The Role of Phytosterols in Host Plant Utilization by Cactophilic *Drosophila*

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The Cactus-*Drosophila* Model System of the Sonoran Desert consists of four endemic species of *Drosophila (D. mojavensis, D. nigrospiracula, D. mettleri* and *D. pachea)* and five species of columnar cacti (agria, organpipe, saguaro, cardón and senita). Extensive collection records indicate that each cactus species has only one species of *Drosophila* as the primary resident. The elimination of six of the twenty possible random combinations of *Drosophila* species and cactus species can be attributed directly to phytosterols. *Drosophila pachea* has a strict requirement for Δ^7-sterols such as 7-cholestenol and 7-campestenol. Since Δ^7-sterols are found only in senita cactus, *D. pachea* cannot use agria, organpipe, saguaro or cardón as host plants. The lipid fractions of agria and organpipe are chemically similar and contain high concentrations of several 3β,6α-dihydroxysterols. Larval viability tests using chemical constituents of organpipe cactus demonstrate that the sterol diols are toxic to *D. nigrospiracula* but not to the resident species, *D. mojavensis.* Agria and organpipe are therefore unsuitable as host plants for *D. nigrospiracula.* These results suggest that phytosterols play a major role in determining host plant utilization by cactophilic *Drosophila* in the Sonoran Desert.
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Dietary sterols are of great importance to members of the class Insecta as insects are unable to synthesize them de novo (1). The biological functions of sterols in insects include incorporation into membranes and roles as precursors for steroid hormones (e.g., ecdysone) and, in some cases, as defensive secretions. In addition, phytosterols in the host plants of cactophilic *Drosophila* of the Sonoran Desert appear to play a major role in host plant utilization.

The Sonoran Desert includes most of the Baja Peninsula and a large portion of northwestern Mexico extending into southwestern Arizona. The Cactus-*Drosophila* Model System of the Sonoran Desert consists of four endemic drosophilids which feed and breed in the necrotic tissue of five species of columnar cacti (Table 1). Although several of the *Drosophila* species shift host plants between Baja California and the mainland, extensive rearing records collected over a 10-yr period indicate that there is essentially only one fly species per cactus species. This phenomenon effectively eliminates interspecific competition, an interaction which may be too ecologically expensive to occur in the stressful environment of the desert. Through investigations of the chemical basis of these host plant relationships, we can gain insight into how the relationships are maintained and, perhaps, how they originally evolved.

Phytosterols in one cactus species, senita, now have been firmly established as primary determinants of the host plant specificity of *D. pachea.* The *Drosophila pachea*-senita investigation began in the mid-1960s with the observation that this species of fly bred only in the rotting stems of senita cactus and could not be reared from standard *Drosophila* media unless a cube of senita cactus were added (2,4-7). The unusual sterols in senita, 4α-methyl-Δ^7-cholesten-3β-ol (lophenol) and Δ^7-stigmasten-3β-ol (schottenol), which had been reported by Djerassi (8) suggested that *D. pachea* may have a unique sterol requirement. The initial sterol utilization tests reported that *D. pachea* could use schottenol, lathosterol and 7-dehydrocholesterol, but that $\Delta^{5,7}$-stigmastadien-3β-ol produced infertile females and cholesterol, lophenol, β-sitosterol, stigmasterol, ergosterol and Δ^7-ergosten-3β-ol did not support larval growth (4). *Drosophila pachea,* then, was the first insect species described that could not

TABLE 1

***Drosophila*-Cactus Relationships and Specificities**

	Host Plants[a]		Rearing records[b]
Species	Baja	Mainland	% Resident species
D. mojavensis	Agria	Organpipe	99.6
D. nigrospiracula	Cardón	Saguaro	99.0[c]
D. mettleri	Cardón soaked soil	Saguaro soaked soil	100[d]
D. pachea	Senita	Senita	99.9

[a]Scientific names: *Stenocereus gummosus* (agria), *S. thurberi* (organpipe), *Pachycereus pringlei* (cardón), *Carnegiea gigantea* (saguaro) and *Lophocereus schottii* (senita).
[b]Data modified from Fellows and Heed (2).
[c]May include *D. mettleri.*
[d]Data from Heed (3).

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use cholesterol. Unfortunately, the synthetic schottenol used in the original study later was shown to be contaminated with ca. 40% Δ^7-campesten-3β-ol. Additional experiments have demonstrated that pure schottenol also does not support larval growth of *D. pachea* (9).

A more complete understanding of the *D. pachea*-senita relationship was delayed until the late 1970s and required further characterization of the sterols associated with this plant. Senita cactus apparently is a plant species with an interrupted sterol biosynthetic pathway which results in the accumulation of intermediate forms (10). The absence of Δ^5-sterols and the presence of 4α-methyl, Δ^7- and $\Delta^{8,14}$-sterols as the principal sterols support the statement that the later steps in the pathways to typical phytosterols (campesterol, sitosterol and stigmasterol) are either inhibited or absent in this cactus. In addition to schottenol and lophenol, six other sterols have been identified: Δ^7-cholesten-3β-ol, Δ^7-campesten-3β-ol, α-spinasterol, $\Delta^{8,14}$-cholestadien-3β-ol, 4α-methyl-$\Delta^{8,14}$-cholestadien-3β-ol (locereol) and 24-methylene lophenol. Given the structures listed above, inhibition appears to involve Δ^{14}-reductase, Δ^5-dehydrogenase and 4α-methyl hydroxylase systems. Sterol biosynthetic intermediates have been isolated from rat liver homogenate and cultures of *Chlorella*, bramble cells and yeasts when certain nitrogenous compounds are added to the medium (10). Senita cactus does contain high concentrations of alkaloids, e.g., lophocereine and pilocereine (11), and it was hypothesized that senita alkaloids may inhibit normal phytosterol biosynthesis and cause the observed accumulation of sterol intermediates. However, six species of cactophilic yeasts grown on a complete medium supplemented with a comparable concentration of senita alkaloids produced typical yeast sterols, principally ergosterol with traces of zymosterol (12). No sterol intermediates comparable to those in senita were detected. Alkaloids, then, do not appear to be the inhibitors which interrupt sterol biosynthesis in the cactus, and the cause of this interruption remains to be determined.

Of the eight sterols identified in senita, only two are known to support the growth of *D. pachea* through two generations. These are 7-cholestenol (lathosterol) and 7-campestenol (9). Knowledge of which sterols can and cannot be used by *D. pachea* provides a basis for speculation as to the metabolic deficiencies of this species. Based on a study of sterols in crickets (13), the sequence of double bond changes in the B ring during sterol metabolism in insects has been postulated to be $\Delta^5 \rightarrow \Delta^0 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$. The overall transformation of $\Delta^5 \rightarrow \Delta^{5,7}$ may be obligatory in insects which ingest Δ^5 sterols because the molting hormones are 6-keto-Δ^7 derivatives. Because *D. pachea* cannot complete its life cycle on cholesterol (Δ^5) or cholestanol (Δ^0), it appears that the $\Delta^0 \rightarrow \Delta^7$ step in the pathway is blocked. This hypothesis is supported by the observation that *D. pachea* does convert dietary lathosterol (Δ^7) to the $\Delta^{5,7}$, i.e., 7-dehydrocholesterol (14).

With respect to the sterol side chain, removal of alkyl groups at C-24 also is a necessary step in the production of ecdysone. Apparently, *D. pachea* lacks the ability to demethylate ergostane derivatives (24β-methyl) or deethylate stigmastane derivatives (24α-ethyl) as it is unable to utilize ergosterol, Δ^7-ergosten-3β-ol or schottenol. Campestane derivatives (25α-methyl), however, can be used. *Drosophila pachea* must be able either to remove an α-methyl group from C-24 or to produce and use a 24α-methyl ecdysone derivative. Makisterone A (22R-2β,3β,14,20,22,25-hexa-hydroxy-5β-campest-7-en-6-one) is a 24α-methyl derivative and has been reported as the main molting hormone in embryos of the milkweed bug (15), an insect that does not dealkylate plant sterols.

This paper deals with the role of phytosterols in the host plant utilization of organpipe and agria cacti. Extensive field and laboratory studies have demonstrated that *D. nigrospiracula* larvae mature in necrotic tissues of saguaro cactus (Table 1) but cannot do so in rotting organpipe and agria tissue. For example, progeny production of *D. nigrospiracula* females on organpipe substrate was less than 5% of their production on saguaro (2). The resident drosophilid for organpipe and agria is *D. mojavensis*.

The chemistry of organpipe has been well-characterized. Approximately 28% of the dry weight of the plant is composed of triterpene glycosides, while lipids make up about 11% (9). The glycosides are glucose-rhamnose tetrasaccharides of oleanolic acid, thurberogenin and queretaroic acid (16). The lipids are composed mainly of C_6 to C_{12} fatty acid esters of nine mono-, di- and trihydroxy triterpenes (17–19) and of five 3β,6α-dihydroxy sterols (20). The major neutral triterpenes are betulin and calenduladiol. In one sample of fresh tissue, hydrolysis of the lipids and compositional analysis gave 2.6% sterol diols, 5.1% neutral triterpenes, 3% medium chain fatty acids and .07% plant phytosterols (cholesterol/campesterol/sitosterol, 1:2:7), all expressed as percent of the cactus dry weight.

The sterol diol category of organpipe consists of five dihydroxy sterols which fall into a logical biosynthetic sequence. These five are cyclostenol (14α-methyl-9,19-cyclo-5α-cholestan-3β,6α-diol), stenocereol (14α-methyl-5α-cholesta-8,24-dien-3β,6α-diol), macdougallin (14α-methyl-5α-cholest-8-en-3β,6α-diol), thurberol (5α-cholesta-8,14-dien-3β,6α-diol) and peniocerol (5α-cholest-8[9]-en-3β, 6α-diol). The putative sequence starts with either cyclostenol or stenocereol and ends with peniocerol. The $\Delta^8 \rightarrow \Delta^7$ isomerization which normally follows in sterol biosynthesis presumably is inhibited by the 6α-OH group of peniocerol, and the biosynthesis terminates with this diol. Alkylation of the side chain also must be prevented because all of the sterol diols have the cholesterol side chain.

Agria cactus has not been characterized as well as organpipe, but appears to be very chemically similar. Approximately 36% of the dry weight of the tissue is triterpene glycosides and about 7% is lipids. Hydrolysis of the triterpene glycosides gave a 2:1 ratio of glucose to rhamnose and a 4:1 ratio of sugars to aglycones. The aglycones are represented by one neutral and two acidic triterpenes, none of which are found in organpipe. Examination of the lipid fraction showed only small amounts of normal phytosterols but a relatively large concentration of sterol diols similar to those in organpipe and of three pentacyclic triterpenes. Like in organpipe, the triterpenes and sterol diols are monoesterified to medium chain fatty acids (9).

The experiments described herein represent an attempt to determine which chemical constituents of organpipe and agria are responsible for the apparent inability of *D. nigrospiracula* to utilize these cacti as larval substrates. Because agria and organpipe are chemically similar, only chemical constituents of organpipe were used.

MATERIALS AND METHODS

Both species of *Drosophila* were obtained from the laboratory of W. B. Heed, Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona. Organpipe chemical constituents were extracted from fresh tissue as follows: cactus tissue was homogenized with an equal volume of MeOH. This slurry was filtered and the residue was extracted exhaustively with $CHCl_3$-MeOH (2:1, v/v). The MeOH and $CHCl_3$-MeOH filtrates were combined, evaporated and distributed between water and ether. Triterpene glycosides were purified by extraction from aqueous solution with n-butanol and precipitated with ether. Aglycones (acidic triterpenes) were obtained by hydrolysis of the saponins with 3N H_2SO_4 in H_2O-PrOH (2:1, v/v) on a steam bath for 24 hr followed by ether extraction. The ether soluble lipids were hydrolyzed with alkali, and betulin and calenduladiol (major neutral triterpenes) subsequently were crystallized from the nonsaponifiable fraction with 95% EtOH. A mixture of sterol diols was obtained from the nonsaponifiable fraction by column chromatography using silica gel.

Medium for testing larval viability was prepared by adding the chemical constituent to homogenized saguaro rot. However, water-insoluble compounds first were dissolved in ether and added to dried saguaro powder. The ether then was evaporated and the powder rehydrated and added to homogenized saguaro rot. In all cases, the resulting mixture was blended to insure homogeneity and divided into six portions in half-pint milk bottles. The quantities of organpipe constituents used were calculated as a percentage of the dry weight of the media. Viability tests for each species were set up in triplicate using 50 first-instar larvae per bottle. Larval density was decided on the basis of a preliminary experiment which showed that 50 larvae per test yielded a higher percent adult emergence than 100, 200 or 300 larvae per test. Larvae were given sufficient time (ca. 30 days) to develop into adult flies. The number of adult flies that eclose in each bottle is a measure of larval viability in the media. Control bottles containing saguaro rot without organpipe constituents were set up for each test of a particular constituent to control for possible differences between batches of larvae or saguaro rot. The data were analyzed using a one-way analysis of variance (ANOVA) with replication (21).

Analyses of fresh tissue, "young" rots and "old" rots of organpipe were performed by blending individual samples (several kg) with two vol of methanol. The mixtures were filtered and the residues extracted with fresh methanol and acetone and dried. The pooled extracts were evaporated, and the aqueous mixtures remaining were acidified with HCl, extracted with ether and evaporated to dryness. The ether extracts were extracted with $NaHCO_3$ solution to remove acids and quantitatively analyzed by gas-liquid chromatography (GLC) (5% OV-101, 250 C) to determine sterol diol content. The bicarbonate solutions were acidified, and the fatty acids were extracted with petroleum ether, methylated and analyzed by GLC (20% DEGS, 180 C).

RESULTS AND DISCUSSION

Average percent larval viabilities of *D. mojavensis* and *D. nigrospiracula* on necrotic saguaro and organpipe homogenate are given in Table 2. From the data in this table it is clear that while *D. mojavensis* does equally well on either substrate, *D. nigrospiracula* suffers an enormous loss of viability when forced to use organpipe as a substrate. This substantiates a previous study (2) and demonstrates that organpipe is indeed an unsuitable host plant for *D. nigrospiracula.*

TABLE 2

Average Percent Viability ± Standard Deviation on Cactus Homogenates

	Average viability	
Substrate	*D. mojavensis*	*D. nigrospiracula*
Saguaro	94.0 ± 4.0	85.3 ± 3.1
Organpipe	93.3 ± 2.3	20.7 ± 2.3
F (df = 1,4)	0.063	855.364
P	n.s.	⩽0.001

Results of one-way ANOVA (F) and probability (P) are given. n.s. = Not significant.

The effects of organpipe constituents on the viability of these two *Drosophila* species are shown in Table 3. In all cases, one-way analysis of variance tests were done on each species separately because comparisons between species for each treatment are not as ecologically relevant as the effects of treatments within a species. Addition of triterpene glycosides to saguaro homogenate had no effect on *D. nigrospiracula,* but the statistical analysis did show a significant effect on *D. mojavensis* at the .05 level. Although the effect is statistically significant, the drop in viability of *D. mojavensis* from 92% (control) to 80% (40% triterpene glycosides) is not particularly severe. This reduction in viability is less than half of the corresponding change in *D. nigrospiracula* (97.3% to 68%), which was not statistically significant. It appears that the significance of the effect of triterpene glycosides on *D. mojavensis* is due mainly to the low variance between replicates within treatments, particularly the ones involving 20% and 40% dry weight triterpene glycosides, and is of minimal biological significance.

No significant effect on viability was observed for either species when triterpene acids (aglycones), crude lipids or the two major neutral triterpenes were added to the saguaro homogenate. The first four compounds in Table 3, therefore, cannot be responsible for the exclusion of *D. nigrospiracula* from organpipe.

Drastic effects on both species were observed when free fatty acids were added to the medium. However, their effect on *D. nigrospiracula* was manifested at a lower concentration compared to *D. mojavensis.* The lowest concentration used (0.5% dry weight) reduced the viability of *D. nigrospiracula* to about 7% that of the control but did not appear to affect *D. mojavensis.* Higher concentrations reduced the viability of both species to zero or near zero.

The addition of free sterol diols to the medium had no significant effect on *D. mojavensis* even at the highest concentrations (10% dry weight). They did, however, affect *D. nigrospiracula* at concentrations of 1% dry weight

TABLE 3

Average Percent Viability ± Standard Deviation on Saguaro Homogenate + Organpipe Constituents

Substrate (Saguaro + constituent)	Average viability	
	D. mojavensis	*D. nigrospiracula*
Triterpene glycosides		
Control	92.0 ± 6.9	97.3 ± 10.3
10% dry weight	94.0 ± 7.2	87.3 ± 21.4
20% dry weight	78.7 ± 2.3	81.3 ± 3.1
40% dry weight	80.0 ± 2.0	68.0 ± 10.4
F (df = 3,8)	6.939	2.653
P	<0.05	n.s.
Triterpene acids		
Control	82.7 ± 12.2	55.3 ± 22.1
2.5% dry weight	74.7 ± 4.2	67.3 ± 14.2
5.0% dry weight	72.7 ± 4.2	64.0 ± 15.6
F (df = 2,6)	1.370	0.370
P	n.s.	n.s.
Crude lipids		
Control	86.0 ± 11.1	83.3 ± 7.0
5% dry weight	82.0 ± 10.0	89.3 ± 1.2
10% dry weight	76.7 ± 4.6	80.7 ± 4.6
F (df = 2,6)	0.804	2.463
P	n.s.	n.s.
Neutral triterpenes: betulin-calenduladiol		
Control	80.0 ± 7.2	65.3 ± 11.7
5% dry weight	82.0 ± 3.5	62.7 ± 10.1
10% dry weight	80.7 ± 2.3	64.7 ± 25.4
F (df = 2,6)	0.135	0.020
P	n.s.	n.s.
Fatty acids		
Control	86.0 ± 9.2	79.3 ± 12.2
0.5% dry weight	85.3 ± 8.1	5.3 ± 4.2
1.0% dry weight	8.0 ± 4.0	0.0 ± 0.0
2.0% dry weight	1.3 ± 1.2	0.0 ± 0.0
F (df = 3,8)	158.003	108.723
P	≪0.001	≪0.001
Sterol diols		
Control	92.0 ± 7.2	68.7 ± 19.6
0.5% dry weight	76.7 ± 9.5	54.7 ± 23.2
1.0% dry weight	78.0 ± 5.3	26.0 ± 19.7
2.0% dry weight	82.7 ± 15.5	4.7 ± 5.0
5.0% dry weight	73.3 ± 8.1	0.0 ± 0.0
10.0% dry weight	87.3 ± 17.0	0.0 ± 0.0
F (df = 5,12)	1.166	11.987
P	n.s.	<0.001

Results of one-way ANOVA (F) and probability (P) are given. n.s. = Not significant.

TABLE 4

Average Percent Dry Weight of Several Constituents of Organpipe During the Rotting Process

Tissue	No. samples	Lipids	Free sterol diols	Free fatty acids
Unrotted	4	16.0	0.43	0.41
"Young" rot	3	19.7	1.23	0.65
"Old" rot	3	23.7	1.87	0.35

or greater, and viability of this species is essentially zero at concentrations of 2% or greater.

These results indicate that only two organpipe constituents, fatty acids and sterol diols, have any toxic effect at reasonably natural concentrations. The explanation as to why these compounds are toxic, when the crude lipid fraction which contains these compounds is not, involves the fact that only small amounts of fatty acids and sterol diols are in a free form in the lipid fraction. In fresh tissue, the majority of these compounds are esterified to each other. The data in Table 3 indicate that the esterified form is not toxic. During the rotting process, however, some of these ester bonds may be broken by the action of microbial enzymes. This leads to an interesting question: just how much of the free C_6-C_{12} fatty acids and 3β,6α-dihydroxy sterols is present in necrotic organpipe tissues? To answer this question, a small number of samples of fresh tissue, "young" rots and "old" rots were analyzed; the results are presented in Table 4. Rots were designated as "young" or "old" based on color. Fresh organpipe tissue is light greenish-yellow, and rots develop through various color stages from orange to brown to black as the microorganisms consume the carbohydrate portion and oxygen converts phenolics to insoluble dark pigments. It can be seen in Table 4 that both the lipid fraction and the concentration of free sterol diols increase during the rotting process. The increase in the lipid fraction is due mainly to the release of the aglycones from the triterpene glycosides by microbial hydrolysis (9). Free sterol diols also are released during rotting by hydrolysis of the fatty acid esters. Free fatty acids, however, do not increase in concentration during rotting even though they are being released by hydrolysis. There are two possible explanations for this lack of increase. First, two frequently encountered cactophilic yeasts, *Candida ingens* and *Pichia mexicana*, produce extracellular lipases, and *C. ingens* can use free fatty acids as carbon sources (22). The growth of these two yeast species would, therefore, reduce the concentration of free fatty acids or prevent their accumulation in necrotic organpipe tissues. Second, free fatty acids are known to be quite chemically reactive. It is possible that the released fatty acids are being complexed with other components of the lipid fraction, e.g., triterpenes. This statement is supported by the observation that effect of fatty acids on both *Drosophila* species was reduced when they were added to saguaro homogenate along with 10% (dry weight) crude organpipe lipids. In the same procedure used to produce the data in Table 3, 2% fatty acids (plus 10% organpipe lipids) had no significant effect on *D. mojavensis* (average percent viability: control = 82.0, 2% = 88.0). Average percent viability of this species on 2% fatty acids without lipids was 1.3 (Table 3). The viability of *D. nigrospiracula* increased from zero on medium with 1% fatty acids to 36.6% on medium with 1% fatty acids plus 10% lipids.

The actual physiological mechanism of the toxic effect of sterol diols on *D. nigrospiracula* is unknown. In a pilot study using axenic cultures, neither species could use organpipe sterol diols as the sole source of dietary sterols because neither could survive for two generations on sterol-deficient medium supplemented with sterol diols (Kircher, H. W., unpublished data). Apparently, *D. mojavensis* has some means of excluding or detoxifying these compounds which is lacking in *D. nigrospiracula*. *Drosophila mojavensis*, which have been reared from an

organpipe necrosis, do not contain sterol diols in their tissues (23).

Also unknown is the relative importance of fatty acids vs sterol diols in the host plant relationships of these two species. Unquestionably, sterol diols are involved as determinants of host plant utilization. Both acids and diols are effective in preventing the development of *D. nigrospiracula* from larvae to adults. The tolerance of *D. mojavensis* and the concentration of these compounds in natural rots is sufficient to explain why *D. mojavensis* and not *D. nigrospiracula* can use necrotic organpipe and agria cactus tissues as breeding sites.

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