	0
Pichia heedii	0.010	0	0	0

isolated from virtually every rot examined, but never be the most frequent yeast within any of them. In this case, the yeast would have a high frequency of isolation but a low relative frequency. The right-hand column in Table 2 lists the average total yeasts per gram of substrate or per larva. In the substrates, the yeasts range between 0.2×10^6 and 26.0×10^6 cells per gram. The average total concentration of yeasts in larvae range from about 500 to 50,000 per larva.

The most important point brought out in Table 2 is that in four of the five substrates tested, the *D. mojavensis* larvae did not contain a random sample of the yeasts present in the substrates. In agria from northern Baja California, the larvae contained significantly greater frequencies of *P. cactophila* and *P. amethionina* than the substrates. The frequencies of these species increased at the expense of *K. marxianus*, *C. opuntiae*, and *C. cereanus*. In agria from southern Baja, *P. cactophila* and *P. amethionina* again were in greater frequency in the larvae, although neither increase from substrate to larvae was statistically significant by itself. *C. sonorensis* was in significantly greater frequency in the substrate than in the larvae. Comparing the cochal substrate to larvae, *P. cactophila* increased significantly, whereas *C. sonorensis* and *P. amethionina* decreased. In organpipe, *P. cactophila* was more frequent in larvae and *C. sonorensis* was more frequent in the substrate. The only substrate–larvae comparison that did not show significant differences was that involving barrel cacti. The major trends, then, in the data presented in Table 2 are: (*i*) four of the five substrate–larvae comparisons involving *P. cactophila* show it to be in significantly greater frequency in the larvae and (*ii*) three of the four comparisons involving *C. sonorensis* indicate that it is typically in greater frequency in the substrate than in the larvae.

Aspects of the biology of larvae that may be responsible for the above phenomenon include morphology, physiology, and behavior. First, the feeding apparatus of a larva is morpholog-

Table 2. Comparisons of the yeast florae in natural substrates and larval guts of *D. mojavensis*

	Replicates	Yeast species*							log(total yeasts)†
		P.c.	*C.s.*	*P.a.*	*K.m.*	*C.o.*	*C.c.*	*C.i.*	
Agria‡	4	8.0 ± 0.6	8.4 ± 2.8	14.7 ± 2.3	19.6 ± 2.0	47.4 ± 2.3	1.9 ± 0.4	—	6.300
Larvae	4	36.7 ± 3.5	14.7 ± 5.5	29.8 ± 2.7	11.6 ± 2.0	7.0 ± 1.2	0.2 ± 0.2	—	2.658
t_s		9.614	1.041	4.210	2.889	15.003	3.991		
P		<0.001	NS	<0.01	<0.05	<0.001	<0.001		
Agria§	3	38.8 ± 5.3	14.1 ± 1.6	41.1 ± 4.7	—	—	—	6.0 ± 2.6	6.402
Larvae	3	44.2 ± 10.3	5.1 ± 1.2	49.1 ± 11.2	—	—	—	1.6 ± 1.1	3.994
t_s		0.464	4.518	0.623				1.497	
P		NS	<0.05	NS				NS	
Cochal	4	76.0 ± 4.5	15.1 ± 2.7	8.6 ± 1.7	—	—	—	—	6.509
Larvae	4	96.8 ± 1.3	1.3 ± 0.5	1.8 ± 0.7	—	—	—	—	4.216
t_s		5.286	6.252	4.270					
P		<0.01	<0.001	<0.01					
Organpipe	3	95.4 ± 1.9	4.5 ± 1.9	—	—	—	—	—	7.415
Larvae	3	99.4 ± 0.3	0.6 ± 0.3	—	—	—	—	—	4.628
t_s		2.569	2.664						
P		<0.1	<0.1						
Barrel	4	34.8 ± 3.4	—	65.3 ± 3.4	—	—	—	—	5.452
Larvae	4	33.7 ± 4.6	—	66.3 ± 4.6	—	—	—	—	3.440
t_s		0.209		0.215					
P		NS		NS					

Numbers for substrates and larvae are relative percentages averaged over replications ± SEM. Statistical tests (t_s) of the differences between substrates and larvae were performed on arcsine-transformed data. NS, not significant.

* Species from left to right are: *Pichia cactophila*, *Candida sonorensis*, *Pichia amethionina*, *Kluyveromyces marxianus*, *Clavispora opuntiae*, *Cryptococcus cereanus*, and *Candida ingens*.

† Averaged over replicates. Units for substrates and larvae are per gram of tissue and per larva, respectively.

‡ From northern Baja California.

§ From southern Baja California.

ically complex, with rows of fringes and grooves. These structures could serve as a filter and provide for nonrandom ingestion. This explanation, however, seems unlikely because one would expect the nonrandom ingestion to be correlated with yeast cell size (either directly or inversely). No such correlation has yet been observed.

The physiological explanation involves differential digestion. If several yeast species are ingested in the same relative proportions as they occur in the substrate, but some species are digested much faster than others, perhaps due to differences in cell wall structure, the relative proportions of the digestion-resistant yeast species would be greater in the guts of larvae than in the substrate. The results of experiments designed to test this idea are presented in Table 3. *P. cactophila* and *C. opuntiae* were chosen for this test because they exhibited the largest change in relative frequency from substrate to larvae (Table 2). The virtual disappearance of these two yeast species from the gut of *D. mojavensis* larvae takes only about 10 hr. The data were analyzed by calculating the regression of the natural logarithm of the number of yeast cells as a function of time (in hours). The

Table 3. Digestive decrease in the number of yeast cells in the guts of *D. mojavensis* larvae

Time after cessation of feeding,* hr	No. of yeast cells					
	P. cactophila			*C. opuntiae*		
	1	2	3	1	2	3
0.01	18,830	13,405	32,080	18,220	9,800	25,110
4.17	6,210	8,210	9,165	—	—	—
4.50	—	—	—	1,950	1,340	4,990
6.17	432	1,458	1,138	—	—	—
6.50	—	—	—	484	17	31
8.17	236	369	250	—	—	—
8.50	—	—	—	211	321	90
10.17	278	237	172	—	—	—
10.50	—	—	—	11	138	80

Three replications for each species are presented. Regression equation: ln(number of cells) = ln(a) + b(hr)

		P. cactophila	*C. opuntiae*
Parameters:	Slope (b)	−0.4944	−0.5840
	Intercept (a)	26,325.99	15,899.55
Null hypothesis: $b_1 = b_2$		$t = 0.82$ (df = 26, $P > 0.05$)	

* Midpoint of a 20-min procedure (except for initial time point).

Table 4. Laboratory demonstration of larval preference for yeast species

Experiment			No. of larvae observed, by replicate							
Yeast species	*D. mojavensis* strain	Location of larvae	1	2	3	4	5	Total	χ^2 (df = 1)	*P*
*P. cactophila** vs.	A761	*P. cactophila*	70	89	61	134	—	354	124.37	<<0.001
C. opuntiae†		*C. opuntiae*	16	33	28	36	—	113		
P. cactophila‡ vs.	A567	*P. cactophila*	47	43	25	51	61	227	134.80	<<0.001
C. sonorensis§		*C. sonorensis*	2	4	8	15	9	38		
P. cactophila‡ vs.	A700	*P. cactophila*	30	26	21	—	—	77	40.01	<<0.001
C. sonorensis§		*C. sonorensis*	3	2	11	—	—	16		

Numbers represent the total number of larvae feeding on a yeast type cumulative over the five different observation times after introduction of larvae.
* Strain 79-267.1.
† Strain 79-267.12.
‡ Strain 78-32.
§ Strain 78-34.

slopes of the two regression lines were compared by using a *t* test (null hypothesis: $b_1 = b_2$). The calculated *t* value was 0.82 (Table 3), which indicates that there is no significant difference in the rates of digestion.

Larval behavior may also be a causal factor. That is, nonrandom ingestion might be the result of selective feeding. If the structure of the substrate is such that patches of yeasts exist, then the phenomenon shown in Table 2 could be produced by larvae merely preferring to feed (or feeding for a longer period) in patches of certain yeasts. Patchy substrate structure is consistent with the colonial type growth of yeasts. In order to demonstrate that larvae have the ability to feed selectively, preference tests for yeast species were performed in the laboratory. The techniques used in these tests were similar to those used by Cooper (28). As before, the yeasts that were used, *P. cactophila*, *C. opuntiae*, and *C. sonorensis*, were chosen because they showed significant changes in relative frequency from substrate to larvae. The data in Table 4 show conclusively that larvae do prefer certain yeasts. Larvae of *D. mojavensis* are more frequently observed feeding in patches of a preferred yeast (i.e., *P. cactophila*) than in patches of the other species. All differences in the numbers observed feeding in patches of different yeast species (Table 4) are statistically significant. It is by no means trivial that the preferred yeast in these laboratory tests, *P. cactophila*, is also the species that is consistently in higher relative frequency in larval guts than in natural substrates (Table 2).

Thus, *D. mojavensis* larvae are behaviorally capable of nonrandom ingestion by spending more time feeding in patches of preferred yeasts. This phenomenon of selective feeding in the Sonoran Desert may be related to the degree of polyphagy. *D. mojavensis* is the only cactophilic *Drosophila* to exhibit selective feeding in natural substrates (unpublished data) and is the only polyphagous species. Being polyphagous, *D. mojavensis* has had the evolutionary latitude to specialize on a widely distributed yeast, *P. cactophila*. Intrinsic in this explanation is the fact that *P. cactophila* must necessarily be an advantageous food source. The other desert-adapted *Drosophila* species are more or less restricted to a single host plant and cannot ecologically afford to specialize. They therefore, are yeast generalists—eating whatever is available. This concept of ecological specialism (plants) versus physiological generalism (yeasts) was proposed by McNaughton and Wolf (29). In this case, *D. mojavensis* would be considered an ecological generalist and a physiological specialist. *D. nigrospiracula*, *D. mettleri*, and *D. pachea* are ecological specialists but physiological generalists. Preliminary data on polyphagous species outside the Sonoran Desert (*D. arizonensis*, *D. pseudoobscura*, and *D. melanogaster*) show them also to be physiological specialists (unpublished data).

The observations reported in this paper also have direct applications to the field of optimal foraging theory. Unfortunately, our knowledge is too incomplete to say that the larvae are foraging optimally. For example, optimal foraging could involve selective feeding on an exceptionally nutritious yeast species. However, the relative nutritional values of the yeasts are, as yet, unknown. With additional information, the larvae and the yeasts could certainly be used as a model system for the examination of specific foraging theories. The frequency, density, and size of the food patches (yeasts) are all experimentally manipulatable. Laboratory tests show that the selective feeding behavior of *D. mojavensis* larvae depends on all three parameters. Experimentation along these lines may provide insights into the nature of optimal foraging behavior as well as contribute to the understanding of insect–yeast–host plant relationships and their evolution.

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