

# Phytosterol Metabolism and Absorption in the Generalist Grasshopper, *Schistocerca americana* (Orthoptera: Acrididae)

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A series of experiments, using GLC, RP-HPLC, and GC-MS techniques, were performed to examine the metabolic fate and absorption of different dietary sterols in the grasshopper *Schistocerca americana*. In the first experiment, grasshoppers were reared on diets containing different sterols presented singly. Cholesterol was the dominant tissue sterol recovered from cholesterol and “soybean sitosterol” fed grasshoppers but among the grasshoppers fed diets with stigmasterol and spinach sterols (both unsuitable for growth and development), the amount of cholesterol recovered was not different from that of newly hatched grasshoppers. In the second experiment, grasshoppers were given diets containing mixtures of soybean sitosterol and stigmasterol and the metabolic fate of these dietary sterols was recorded. Results from this experiment suggest that the presence of an unsuitable dietary sterol does not interfere with cholesterol production from sitosterol. They also demonstrate that large quantities of unmetabolized dietary sterols with C-24 ethyl groups are recovered from grasshoppers fed diets containing stigmasterol. Finally, tissue sterol profiles of grasshoppers with and without their midguts were compared. Results suggest that the midgut is the major tissue where unmetabolized dietary sterols accumulate. How these sterol metabolic constraints impact development and survival is discussed as well as the impact they might have on grasshopper feeding behavior. Arch. Insect Biochem. Physiol. 42:13–25, 1999. © 1999 Wiley-Liss, Inc.

**Key words:** cholesterol; grasshoppers; metabolic constraints; phytosterols; *S. americana*

\*Abbreviations used: KW = Kruskal-Wallis test; GLC = gas liquid chromatography; RP-HPLC = reversed phase high performance liquid chromatography; GC-MS = gas chromatography-mass spectrometry; 20-OH ecdysone = 20-hydroxyecdysone.

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## INTRODUCTION

Insects, as well as all other arthropods, are unable to biosynthesize sterols and must acquire these essential nutrients from their food (Clayton, 1964; Dadd, 1985). Cholesterol is the common sterol found in insects and serves in at least two significant physiological processes: (1) it is a structural component of cell membranes, and (2) it is a required precursor for the insect molting hormone 20-OH ecdysone (Grieneisen, 1994). Cholesterol has been shown to support normal development in most carnivorous and herbivorous insects (Dadd, 1977), but since it is rarely found above trace concentrations in plants (Salt et al., 1991; Patterson, 1994; but see Garg et al., 1987), herbivorous insects must either use the plant sterols present or metabolize them (Svoboda et al., 1994).

Physiological constraints in some herbivorous insects, however, can limit which phytosterols will support normal development (reviewed by Bernays, 1992). Among the grasshoppers (Orthoptera: Acrididae) that have now been examined, which include eight species representing four different subfamilies, development to the adult stage is always best on diets containing  $\Delta^5$ -sterols (e.g., cholesterol and sitosterol); on diets containing  $\Delta^7$  and/or  $\Delta^{22}$ -sterols (e.g., stigmasterol, lathosterol, or spinasterol), however, development is always poor (Dadd, 1960; Behmer, 1998). Interestingly, sterols with  $\Delta^7$  and/or  $\Delta^{22}$ -configurations can prevent development in the grasshopper *Schistocerca americana* even when a sufficient amount of suitable sterol (e.g., sitosterol) is present (Behmer and Elias, 1999a).

In a recent biochemical study, radiolabeled cholesterol was recovered from the grasshopper, *Locusta migratoria*, after it had been fed dietary [ $^{14}\text{C}$ ] sitosterol (Rath et al., 1993). Since sitosterol supports normal development in all grasshoppers so far studied, their failure to develop on diets that contain  $\Delta^7$  and/or  $\Delta^{22}$ -sterols may reflect an inability to metabolize them to cholesterol or absorb them as they are. In the current paper, we investigate the metabolic fate and absorption of different dietary sterols in the grasshopper *S. americana*. In our first experiment, grasshoppers were fed artificial diets containing different sterols that were presented singly and the metabolic fate of these dietary sterols was determined. In

our second experiment, grasshoppers were fed diets containing "soybean sitosterol" (a suitable sterol mixture) combined with stigmasterol (an unsuitable sterol) at different concentrations. This was done to determine whether unsuitable sterols interfered with cholesterol production from sitosterol. In our final experiment, absorption of unmetabolized dietary sterols into the hemocoel was measured. We suggest, based on the combined results from this study, that the failure of grasshoppers to complete development on diets with  $\Delta^7$  and/or  $\Delta^{22}$ -sterols is most likely associated with the accumulation of unmetabolized dietary sterols in the midgut tissues. Results from this study are also used to discuss how sterol metabolic constraints might impact grasshopper feeding behavior.

## METHODS

### Experimental Insect

*S. americana* (Drury), Orthoptera: Acrididae, is a polyphagous grasshopper occurring throughout the south and eastern United States and Mexico (Harvey, 1981). It is recorded as feeding on a wide range of cultivated and naturally occurring plant species (Kuitert and Connin, 1952). Grasshopper eggs were collected from a laboratory colony that was being reared and maintained under standard laboratory conditions (Behmer, 1998). The eggs were incubated at 35°C until hatch and newly emerging grasshoppers were individually transferred to rearing containers.

### Dietary Sterols and Artificial Diet

The sterols selected for this study represent different permutations of saturation at C-5, C-7, and C-22 (Fig. 1). Cholesterol, a  $\Delta^5$ -sterol, is the dominant sterol found in animals, including insects. The remaining sterols used in this study are synthesized by and accumulate solely in plants; they are also the most common sterols that grasshoppers are likely to encounter in the field. Sitosterol, a C-24 ethyl  $\Delta^5$ -sterol, is the predominant sterol found in grasses and is common in many angiosperm species where it often comprises more than 80% of the total sterol profile (Nes and McKean, 1977). Stigmasterol, a C-24 ethyl  $\Delta^{5,22}$ -sterol, is a common sterol that can comprise up to 40% of the total sterol profile in mem-

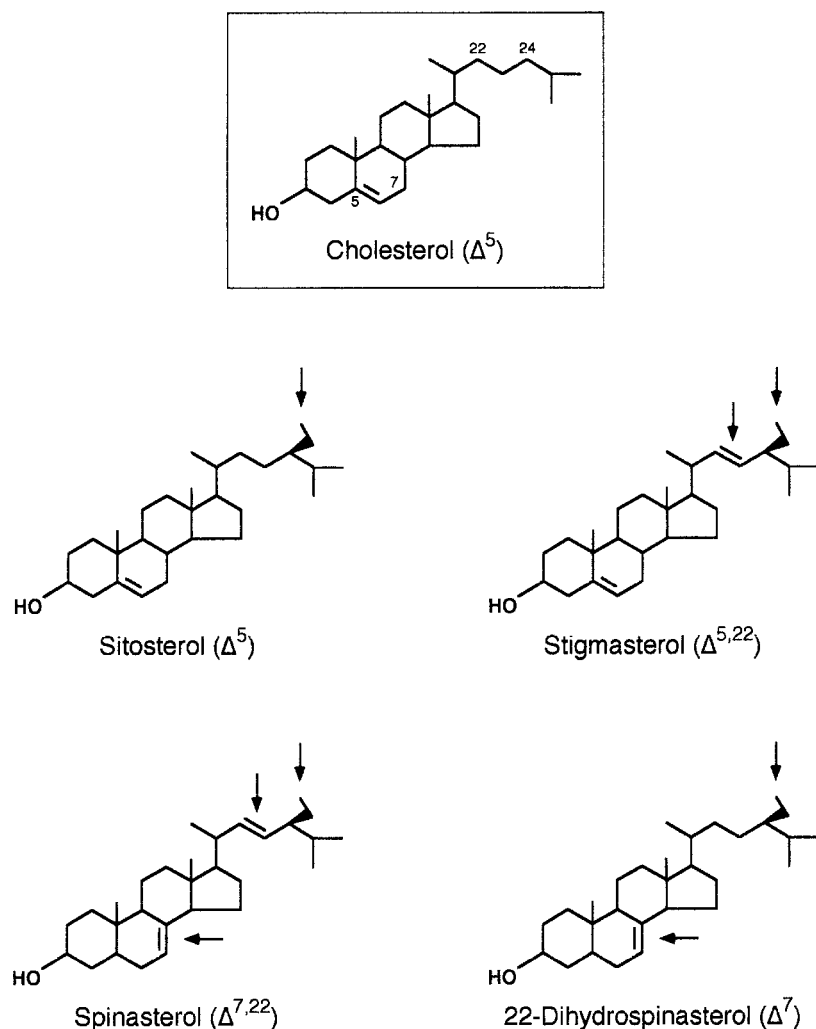


Fig. 1. Sterol structures of interest. Cholesterol has a double bond at position 5 ( $\Delta^5$ ); it is the dominant tissue sterol found in insects. Sitosterol, stigmasterol, spinasterol, and 22-dihydrospinasterol are found in plants. The arrows indicate structural differences from cholesterol. Campesterol, 22-dihydrobrassicasterol, and lathosterol are not shown. The former are  $\Delta^5$ -sterols similar to sitosterol but have a methyl, rather than ethyl group, at C-24. The latter is a  $\Delta^7$ -sterol that is similar to 22-dihydrospinasterol but lacks the ethyl group at C-24.

bers of the Solanaceae (Nes, 1977; Nes and McKean, 1977). Spinasterol, a C-24 ethyl  $\Delta^{7,22}$ -sterol, and dihydrospinasterol, a C-24 ethyl  $\Delta^7$ -sterol, are the dominant sterols in plants belonging to the families Chenopodiaceae and Amaranthaceae (Salt et al., 1991).

Cholesterol, sitosterol, and stigmasterol were purchased from Sigma Chemical (St. Louis, MO). Spinasterol and dihydrospinasterol were isolated from spinach, *Spinacia oleracea*, using standard lipid extraction techniques and preparative thin layer chromatography; the amount collected was quantified using RP-HPLC (Heupel, 1989). All individual sterols were examined analytically by GLC, RP-HPLC, and GC-MS. Cholesterol and stigmasterol were shown to be at least 98% pure. Sitosterol, which was derived from soybean, was a mixture of 60% sitosterol, 27% campesterol, and 13% dihydrobrassicasterol (the latter two are C-

24 methyl  $\Delta^5$ -sterols); all of these sterols support normal growth and development in grasshoppers (Dadd, 1960). Throughout the rest of the paper this sterol mixture is referred to as "soybean sitosterol." Insufficient amounts of spinasterol and dihydrospinasterol were collected to test individually so they were combined in a 3:2 ratio, which is the observed ratio in spinach plants. This combination is collectively referred to as spinach sterol throughout the rest of the paper.

For all the following experiments, grasshoppers were reared on an artificial diet similar to the one used by Simpson et al. (1988). It contained the following ingredients: 28% protein (a 3:1:1 mixture of low fat, vitamin-free casein, bacteriological peptone, and egg albumen); 28% digestible carbohydrate (a 1:1 mixture of sucrose and white dextrin); 39.7% cellulose; 2.4% Wesson's salts; 0.5% linoleic acid; 0.5% linolenic acid; 0.3% ascorbic acid; 0.1%

ferulic acid; 0.2% phenylalanine; 0.2% vitamin cocktail (Dadd, 1961). The type and amount of sterol added to the diet varied depending on the treatment (see experimental protocols below). From previous analyses of individual dietary ingredients, we knew there was sterol contamination in both the albumen (16.6  $\mu\text{g}$  cholesterol/g) and casein (1.3  $\mu\text{g}$  cholesterol/g). On a dry weight basis of all ingredients, however, this contamination was very minor,  $\sim 0.0018\%$ . For all experiments, the diet was suspended in a 1% agar solution in a 1:4 dry ingredients :water ratio and presented to grasshoppers as small cubes. Grasshoppers received fresh diet daily.

### Experimental Protocols

In the first experiment, grasshoppers were reared on diets containing different dietary sterols and their tissue sterols were measured. There were four treatments: (1) cholesterol (suitable), (2) soybean sitosterol (all suitable), (3) stigmasterol (unsuitable), and (4) spinach sterols (all unsuitable). A sterol concentration of 0.1% was used for all treatments, which slightly exceeds the minimum amount of suitable sterol *S. americana* requires for development (Behmer and Elias, 1999a). After individual grasshoppers had molted to the third stadium, they were transferred for 2 days to a diet that was 100% sterol-free (no albumen, casein, or sterol); this treatment cleared the lumen of the alimentary tract of all dietary sterols and allowed us to quantify sterols in whole animals. On the third day, individual grasshoppers were weighed and then frozen at  $-20^{\circ}\text{C}$  until the sterol extraction was performed. Because we did not use tracer experiments in this study, we needed to establish what amount of the total cholesterol pool recovered from third stadium grasshoppers would have been maternal in origin. This was done by collecting newly hatched grasshoppers and freezing them at  $-20^{\circ}\text{C}$  until their sterols were extracted. Like all the other hatchlings used throughout this study, the grasshoppers used to quantify amounts of maternally inherited cholesterol came from females that had been maintained on a diet of 7–10-day-old wheat, Romaine lettuce, and wheat bran.

For the second experiment, tissue sterols were measured from grasshoppers reared on diets containing mixtures of suitable and unsuitable sterols. Here we wanted to determine how

mixtures of suitable and unsuitable sterols interacted to influence cholesterol amounts and tissue sterol profiles. There were four treatments: (1) a diet with soybean sitosterol at a dry weight concentration of 0.05%, (2) a “low mixture” diet with equal amounts of soybean sitosterol and stigmasterol (0.025% each) for a total sterol concentration of 0.05%, (3) a “high mixture” diet with equal amounts of soybean sitosterol and stigmasterol (0.05% each) for a total sterol concentration of 0.1%, and (4) a diet with stigmasterol at a dry weight concentration of 0.05%. These concentrations were based on those used in the previous study (Behmer and Elias, 1999a). The grasshoppers were reared and treated as previously described, with the exception of those reared on the low mixture treatment. These grasshoppers were collected during the late second stadium because high mortality precluded them from reaching the third stadium. Cholesterol amounts reported for newly hatched grasshoppers from the first experiment were used as a measurement to estimate how much of the recovered cholesterol from grasshoppers on the different mixtures was maternal in origin.

In the third and final experiment, we wanted to determine whether sterol composition in the whole body was indicative of sterols taken up from the midgut. Grasshoppers for this experiment were fed diets containing soybean sitosterol or spinach sterols presented alone or in mixtures. There were four different treatments: (1) a diet with soybean sitosterol at a dry weight concentration of 0.05%, (2) a low mixture diet with equal amounts of soybean sitosterol and spinach sterols (0.025% each) for a total sterol concentration of 0.05%, (3) a high mixture diet with equal amounts of a soybean sitosterol and spinach sterols (0.05% each) for a total sterol concentration of 0.1%, and (4) a diet with spinach sterols at a dry weight concentration of 0.05%. In contrast to the previous two experiments, grasshoppers were reared on the different sterol treatments until the start of the fourth stadium; they were fed the same diet for one day following this molt. On the second day of the fourth stadium, grasshoppers had their alimentary tracts removed and the remaining cadaver was frozen at  $-20^{\circ}\text{C}$  until the sterols were extracted.

## Isolation of Sterols

Individual cadavers were homogenized in 20-ml vials using liquid nitrogen and a glass stirring rod; the homogenized samples were subsequently suspended in 95% methanol for at least 72 h. After the methanol extraction, solutions were evaporated to dryness under N<sub>2</sub>, resuspended in 5 ml of hexane/toluene, and run through preconditioned silica columns (Silica-Gel G, Sigma). These columns were disposable Pasteur pipettes that had their tips removed and were packed with a small amount of glass wool and approximately 1 g of silica. The hexane/toluene fraction containing the tissue sterols was added to the column and washed with 6 column volumes of hexane/toluene (1:1) followed by 12 column volumes of ether. The sterol reproducibly eluted with the ether fraction. The ether fraction was then evaporated under N<sub>2</sub> and resuspended in approximately 4 ml of methanol until analysis. Because we only had a small amount of sample from which to analyze tissue sterol profiles, we were unable to test for the possible presence of steryl esters.

## Identification and Quantification of Sterols

The sterols isolated from grasshoppers were characterized and quantified by GLC, RP-HPLC, and GC-MS. Sterols were quantified by analytical RP-HPLC on a Supelco C18 ODS column (25 cm × 4.6 mm) using acetonitrile/isopropyl alcohol (8:2, v/v; 1 ml/min) at 45°C and were detected using a Waters 486 variable wavelength detector. Sample injection was performed by a Waters 717 Autosampler and data presented on a Waters 746 data module. The known sterols were structurally identified through gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5890 gas chromatograph and a Hewlett-Packard 5970 mass spectrometer. GC separation of isolated sterols was achieved on a HP-1 column (15 m × .3 mm) with a 0.25-mm film thickness. Oven temperature was programmed from 200 to 280°C at 20°C/min and the carrier gas was helium at a velocity of 30 cm/s. The MS was operated at an ionizing potential of 70 eV, the ion source maintained at 280°C, and the injector port was maintained at 250°C. Structural and chromatographic values (retention times relative to cholesterol) agreed with authentic standards and previous reports for cholesterol, sitosterol, campesterol, dihydrobrassicaterol, stigmasterol, spin-

asterol, and 22-dihydrospinasterol (Armarengo et al., 1973) and lathosterol (Nes et al., 1977).

## Statistical Analysis

All statistical comparisons were performed using the Kruskal-Wallis test, a nonparametric equivalent to a one-way ANOVA (Abacus Concepts, *StatView Reference*; Abacus Concepts, Inc., Berkeley, CA, 1996). This test was used because replication was always unequal and in some cases the differences in sample size among treatments were quite large. An additional benefit of using this test is that it makes no assumptions about the underlying distribution of the data. When significant differences were detected, a Tukey-type comparison for medians was employed, with an  $\alpha$  value set at 0.05, to determine which treatments differed significantly from one another (Zar, 1996).

## RESULTS

### Experiment 1

**Tissue sterols identified from grasshoppers on the single sterol diets.** In the newly hatched and cholesterol fed grasshoppers, cholesterol was the only sterol recovered. In contrast, grasshoppers on the other treatments all contained mixtures of sterols. For example, cholesterol and unmetabolized sitosterol and campesterol were recovered from grasshoppers fed the soybean sitosterol diet. In grasshoppers fed the stigmasterol diet, cholesterol and stigmasterol were recovered. Finally, cholesterol, lathosterol, spinasterol, and 22-dihydrospinasterol were all identified from grasshoppers reared on the spinach sterol diet.

**Quantitative tissue sterol comparisons: experiment 1.** Sterol treatment influenced both insect performance and tissue sterol profiles. First, two-day-old third stadium grasshoppers reared on the cholesterol and soybean sitosterol diets had a greater median mass than those fed the stigmasterol or spinach sterol diets (Kruskal-Wallis test (KW):  $df = 3$ ,  $H$ -value = 26.358,  $P < 0.01$ ; Table 1A); a similar negative effect of unsuitable sterols on weight gain has also been reported for other acridids (Behmer, 1998). Second, and perhaps more interestingly, quantitative differences in tissue cholesterol levels were observed among the different treatments. For instance, cholesterol amounts ( $\mu\text{g/insect}$ ) recovered from grass-

TABLE 1. Dry Mass of Grasshoppers When Analyzed for Sterol Content\*

Treatment	Developmental stadium	Dry mass ( $\pm$ MAD) (mg)
A. Single sterol diet comparison		
Cholesterol	Early third	13.6 ( $\pm$ 1.4) <sup>a</sup>
Soybean sitosterol	Early third	12.6 ( $\pm$ 0.9) <sup>a</sup>
Sigmasterol	Early third	9.2 ( $\pm$ 1.1) <sup>b</sup>
Spinach sterols	Early third	9.0 ( $\pm$ 2.1) <sup>b</sup>
B. Mixed sterol diet comparison		
Soybean sitosterol	Early third	12.6 ( $\pm$ 1.0) <sup>a</sup>
Low mixture	Late second	5.6 ( $\pm$ 0.1) <sup>b</sup>
High mixture	Early third	13.0 ( $\pm$ 1.5) <sup>a</sup>
Sitmasterol	Early third	8.8 ( $\pm$ 1.1) <sup>b</sup>

A: Grasshoppers reared on single sterol diets.

B: Grasshoppers reared on diets containing soybean sitosterol either singly or combined with sigmasterol at different concentrations. Developmental stage denotes the age of grasshoppers when the tissue sterols were taken. Different letters indicate significant differences among the treatments.

hoppers fed the cholesterol or soybean sitosterol diets were significantly greater than amounts recovered from newly hatched grasshoppers or those fed either the stigmasterol or the spinach sterol diets (KW:  $df = 4$ ,  $H$ -value = 37.430,  $P < 0.01$ ). Cholesterol amounts recovered from the newly hatched grasshoppers were, interestingly, similar to those recovered from grasshoppers fed the stigmasterol or spinach sterols diets (Fig. 2A). The relative amount of cholesterol ( $\mu\text{g}/\text{mg}$  dry body mass) was also significantly different among treatments (KW:  $df = 4$ ,  $H$ -value = 36.520,  $P < 0.01$ ), being lowest in the stigmasterol and spinach sterol fed grasshoppers and, somewhat unexpectedly, highest in the newly hatched grasshoppers (Fig. 2B).

We also observed differences among the treatments in both total and relative sterol amounts (KW:  $df = 3$ ,  $H$ -value = 9.773,  $P < 0.05$ , and  $df = 4$ ,  $H$ -value = 18.755,  $P < 0.01$ , respectively). For example, total sterol amounts ( $\mu\text{g}/\text{insect}$ ) were greatest in grasshoppers fed the cholesterol and soybean sitosterol diet (Table 2). Total relative sterol amounts ( $\mu\text{g}/\text{mg}$  dry body mass), meanwhile, were highest in the newly hatched grasshoppers. Among the grasshoppers reared on the different treatments to the third stadium, however, no significant difference in total relative amount was observed. Finally, there was a significant difference in the percent cholesterol among the different treatments (KW:  $df = 4$ ,  $H$ -value = 65.672,  $P < 0.01$ ); it was significantly lower in the grasshoppers fed stigmasterol and spinach sterols compared to grasshoppers fed the other diets (Table 2).

## Experiments 2 and 3

**Tissue sterols identified from grasshoppers on the mixed sterol diets.** Four sterol peaks were identified by GLC, RP-HPLC, and GC-MS from grasshoppers that were reared on diets with soybean sitosterol plus stigmasterol (experiment 2). These peaks corresponded to cholesterol, stigmasterol, campesterol, and sitosterol. Four sterol peaks were also identified by GLC and GC-MS for the grasshoppers reared on the diets with soybean sitosterol plus spinach sterols (experiment 3). The first three peaks corresponded to cholesterol, lathosterol, and campesterol. The fourth peak was a mixture of sitosterol and spinasterol. We were unable to separate these two sterols since they co-migrate under standard GLC conditions.

**Quantitative tissue sterol comparison: experiment 2.** Insect performance and tissue sterol profiles, much like the first experiment, were influenced by the different sterol treatments. For example, grasshoppers reared on the soybean sitosterol and high mixture treatments had greater mass than those reared on the low mixture treatment (KW:  $df = 2$ ,  $H$ -value = 6.269,  $P < 0.05$ ; Table 1B). More substantially, though, the amount of cholesterol ( $\mu\text{g}/\text{insect}$ ) recovered from the grasshoppers differed significantly among treatments (KW:  $df = 4$ ,  $H$ -value = 23.961,  $P < 0.01$ ). It was highest in grasshoppers fed the soybean sitosterol diet, intermediate on the high mixture diet, and lowest in those given the low mixture and stigmasterol diets (Fig. 3A). Interestingly, the amount of cholesterol recovered from the low mixture and stigmasterol fed grasshoppers was not signifi-

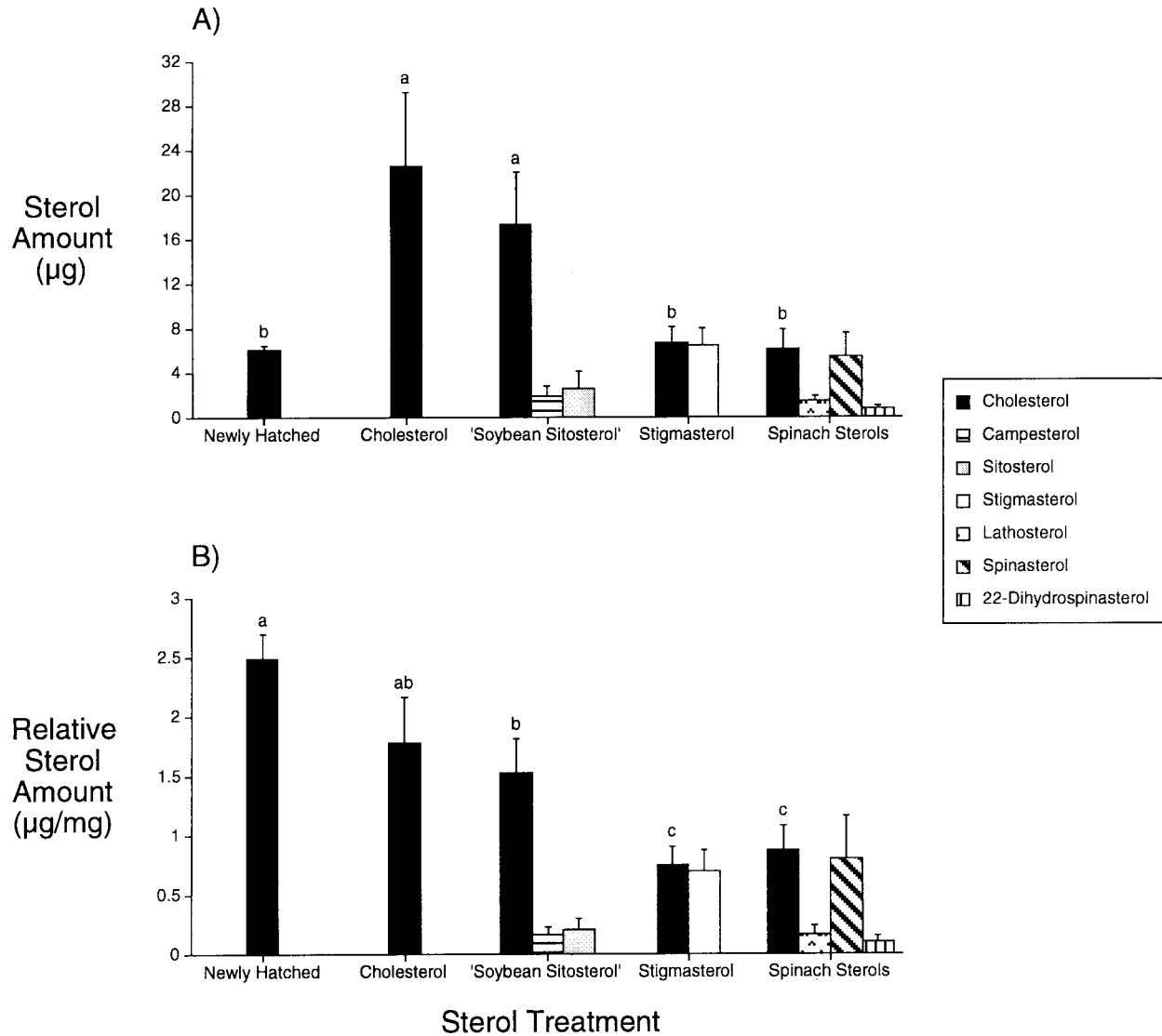


Fig. 2. Sterol profiles from 2-day-old third stadium *S. americana* that were reared on diets containing different dietary sterols presented singly and from newly hatched grasshoppers that originated from females reared on 7–10-

day-old wheat, Romaine lettuce and wheat bran. **A:** Total sterol amounts. **B:** Relative sterol amounts. Data are presented as medians ( $\pm$ MAD). Different letters indicate significant differences among the treatments.

cantly different from the amount recovered from the newly hatched grasshoppers. A significant difference in the relative amount of cholesterol ( $\mu\text{g}/\text{mg}$  dry body mass) was also observed among grasshoppers from the different treatments (KW:  $df = 4$ ,  $H\text{-value} = 29.456$ ,  $P < 0.01$ ). It was lowest in the stigmasterol fed grasshoppers and, as in the first experiment, highest in the newly hatched grasshoppers (Fig. 3B). There was, however, no statistical difference in cholesterol amounts ( $\mu\text{g}/\text{insect}$ ) among the grasshoppers reared on the high mixture, low mixture, and stigmasterol diet. Significantly more C-24 ethyl-

sterols were, however, recovered from grasshoppers fed diets with stigmasterol compared to those fed diets containing only soybean sitosterol (KW:  $df = 3$ ,  $H\text{-value} = 17.141$ ,  $P < 0.01$ ; Table 3). The relative amount of C-24 ethyl-sterols also varied significantly among treatments (KW:  $df = 3$ ,  $H\text{-value} = 27.075$ ,  $P < 0.01$ ). They were highest in the low mixture and stigmasterol treatments and lowest in the soybean sitosterol treatment (Fig. 3B).

When all the tissue sterols were summed and compared, relative amounts, but not total amounts, differed significantly among treatments

TABLE 2. Tissue Sterols of *S. americana*\*

Treatment	Total sterol amount (µg/insect)	Total relative sterol amount (µg/mg)	Sterol	Diet profile (%)	Tissue profile (%)
A. Baseline tissue sterols					
Newly hatched	6.112 (±0.341)	2.480 (±0.216) <sup>a</sup>	Cholesterol		100.0 (±0.0)
B. Different dietary sterols					
Cholesterol	22.560 (±6.653) <sup>a</sup>	1.761 (±0.388) <sup>a,b</sup>	Cholesterol	100.0	100.0 (±0.0) <sup>a</sup>
Soybean sitosterol	23.442 (±8.853) <sup>a</sup>	1.810 (±0.576) <sup>a,b</sup>	Cholesterol	0.0	81.0 (±6.9) <sup>b</sup>
			Campesterol	27.0	9.4 (±1.7)
			Sitosterol	60.0	9.4 (±6.1)
			22-dihydro-brassicasterol	13.0	<1.0 (±0.0)
Sitgmasterol	13.314 (±2.203) <sup>b</sup>	1.381 (±0.320) <sup>b</sup>	Cholesterol	0.0	54.0 (±3.2) <sup>c</sup>
			Stigmasterol	99.0	46.0 (±3.2)
Spinach sterols	16.102 (±5.614) <sup>a,b</sup>	2.067 (±0.602) <sup>a,b</sup>	Cholesterol	0.0	45.7 (±9.4) <sup>c</sup>
			Lathosterol	0.0	10.3 (±1.1)
			Spinasterol	60.0	37.0 (±5.4)
			22-dihydrospinasterol	40.0	6.7 (±2.1)

A: Newly hatched grasshoppers.

B: Grasshoppers reared on diets containing different sterols. The data are presented as median (±MAD). For tissue profile only percent cholesterol is compared. Different letters indicate significant differences among the treatments ( $\alpha = 0.05$ ).

(KW:  $df = 4$ ,  $H$ -value = 22.063,  $P < 0.01$ , and  $df = 3$ ,  $H$ -value = 6.352,  $P = 0.096$ , respectively). Total relative amounts (µg/mg dry body mass) were highest in the newly hatched grasshoppers but among the other treatments there were no significant differences (Table 3). Finally, cholesterol, expressed as a percent of the total tissue sterol, was highest in the soybean sitosterol fed grasshoppers and equally lowest in the low mixture and stigmasterol fed grasshoppers (KW:  $df = 3$ ,  $H$ -value = 32.210,  $P < 0.01$ ; Table 3).

**Quantitative tissue sterol comparison: experiment 3.** No difference in the percent cholesterol was observed among the different treatments for insects without midguts (KW:  $df = 3$ ,  $H$ -value = 7.271,  $P = 0.064$ ; Fig. 4), but clearly unsuitable sterols were taken up into the haemocoel (between 20–25%). A comparison of intact grasshoppers to those that had their alimentary tract removed, however, revealed an interesting difference in sterol composition. The tissue sterol profile from intact grasshoppers reared on the spinach sterol diet was approximately 50% cholesterol and 50%  $\Delta^7$ -sterol (i.e., lathosterol, spinasterol, and 22-dihydrospinasterol; Table 2); the sterol profile of grasshoppers reared on the same diet with their midguts removed, however, was approximately 83% cholesterol and 17%  $\Delta^7$ -sterol (i.e., lathosterol and spinasterol).

## DISCUSSION

Phytosterols typically contain an alkyl group at C-24 (Salt et al., 1991; Patterson, 1994) yet cholesterol is the dominant tissue sterol in the phytophagous insects that have been examined (Svoboda and Thompson, 1985). For most phytophagous insects, including grasshoppers, dealkylation of alkyl groups at C-24 is a key step in the accepted pathway to the production of cholesterol (Svoboda and Feldlaufer, 1991; Ikekawa et al., 1993). In the present work, this is shown by: (1) high quantities of cholesterol recovered from the grasshoppers fed the soybean sitosterol diet, and (2) significant quantities of lathosterol from the spinach sterol fed grasshoppers (lathosterol is a non-dietary sterol that would have been produced via dealkylation of the ethyl group at C-24 on the dihydrospinasterol side chain). In grasshoppers, however, dealkylation cannot occur if a double bond occurs at position 22 on the side chain. In the current study both unmetabolized stigmasterol ( $\Delta^{5,22}$ ) and spinasterol ( $\Delta^{7,22}$ ) were recovered in large quantities from the stigmasterol and spinach sterol fed grasshoppers, respectively. Although cholesterol was recovered from both the stigmasterol and spinach sterol fed grasshoppers, it was likely maternal in origin; quantities measured in both the stigmasterol and spinach sterol fed grasshoppers were no greater than those

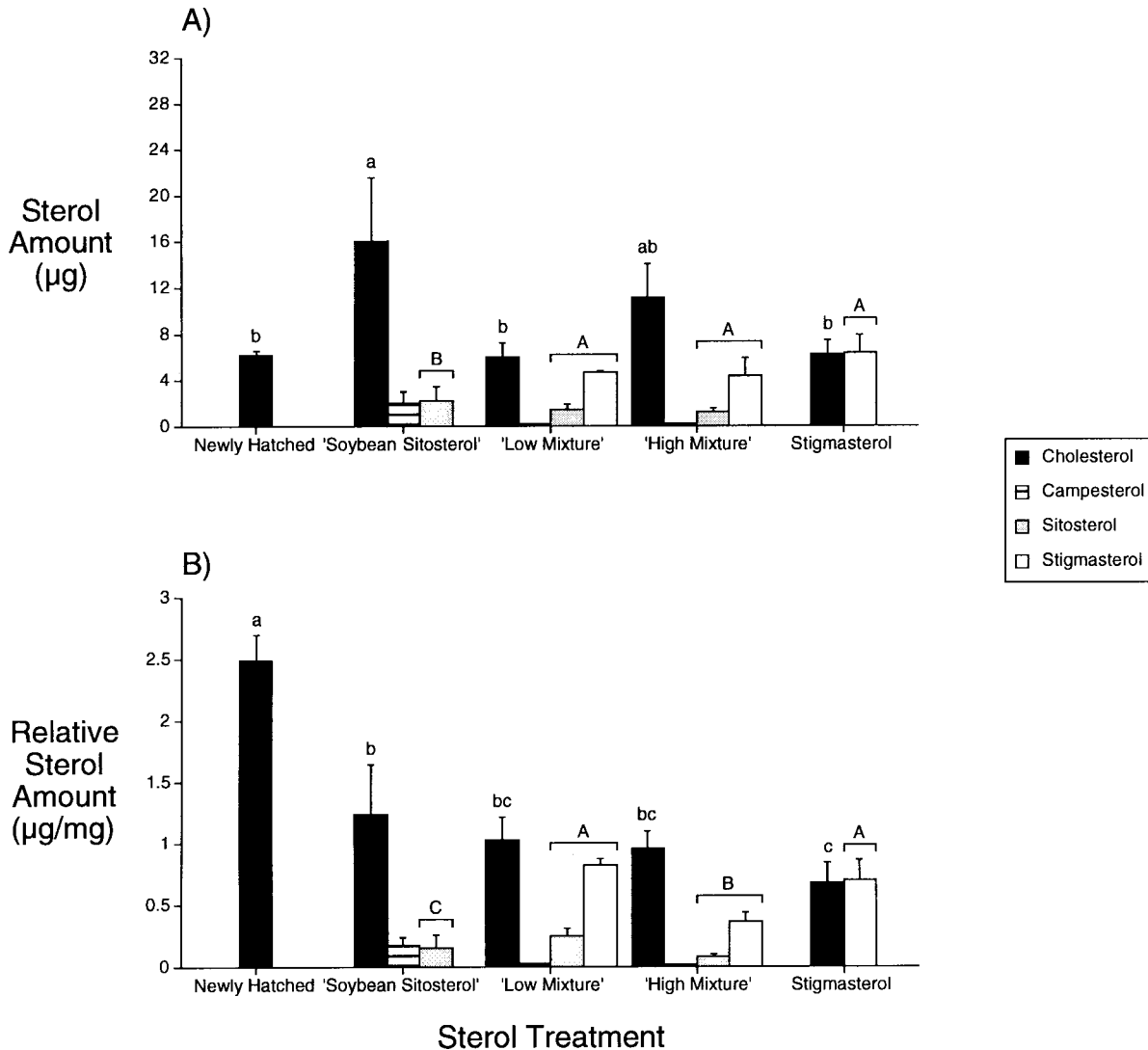


Fig. 3. Sterol profiles from 2-day-old third stadium *S. americana* that were reared on diets containing soybean sitosterol or stigmasterol either alone or combined at different concentrations and from newly hatched grasshoppers that originated from females reared on 7–10-day-old wheat, Romaine lettuce and wheat bran. **A:** Total sterol amounts. **B:** Relative sterol amounts. Data are presented as medians

( $\pm$ MAD). Different lowercase letters indicate significant differences in cholesterol among the treatments while uppercase letters indicate significant differences in the levels of sterols with ethyl groups at C-24 on the side chain. Amounts reported for newly hatched grasshoppers are the same as those shown in Figure 2.

of newly emerged grasshoppers. Tracer experiments will be needed, however, to confirm this supposition.

Results also indicate that no metabolism of the B ring occurred when dietary sterols contained a double bond at position 5. When *S. americana* was reared on the soybean sitosterol or stigmasterol diets, all of the recovered tissue sterols had  $\Delta^5$ -configurations. It also seems that no, or at most very little, metabolism of the B ring occurs when dietary sterols contain double

bonds at position 7. When grasshoppers were reared on the spinach sterol diet (all  $\Delta^7$ -sterols), slightly more than half of the recovered tissue sterols had  $\Delta^7$ -configurations. The  $\Delta^5$ -sterol (cholesterol) that was recovered from these insect was, in all likelihood, maternal in origin. Although very few studies have examined metabolism of the B ring, variation among different species seems to exist. The corn earworm, *Helicoverpa zea*, is a phytophagous insects that does very little metabolism of the B-ring (Ritter, 1984). In contrast, the

TABLE 3. Tissue Sterols from *S. americana*\*

Treatment	Total sterol amount (µg/insect)	Total relative sterol amount (µg/mg)	Sterol	Diet profile (%)	Tissue profile (%)
A. Baseline tissue sterols					
Newly hatched	6.112 (±0.341)	2.480 (±0.216) <sup>a</sup>	Cholesterol		100.0 (±0.0)
B. Different dietary sterols					
Soybean sitosterol	21.557 (±8.617) <sup>a</sup>	1.645 (±0.474) <sup>b</sup>	Cholesterol	0.0	77.6 (±5.4) <sup>a</sup>
			Campesterol	27.0	12.6 (±3.7)
			Sitosterol	60.0	9.8 (±1.4)
			22-dihydro-brassicasterol	13.0	<1.0 (±0.0)
Low mixture	12.387 (±2.343) <sup>a</sup>	2.212 (±0.466) <sup>a,b</sup>	Cholesterol	0.0	59.6 (±2.8) <sup>b,c</sup>
			Stigmasterol	50.0	32.4 (±4.7)
			Campesterol	13.5	1.2 (±0.2)
			Sitosterol	30.0	15.2 (±8.2)
			22-dihydro-brassicasterol	6.5	<0.0 (±0.0)
High mixture	16.986 (±5.162) <sup>a</sup>	1.381 (±0.320) <sup>b</sup>	Cholesterol	0.0	66.6 (±1.6) <sup>b</sup>
			Stigmasterol	50.0	26.6 (±1.1)
			Campesterol	13.5	0.9 (±0.4)
			Sitosterol	30.0	5.7 (±0.6)
			22-dihydro-brassicasterol	6.5	<0.0 (±0.0)
Stigmasterol	12.960 (±2.348) <sup>a</sup>	1.433 (±0.343) <sup>b</sup>	Cholesterol	0.0	54.6 (±3.4) <sup>c</sup>
			Sitgmasterol	100.0	46.4 (±3.3)

A: Newly hatched grasshoppers.

B: Grasshoppers reared on diets containing soybean sitosterol (a suitable sterol) or stigmasterol (an unsuitable sterol) alone or in combination. The data are presented as medians (±MAD). For tissue profile only percent cholesterol is compared. Different letters indicate significant differences among the treatments ( $\alpha = 0.05$ ).

german cockroach, *Blattella germanica*, is capable of reducing a double bond at position 7 (Clark and Bloch, 1959).

In previous studies, it was shown that *S. americana* failed to complete development when

it was reared on a diet containing equal concentrations of suitable and unsuitable sterols (Behmer and Elias, 1999a); this occurred even though the concentration of soybean sitosterol in the diet was at a level that alone would support normal

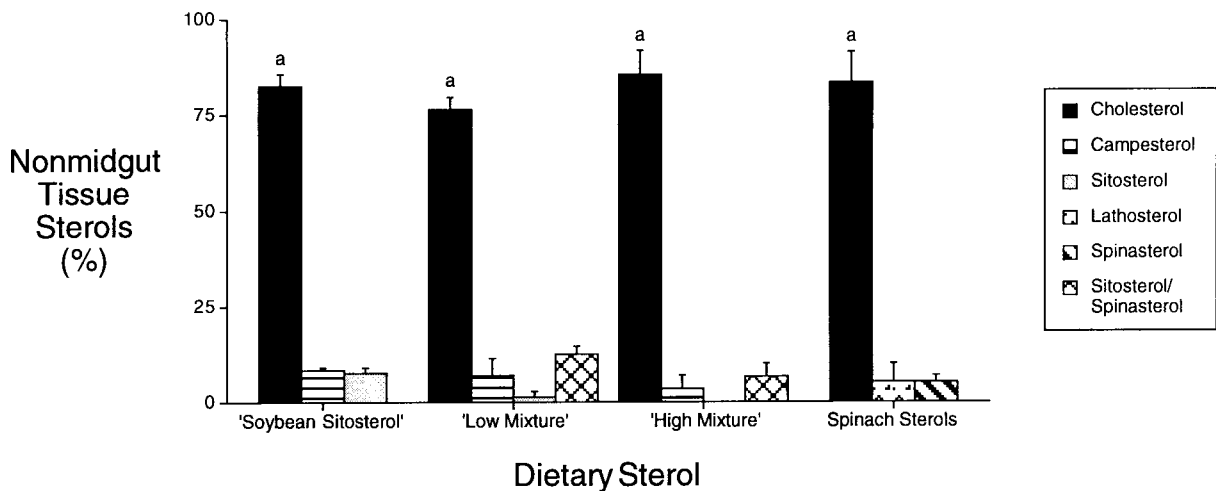


Fig. 4. Sterol profiles from 2-day-old fourth stadium *S. americana* with their midguts removed. In this experiment, grasshoppers were reared on diets containing soybean sito-

sterol or spinach sterols either singly or combined at different concentrations. No significant difference in percent cholesterol was observed among the treatments.

development. It was suggested that the unsuitable dietary sterols may have been inhibiting the dealkylation of the suitable sterols to cholesterol. In the current experiment, however, cholesterol amounts in grasshoppers reared on the high mixture treatments did not differ significantly from those fed the soybean sitosterol diet. Perhaps, instead, the failure of grasshoppers to complete development on mixed sterol diets was associated, either directly or indirectly, with the accumulation of unmetabolized dietary sterols. Unmetabolized dietary sterols comprised approximately 50% of the total sterol profile when grasshoppers were fed the stigmaterol or low mixture diet and more than 32% when they were fed the high mixture treatment; in the soybean sitosterol fed grasshoppers, however, only 22% of the total sterol profile was unmetabolized dietary sterol. It is also interesting to note that when sitosterol and stigmaterol were both present in the diet, the amount of campesterol recovered was reduced compared to the amounts recovered from the soybean sitosterol diet. Perhaps campesterol is more readily metabolized to cholesterol in the presence of stigmaterol. Here again tracer experiments would help to shed light on this intriguing pattern.

The comparison of sterol profiles from intact insects to those with their midguts removed suggests that the unmetabolized dietary sterols tend to accumulate in the midgut tissue. The midgut is a very active tissue and grasshoppers might begin to experience negative effects in at least two ways once unmetabolized sterols, particularly those with ethyl groups at position C-24 on the cholestane side chain (e.g., stigmaterol, spinasterol), begin to accumulate and become incorporated here. First, the structural integrity of individual cell membranes could be compromised because phospholipids might have problems packing tightly around sterols with C-24 ethyl groups. This may cause cell membranes to become leaky (Stien, 1981). Second, as cholesterol content in cellular membranes decreases, the ability of membrane bound proteins to function properly might decline (Hadley, 1985). The contrast of sterol profiles between intact grasshoppers and those without midguts also suggests that there may be differences in uptake specificity of sterols into the midgut tissue and into the hemolymph. Mixed bile salt micelles and small unilamellar vesicles are

the natural carriers from which sterols are absorbed from the gut lumen (Carey and Hernell, 1992); uptake mechanism of this nature tend to be non-specific. In contrast, uptake and transport of sterols into the hemolymph is known to be facilitated by lipophorin (reviewed by Blacklock and Ryan, 1994). Little is known about the mechanisms involved in the loading of sterols from the midgut into lipophorin, but a sterol specific transfer particle, which preferentially loads cholesterol, might be involved.

As mentioned previously, cholesterol amounts in the grasshoppers reared on the stigmaterol and spinach sterol diets were similar to those of newly hatched grasshoppers. Since our results seem to indicate that *S. americana* cannot metabolize either of these dietary sterols to cholesterol, this suggests that grasshoppers may have a remarkable ability to retain the cholesterol that was maternally allocated to them. The midgut is a very substantial tissue and in some insects, such as *Periplaneta americana*, there is significant turnover of old or damaged cells (Bignell, 1981). Perhaps there are mechanisms that allow grasshoppers to recycle the cholesterol, which would otherwise be lost in these old or damaged cells. It is also interesting to note that relative amounts of cholesterol were highest in newly hatched grasshoppers. If a minimum relative amount of cholesterol is required for development to proceed, the high starting concentration of cholesterol in the newly hatched grasshoppers may act as a buffer and reduce their need to acquire only suitable sterols during early development. The high relative amount of sterols recovered from low mixture fed individuals probably reflects the high starting concentration of cholesterol in newly hatched individuals, especially since grasshoppers from this treatment were so small when they were collected for the analysis.

Although constraints on sterol metabolism can have severe consequences in grasshoppers, it seems they are capable of detecting unsuitable sterols in their foods; short-term behavioral studies indicate that sixth stadium *S. americana* nymphs can develop learned aversions to plants and artificial diets with unsuitable sterols after only a single meal (Champagne and Bernays, 1991; Behmer and Elias, 1999b). Additional experiments suggest that the injection of unsuitable dietary sterols into the hemolymph can play a significant role in

the development of this aversion response (Behmer et al., 1999). Results from the current study indicate that most of the unsuitable dietary sterols accumulate in the midgut but clearly some are absorbed into the hemocoel. How quickly this absorption occurs, though, is not known. Ingested sterols have been detected in the fat body of *H. zea* within a few hours (Kuthiala and Ritter, 1988), but the aversion response of *S. americana* to foods with unsuitable sterols would suggest that sterol absorption may occur quite rapidly, perhaps in less than one hour. Further studies will be required, however, to determine just how quickly sterols are absorbed into the hemolymph.

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