

Phytosterol structure and its impact on feeding behaviour in the generalist grasshopper *Schistocerca americana*

SPENCER T. BEHMER and DAMIAN O. ELIAS

Department of Entomology and Center for Insect Science, University of Arizona, Tucson, U.S.A.

Abstract. Sixth-stadium nymphs of the grasshopper *Schistocerca americana* (Drury) (Orthoptera: Acrididae) were observed in a series of experiments designed to measure feeding behaviour in response to suitable and unsuitable phytosterols. In the first experiment, grasshoppers were presented with artificial diet that contained either sitosterol, a suitable phytosterol, or a spinach lipid extract which contained only unsuitable sterols as well as other spinach lipids. The diet with the spinach lipid extract, but not the sitosterol diet, evoked a deterrent response. To determine if the spinach sterols were responsible for the deterrent response, a second experiment was performed where the spinach lipid extract was separated into three lipid classes, including desmethyl sterols, dimethyl sterols and the remaining spinach lipids. Grasshoppers presented with artificial diet containing the desmethyl sterols (the end-product sterols in spinach) exhibited deterrent responses. Finally, feeding behaviour to a suite of different sterols, including cholesterol (suitable), stigmasterol (unsuitable), and lathosterol (unsuitable), was observed; these sterols were selected because they show variation in the position of double bonds. Grasshoppers presented with diets containing unsuitable sterols again exhibited deterrent responses. Overall, the deterrent effect was strongest when sterols with a double bond at position 22 were in the diet.

Key words. Aversion learning, feeding behaviour, insect nutrition, lipids, Orthoptera, *Schistocerca americana*, spinach, sterols.

Introduction

All insects, whether they are predaceous or herbivorous, share an inability to biosynthesize sterols that are necessary in lipid biostructures and serve as precursors to steroid hormones (Clayton, 1964; Svoboda & Thompson, 1985). Cholesterol appears to be the most common sterol in insect tissue, and it is the precursor for the moulting hormone 20-OH ecdysone (Grieneisen, 1994). For herbivorous insects, however, only trace levels of cholesterol are found in their host plants (Nes & McKean, 1977; Salt *et al.*, 1991; but see Garg *et al.*, 1987). Herbivorous insects typically metabolise phytosterols to cholesterol in order to meet their sterol nutritional requirements (Ikekawa *et al.*, 1993).

There are two general variations that arise in phytosterol structure: differences in the nucleus and differences in the side

chain. These variations can have major consequences for herbivorous insects. For example, among the different grasshoppers (Orthoptera: Acrididae) studied to date, which include representatives from four different subfamilies, all are highly restricted with respect to the phytosterols they can use (Dadd, 1960; Behmer, 1998). Phytosterols that possess double bonds in the nucleus at position 7 (Δ^7), or on the side chain at position 22 (Δ^{22}), cannot support normal growth and development in grasshoppers.

In addition to their developmental effect, phytosterols may also influence feeding behaviour. Sitosterol has been suggested to be a feeding stimulant for the silkworm, *Bombyx mori* (Hamamura, 1970), and there is good evidence of a regulatory role for phytosterols in grasshoppers. When spinach, a plant that contains only unsuitable phytosterols, was presented to the grasshopper *Schistocerca americana*, it was initially accepted (Lee & Bernays, 1988). With continued exposure, however, its acceptability declined until it was completely rejected. In a later study, Champagne & Bernays (1991) found that when *S. americana* nymphs were fed spinach leaves with added cholesterol or sitosterol, acceptability did not decline

Correspondence: Spencer T. Behmer, Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, U.K. E-mail: spencer.behmer@zoology.ox.ac.uk

with experience. Further experiments led them to propose that acceptability did not decline because feeding was probably being regulated by a post-ingestive feedback mechanism working in response to the presence of an appropriate sterol. They found no evidence that the grasshoppers were able to taste sterols, however, indicating that chemoreception of the sterols themselves probably did not play a role in this behaviour; it was suggested that associative learning influenced feeding behaviour on spinach. In associative learning, experience results in an animal being able to associate a stimulus having no specific meaning (i.e. it is neutral), with some meaningful positive or negative effect (Bernays, 1995). For grasshoppers that exhibit an aversion response to spinach over successive meals, the meaningful effect could be the unsuitable phytosterols, whereas the neutral one that comes to be associated with the unsuitable phytosterols is some property of the spinach leaf, be it texture or taste.

The impact that unsuitable sterols have on feeding behaviour, however, has not been investigated thoroughly. The declining acceptability of spinach by *S. americana* over successive meals (Lee & Bernays, 1988; Champagne & Bernays, 1991) could be a consequence of attributes other than the phytosterol profile. For example, spinach is known to contain natural products such as ecdysteroids (Greibenok *et al.*, 1991), oxalates (Libert & Franceschi, 1987) and phenols (Huang *et al.*, 1986), all of which may affect acceptability. In this study, the role of phytosterols on feeding behaviour of *S. americana* was investigated using behavioural studies on artificial diets in controlled laboratory conditions. The hypothesis that unsuitable sterols are deterrent was tested in three separate experiments using grasshoppers that had been conditioned to feed on artificial diets. The first experiment was intended to show that lipid components of spinach, which include phytosterols, can trigger food rejection behaviour in grasshoppers (Lee & Bernays, 1988; Champagne & Bernays, 1991). This experiment examined feeding-related behaviours on a diet containing the total spinach lipid extract and compared it with behaviour on a diet with sitosterol, a known usable phytosterol. The second experiment was aimed at determining whether unsuitable phytosterols or some other class of lipid from spinach was responsible for the deterrent response. Finally, the third experiment investigated how sterols with markedly different structures influenced feeding behaviour.

Materials and Methods

Experimental insect

Schistocerca americana (Drury) (Orthoptera: Acrididae) is a polyphagous grasshopper occurring throughout the eastern United States and Mexico (Harvey, 1981). It is recorded as feeding on a wide range of cultivated and naturally occurring plant species (Kuitert & Connin, 1952). Insects were from a laboratory colony reared in Bioquip cages (30 × 30 × 30 cm) on a diet of Romaine lettuce, 7- to 10-day-old wheat seedlings and wheat bran, and maintained under standard laboratory conditions (LD 16:8 h, 24–35:19–22°C). Grasshoppers were

removed daily as they moulted to the sixth stadium and only females were used for the experiments. These were transferred to ventilated 5-litre Plexiglass tubs where they were given Romaine lettuce and wheat bran for 18–24 h.

Experimental protocol

On the second day of the sixth stadium, female grasshoppers that had previously fed on fresh plant material were transferred to individual plastic boxes (11 × 11 × 3 cm) that had screened ventilation holes on two sides. These grasshoppers were then fed (conditioned) for 2 days on artificial diet (for full details see Simpson & Abisgold, 1985) that contained a suitable sterol (cholesterol or sitosterol, depending on the experiment). Two variations of the diet were used: a 14% protein/14% digestible carbohydrate diet (herein called 14:14) and a 7% protein/7% digestible carbohydrate diet (herein called 7:7). Diet 7:7 was nutritionally identical to diet 14:14, except that the levels of protein and carbohydrate were half; the difference was replaced by cellulose. These diets were suspended in a 1% agar solution in a dry:wet ratio of 1:4 and presented to grasshoppers as small cubes. If grasshoppers did not accept the artificial diet readily, as determined by the production of faecal pellets, they were not used in observations. During the 2-day conditioning period, grasshoppers were maintained in a Percival growth chamber at LD 16:8 h and heat regime set at 32:28°C. Grasshoppers were given fresh diet twice daily.

Four-day-old grasshoppers were used for all observations; in *S. americana* the sixth stadium lasts 10–12 days under our rearing conditions, and day 4 corresponds to the time of maximum feeding. On the morning of day 4, grasshoppers were moved from the Percival growth chamber to an observation room held at 31–33°C. Insect boxes were placed side by side and were illuminated from above with a single 15 W fluorescent lamp during the observation period. White partitions were placed between boxes to eliminate any possible visual interaction between grasshoppers. Observations of insect behaviour were initiated at 08.00 hours on each occasion, ≈ 2 h after lights on. Grasshoppers were observed continuously for the duration of each experiment and their behaviour recorded on a laptop computer using the software package The Observer 3.0 (Noldus Information Technology, Inc.). Continuous observations allowed a critical analysis of how feeding behaviour might change with time and/or experience. For all experiments the following behavioural activity was recorded: (1) palpating the food, (2) biting the food, (3) feeding, (4) on the food but not feeding, and (5) off the food.

Experiment 1

This experiment tested whether a lipid component of spinach was the basis for the aversion response previously observed in *S. americana* (Lee *et al.* 1988; Champagne & Bernays, 1991). First, grasshoppers were conditioned for 2 days on a 14:14 diet that contained the suitable sterol cholesterol (0.2% dry weight). Following this conditioning period, grasshoppers were

observed feeding on one of two 14:14 diets containing: a lipid extract from spinach, or sitosterol (the known usable sterol and thus the control). The spinach lipids used in this experiment were extracted from shop-bought spinach, *Spinacia oleracea*, by methods previously described (Heupel, 1989). After this fraction was collected, it was suspended in a known volume of methanol and quantified according to total sterol levels using Gas Chromatography (GC). Sitosterol, which was derived from soybean, was a mixture of 60% sitosterol, 27% campesterol and 13% dihydrobrassicasterol; all are believed suitable for grasshopper development (Dadd, 1960). The sitosterol was purchased from Sigma Chemical (St. Louis, MO). For both treatments, the sterol concentration of the diet was 0.55% dry weight. The observations of grasshopper feeding were continuous and lasted for at least 2 h following the first encounter with the diet; no more than eight individual grasshoppers were recorded by a single observer during a session. Feeding records for ten individuals on the spinach lipid extract diet and eight individuals on the sitosterol diet were taken.

Experiment 2

This experiment tested which major class of lipid was most responsible for the deterrent response exhibited by *S. americana* in the first experiment. Spinach lipids were again collected from shop-bought spinach using the methods of Heupel (1989), but this time the lipid extract was further separated into three different classes using thin layer chromatography (TLC). These different classes included: the desmethyl sterols, the dimethyl sterols and all other spinach lipids. After these fractions were scraped from the TLC plates, they were resuspended in equal volumes of methanol; the desmethyl fraction was then quantified against a sitosterol standard using GC methods. For this experiment, all grasshoppers were conditioned on the 14:14 diet as described in the previous experiment. On day 4, grasshoppers were observed feeding on one of four 14:14 diets containing: sitosterol (the control), desmethyl sterols which were mostly spinasterol and 22-dihydrospinasterol (in a 2:1 ratio as identified by GC-MS) and all unsuitable, dimethyl sterols, or other spinach lipids. Sitosterol and spinach desmethyl sterols were added to the diet at a concentration of 0.55% dry weight. The other two lipid fractions were added to diets in volumes equal to that of the spinach desmethyl sterol extract. In contrast to the previous experiment, individual grasshoppers were observed continuously for 4 h after their first encounter with the diet. Each treatment was replicated ten times.

Experiment 3

This experiment tested how the feeding behaviour of *S. americana* was influenced by variation in the position of double bonds within the sterol structure. In contrast to the 2-day conditioning period previously described, however, grasshoppers in this experiment were given a 7:7 diet containing sitosterol (0.2% dry weight). A 7:7 diet was used to shorten

the interfeed gap between bouts (Simpson, 1995). Additionally, feeding for all grasshoppers in this experiment was first recorded on the sitosterol conditioning diet. This was done to establish that each insect was in a similar state of readiness to feed when presented with the test diet. After each grasshopper had stopped feeding for 5 min, the conditioning diet was removed and replaced with one of four 7:7 diets that contained: cholesterol (Δ^5), lathosterol (Δ^7), stigmasterol ($\Delta^{5,22}$), or sitosterol (the control). Cholesterol (suitable), lathosterol (unsuitable), and stigmasterol (unsuitable) were all purchased from Sigma Chemical (St. Louis, MO); each was $\approx 99\%$ pure. Individuals were observed continuously until they had encountered the test diet on ten separate occasions; at least ten individuals were recorded on each treatment, except for cholesterol ($n = 8$).

Statistical analysis

Feeding is a complex behaviour and was analysed in a number of different ways throughout this paper. First, statistical comparisons were made on various behavioural activities taken over an entire observation period. For experiments 1 and 2, the percentage time feeding and the number of encounters were analysed using the non-parametric Mann–Whitney *U*-test and Kruskal–Wallis test, respectively. Encounters were any palpating, biting or feeding bout. For experiment 3, the total time feeding over the first ten encounters was analysed using the Kruskal–Wallis test. Additionally, for all three experiments, the amount of time spent feeding over the first three encounters on each treatment was compared using the Kruskal–Wallis test. If significant differences were observed over the first three encounters, this might suggest that the grasshoppers were directly tasting sterols. When significant differences among treatments were detected with the Kruskal–Wallis test, a Tukey-type multiple comparison test for differences among medians was employed (Zar, 1996). The median encounter length for individuals on the different treatments was also determined and comparisons made using one-way analysis of variance (ANOVA). For this analysis, data were square-root transformed to meet the underlying assumptions of normality.

Second, individual encounter lengths were categorized (modified from Raubenheimer & Bernays, 1993) and their distributions compared among the different treatments. The categories were: rejections (including palpation, bites followed by no feeding, and bouts < 18 s), short (18–60 s), medium (60–240 s) or long (> 240 s) feeding encounters. Following square-root transformation, multivariate analysis of variance (MANOVA) was performed. Reported *F*-values comes from Wilke's lambda, which is derived from the maximum likelihood technique (Abacus Concepts, 1989). Contrasts were used to determine if there were significant differences in the occurrence of specific encounter types among treatments of interest.

Finally, the effects of experience on acceptability of the different diets were measured. The sequential feeding pattern of individual grasshoppers for each treatment was analysed using Cochran's *Q*-test, a repeated measures analysis designed for dichotomous variables (Zar, 1996). In this case, we recorded

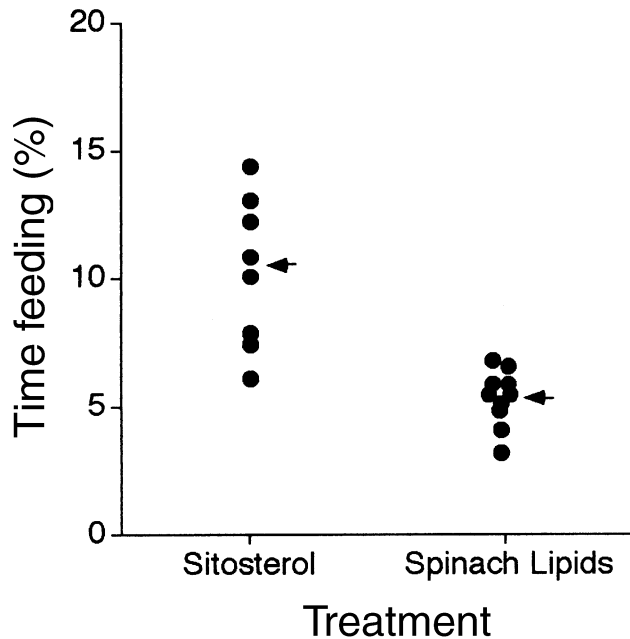


Fig. 1. The percentage of the total time spent feeding over a 2-h period for *S. americana* in experiment 1. Data points represent individual grasshoppers and arrows indicate median values. The difference between the two treatments was significant (MWU-test, $P < 0.01$).

at each encounter whether an individual grasshopper rejected its food (palpation, bites followed by no feeding, or bouts < 18 s). The null hypothesis was that the probability of observing a rejection for any particular treatment is the same for each encounter. For experiment 1, rejection sequences were observed over the first eight encounters; for experiments 2 and 3, rejection sequences were observed over the first ten encounters.

Results

Experiment 1

Over the 2-h observation period, no difference in the number of encounters among the sitosterol or the spinach lipid extract diet was observed (Mann-Whitney U -test (MWU), $Z = -0.447$, $P = 0.657$). Sitosterol-fed grasshoppers had 8.5 ± 3.5 (median \pm MAD) encounters, whereas the spinach lipid extract fed grasshoppers had 9.0 ± 3.0 encounters. Over the first three encounters, no difference in percentage time feeding among the two treatments was observed (MWU, $Z = -0.355$, $P = 0.722$). Over the entire 2-h period, however, the percentage time feeding was significantly less on the diet containing the spinach lipid extract than on the sitosterol control diet (MWU, $Z = -3.376$, $P < 0.01$; Fig. 1).

The median encounter duration was significantly different in the two treatments (ANOVA, d.f. = 1, $F = 30.031$, $P < 0.05$); encounters on sitosterol diet (mean \pm SE: 53.25 ± 27.5 s)

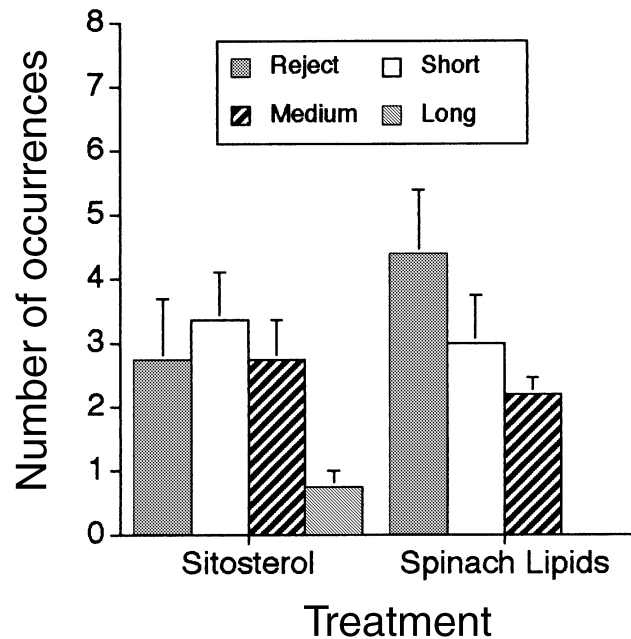


Fig. 2. The mean (\pm SE) number of occurrences of different encounters on either sitosterol or spinach-lipid extract diets. The distributions were significantly different among the two treatments (MANOVA, $P < 0.01$).

were more than twice as long as those on the spinach lipid extract diet (22.5 ± 10.0 s). At a finer scale, a comparison of the distribution of encounter types among the two treatments revealed significant differences (MANOVA, $F = 5.643$, $P < 0.01$). Spinach lipid extract fed grasshoppers had more rejections than those on the sitosterol diet and also had no feeding encounters that lasted greater than 240 s (Fig. 2).

Finally, the pattern of feeding over the first eight encounters was analysed, noting the probability of observing a rejection. Cochran's Q -test revealed significant differences in rejection probabilities over time on the spinach lipid extract diet (d.f. = 7, $\chi^2 = 14.061$, $P < 0.05$); rejection probabilities exceeded 50% in four of the last five encounters (Fig. 3b). This was not the case on the sitosterol diet (d.f. = 7, $\chi^2 = 10.575$, $P > 0.10$), where rejection probabilities remained at or below 50% over the first seven encounters (Fig. 3a).

Experiment 2

The number of encounters over the 4-h period, similar to that shown over the 2-h period in the previous experiment, did not vary among any of the different spinach lipid fractions and the sitosterol control (Kruskal-Wallis test (KW), $H = 1.953$, $P = 0.583$; Table 1). There also was no difference in the time feeding among these treatments over the first three encounters (KW, $H = 1.149$, $P = 0.765$). Grasshopper feeding over the entire 4-h observation was, however, influenced by the type of spinach lipid fraction added to the diet (KW, d.f. = 3, $H = 13.209$, $P < 0.01$). The median percentage time feeding was lower on diets containing one of the three spinach lipid fractions

compared to the sitosterol control diet. Only the difference between the spinach desmethyl sterol diet and the sitosterol diet, however, was significant (Fig. 4).

The median encounter duration was greatest on the sitosterol diet, and lowest on the diet containing spinach desmethyl sterols. The difference among the four treatments, however, was not statistically significant (ANOVA, $F = 1.490$, $P = 0.237$; Table 1). A closer inspection of the distribution of encounter types among the four treatments also failed to reveal a significant difference (MANOVA, $F = 1.180$, $P = 0.310$). There were, however, differences in feeding behaviour when comparisons between the spinach desmethyl sterol diet and the sitosterol diet were made using contrasts. Significantly more rejections (One-tailed contrast, d.f. = 1, $F = 4.580$, $P < 0.05$)

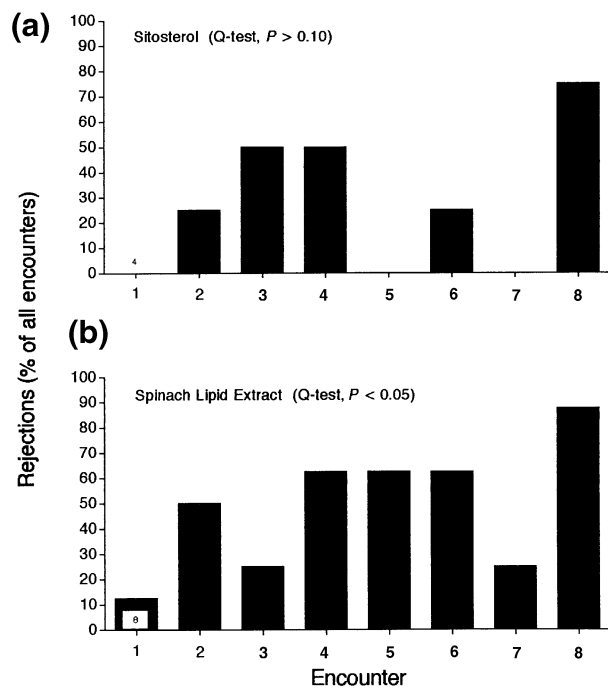


Fig. 3. Rejections, as a percentage of all encounters, for *S. americana* over the first eight encounters in experiment 1. Rejections were defined as palpation, bites followed by no feeding, or encounters lasting less than 18 s. The treatments were: (a) sitosterol, and (b) a spinach lipid extract. Sample size is indicated on the first bar for each treatment.

and fewer long bouts (One-tailed contrast, d.f. = 1, $F = 3.262$, $P < 0.05$) were observed on the spinach desmethyl sterol diet compared to the sitosterol diet (Fig. 5).

Lastly, the sequence of feeding over the first ten encounters was analysed with respect to rejection behaviour. Significant differences in the rejection pattern were observed on the diets containing spinach sterols (desmethyl and dimethyl) but not on the diets containing other spinach lipids or sitosterol (Table 1). The rejection pattern was strongest on the diet containing spinach desmethyl sterols (spinasterol and 22-dihydrospinasterol); rejection probabilities were at least 70% in five of the last six encounters (Fig. 6b). Rejection patterns also differed significantly over time for diets containing the spinach dimethyl sterols (Fig. 6c), although not to the degree observed for the desmethyl sterols. For the diets containing either sitosterol (Fig. 6a) or other spinach lipids (Fig. 6d), however, rejection probabilities did not change as the number of encounters with the diet increased.

Experiment 3

There was no difference in the amount of time spent feeding on the sitosterol, cholesterol, lathosterol or stigmasterol diets

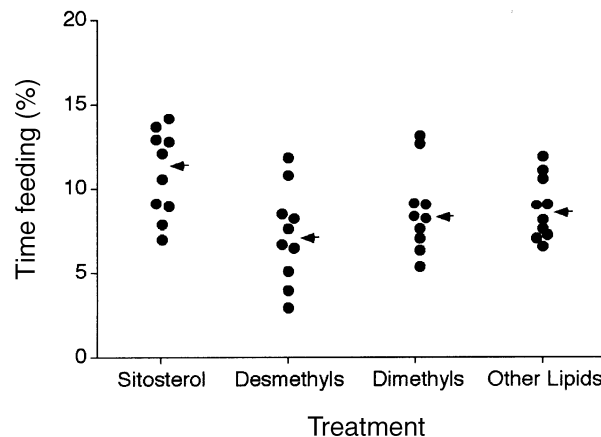


Fig. 4. The percentage of the total time spent feeding over a 4-h period for *S. americana* in experiment 2. Data points represent individual grasshoppers and arrows indicate median values. Only the difference between the sitosterol and the spinach desmethyl sterol diet was significant (Tukey test, $P < 0.05$).

Table 1. Feeding behaviour of *S. americana* in response to the different spinach lipid extract fractions used in experiment 2. Different letters indicate a significant difference between treatments. The rejection sequences were analysed over the first ten encounters. All treatments were replicated ten times.

Treatment	Encounter frequency median (\pm MAD)	Encounter duration (s) mean (\pm SE)	Rejection sequence (<i>Q</i> -test results)		
			d.f.	χ^2	<i>P</i> -value
Sitosterol	26.0 (5.5) a	31.9 (16.0) a	9	15.949	NS
Desmethyls	20.5 (4.5) a	15.2 (6.9) a	9	24.546	**
Dimethyls	19.0 (8.0) a	17.1 (8.4) a	9	17.419	*
Other Lipids	24.0 (7.0) a	21.5 (3.1) a	9	7.305	NS

* $P < 0.05$. ** $P < 0.01$. NS = not significant.

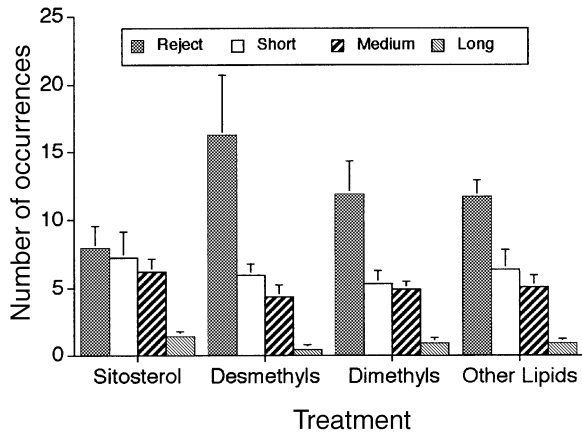


Fig. 5. The mean (\pm SE) number of occurrences of different encounters on the different treatments from experiment 2. The distributions were not significantly different among any of the four treatments (MANOVA, $P = 0.310$).

after the first three encounters (KW, $H = 2.003$, $P = 0.572$). There was, however, a significant difference among the four sterol treatments in time spent feeding after ten encounters (KW, d.f. = 3, $H = 8.874$, $P < 0.05$). Grasshoppers given diets containing stigmaterol, lathosterol and cholesterol fed for a shorter amount of time compared to those given the sitosterol control diet. However, only the difference between the stigmaterol and sitosterol diets was significant (Fig. 7).

There also was a significant difference in the median encounter length among the four sterol treatments (ANOVA, $F = 5.907$, $P < 0.01$). Both stigmaterol- and lathosterol-fed grasshoppers had shorter median encounter lengths than the sitosterol-fed grasshoppers (Table 2). Upon further inspection of the distribution of encounter types, significant differences among the four treatments were also revealed (MANOVA, $F = 2.636$, $P < 0.01$; Fig. 8). More rejections (One-tailed contrast, d.f. = 1, $F = 14.080$, $P < 0.01$) and fewer short (One-tailed contrast, d.f. = 1, $F = 3.055$, $P < 0.05$), medium (One-tailed contrast, d.f. = 1, $F = 4.710$, $P < 0.05$), and long (One-tailed contrast, d.f. = 1, $F = 3.250$, $P < 0.05$) encounters were observed on the unsuitable (stigmaterol and lathosterol) compared to the suitable (sitosterol and cholesterol) sterol diets.

Finally, the pattern of feeding was analysed with respect to observed rejections. Significant differences in rejection patterns were observed on the unsuitable sterol treatments but not on the suitable sterol treatments (Table 2). For stigmaterol, rejection probabilities were greater than 60% for five of the last six encounters (Fig. 9b). For lathosterol, rejection probabilities were greater than 50% on the last eight successive encounters (Fig. 9c). Rejection patterns on the sitosterol (Fig. 9a) and cholesterol (Fig. 9d) diets, however, did not change significantly with time.

Discussion

The first experiment shows that the aversion component is in the lipid fraction and the second experiment indicates that

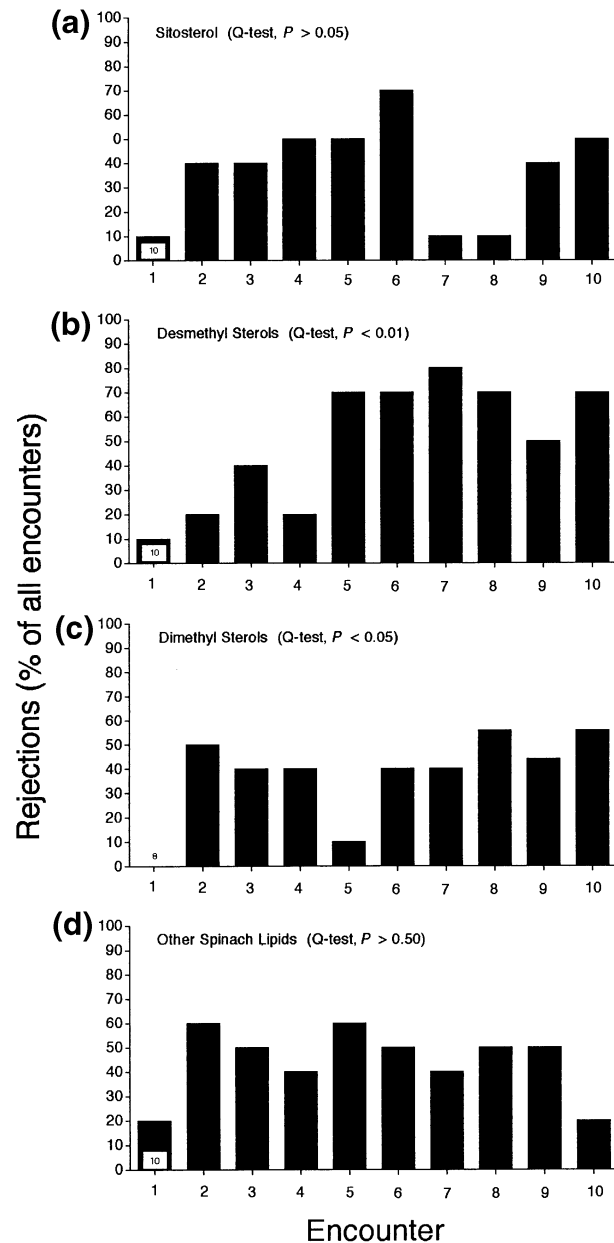


Fig. 6. Rejections, as a percentage of all encounters, for *S. americana* over the first ten encounters in experiment 2. Rejections were defined as palpation, bites followed by no feeding, or encounters lasting less than 18 s. The treatments were: (a) sitosterol, (b) spinach desmethyl sterols, (c) spinach dimethyl sterols, and (d) other spinach lipids. Sample sizes are indicated on the first bar for each treatment.

sterols are indeed involved. In the first experiment, the percentage of the total time spent feeding was significantly lower on the diet with the spinach lipid extract, which contained unsuitable sterols, compared to the sitosterol control diet. Further evidence that these unsuitable sterols contribute to rejection behaviour is seen in the second experiment. The percentage time feeding on the diet with the isolated desmethyl

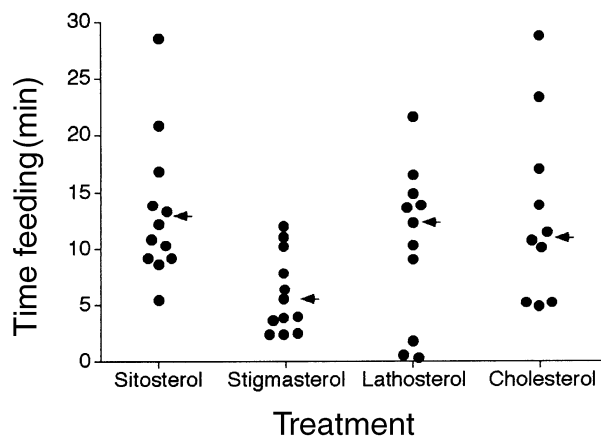


Fig. 7. The percentage of the total time spent feeding over the first ten encounters for *S. americana* in experiment 3. Data points represent individual grasshoppers and arrows indicate median values. Only the difference between the sitosterol and the stigmasterol diet was significant (Tukey test, $P < 0.05$).

Table 2. Feeding behaviour of *S. americana* in response to the different sterols used in experiment 3. Significant differences among treatments are indicated by different letters. The rejection sequences were analysed over the first ten encounters. The sample size for each treatment is indicated in parentheses.

Treatment	Encounter duration (s) median (\pm MAD)	Rejection sequence (Q -test results)		
		d.f.	χ^2	P -value
Sitosterol (11)	44.8 (19.0) a	9	13.125	NS
Stigmasterol (12)	15.6 (7.1) b	9	18.546	*
Lathosterol (12)	14.1 (12.4) b	9	22.703	**
Cholesterol (8)	22.5 (8.0) ab	9	13.742	NS

* $P < 0.05$. ** $P < 0.01$. NS = not significant.

sterols from spinach (which included spinasterol and 22-dihydrospinasterol) was significantly lower compared to the sitosterol control. The difference in the percentage time feeding for the two other lipid fractions, however, was not significantly different from the sitosterol control. Other behavioural activities also point to a regulatory role for spinach sterols. Grasshoppers given the spinach lipid extract diet, when compared to those fed the sitosterol control, had median encounter durations that were shorter, a greater number of rejections, and no encounters that exceeded 4 min. Likewise, significantly more rejections and fewer long encounters were recorded on the desmethyl sterol diet compared to the sitosterol control diet for grasshoppers in the second experiment.

The results from the third experiment, when combined with those from the first two experiments, indicate that, in general, desmethyl sterols that are unsuitable for *S. americana* cause rejection behaviour. Grasshoppers given a diet containing the unsuitable sterol stigmasterol ($\Delta^{5,22}$) ate for significantly less time over the first ten encounters and had median encounter

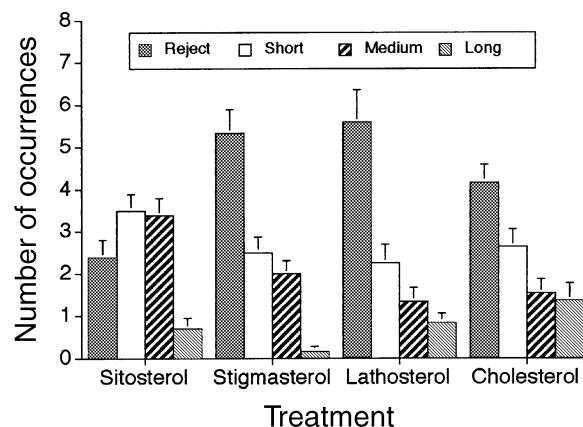


Fig. 8. The mean (\pm SE) number of occurrences of different encounters on the different treatments from experiment 3. The distributions were significantly different among the four treatments (MANOVA, $P < 0.01$).

duration that were much shorter compared to the sitosterol control grasshoppers. For grasshoppers given the unsuitable sterol lathosterol (Δ^7), the median encounter duration was significantly shorter than those given the sitosterol control. Additionally, grasshoppers given diets with unsuitable sterols had more rejections and fewer short, medium and long encounters when compared to the grasshoppers given diets with suitable sterols (sitosterol and cholesterol).

Because it is believed that grasshoppers cannot directly taste sterols (Cook, 1977; Champagne & Bernays, 1991; Behmer, 1998), a possible mechanism regulating the rejection behaviour to unsuitable sterols in these experiments is an indirect nutritional feedback involving associative learning. In the current experiments, the aversion response develops over time, indicating that food which is initially acceptable becomes unacceptable after a particular quantity of unsuitable sterol is ingested. Two separate analyses of the data support this. First, there was no significant difference in the total time feeding over the initial three encounters among any treatments for each of the three experiments. Second, the probability of observing a rejection increased significantly over time for grasshoppers given diets that contained unsuitable desmethyl and dimethyl sterols but not for those given suitable sterols. Both of these responses are consistent with associative learning, where there is often a time delay between sensory patterns associated with food intake and the negative consequences of ingestion (Bernays, 1993).

How and where phytosterols are detected by grasshoppers is still unknown. The existence of a system capable of binding suitable sterols, with a resultant feedback to the brain, has been proposed by Champagne & Bernays (1991). In this system, the resultant feedback is dependent on sterol absorption. They suggest the failure of unsuitable sterols to reach or bind to the putative receptor prevents absorption across the midgut. The failure to absorb unsuitable sterols may cause a metabolic bottleneck that in turn triggers the aversion response. A recent study on phytosterol metabolism in grasshoppers, however, raises doubts about this model. The unsuitable phytosterols

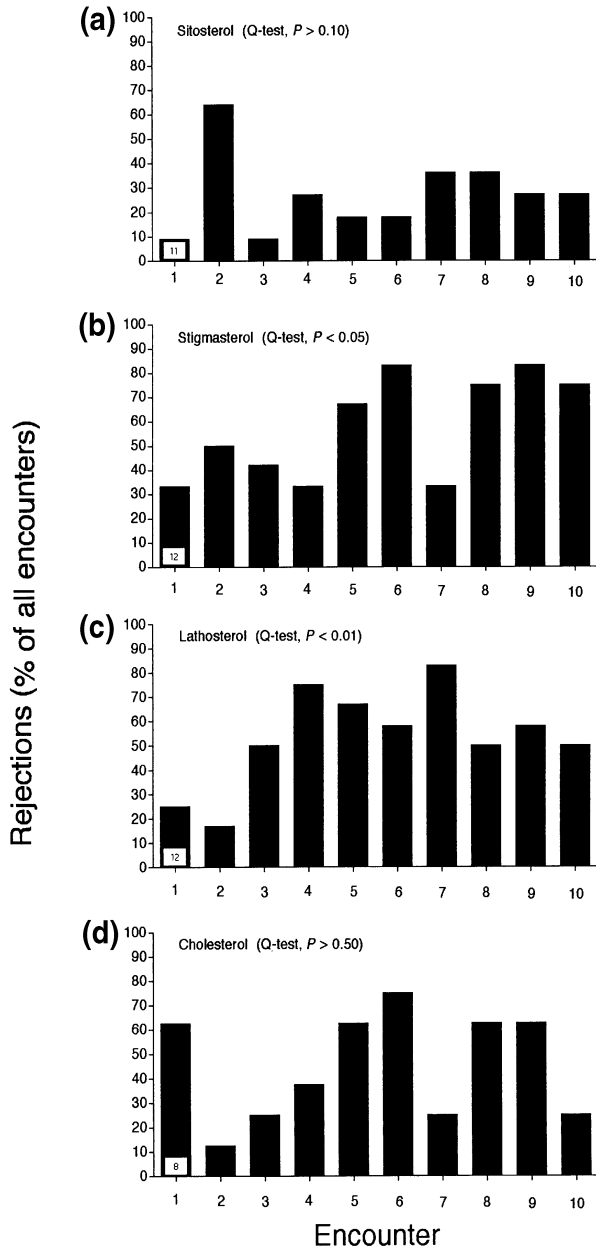


Fig. 9. Rejections, as a percentage of all encounters, for *S. americana* over the first ten encounters in experiment 3. Rejections were defined as palpation, bites followed by no feeding, or encounters lasting less than 18 s. The treatments were: (a) sitosterol, (b) stigmasterol, (c) lathosterol, and (d) cholesterol. Sample sizes are indicated on the first bar for each treatment.

spinasterol and stigmasterol were detected unmetabolised in the bodies of *S. americana* nymphs that had been reared on artificial diet containing these different sterols (Behmer, 1998). Although these findings come from long-term studies, there is no reason to believe that unsuitable phytosterols are not absorbed at rates similar to suitable sterols. A close inspection of the feeding patterns over time indicates that rejection

probabilities are at or above 50% by the fifth encounter on diets containing unsuitable sterols. In most cases, grasshoppers had their fifth encounter within an hour after their first feeding bout. This suggests that unsuitable phytosterols reach the haemolymph quickly following ingestion and that the concentrations needed to elicit rejection behaviour might be low.

In the short term, haemolymph composition, with respect to nutrients such as sugars and amino acids, varies both with time since a meal and with the quantity and quality of previous meals (Abisgold & Simpson, 1987, 1988; Friedman *et al.*, 1991; Simpson & Simpson, 1992). As a result, the haemolymph has the potential to provide constantly updated information about the insect's nutritional state. That unsuitable phytosterols may be acting through the haemolymph to modify feeding behaviour, similarly to sugars and amino acids, was recently investigated by injecting sterols directly into the haemolymph of *S. americana* nymphs. The results, which are reported elsewhere, strongly suggest that the sterols in the haemolymph can play a central role in the development of the aversion response.

It is interesting to note that sterols with a double bond at position 22 on the side chain (e.g. spinasterol and stigmasterol) appear to have the greatest influence on feeding behaviour. Only on the spinach sterol diet, which contained spinasterol ($\Delta^{7,22}$), and the stigmasterol ($\Delta^{5,22}$) diet were all the measured feeding parameters negatively affected. The mechanistic explanation may be related to the configuration of C-24 on the side chain. For both spinasterol and stigmasterol, C-24 shows a *S* configuration, whereas the C-24 of sitosterol, a suitable sterol, shows a *R* configuration. In grasshoppers, this change in configuration blocks dealkylation, thus preventing cholesterol production; it does not, however, prevent absorption into the haemolymph (Behmer, 1998). Lathosterol, which has a double bond in the β -ring at position 7, also influenced feeding in a negative fashion, but not to the extent that Δ^{22} -sterols did. Neither sitosterol nor cholesterol, two Δ^5 -sterols, affected feeding in a negative fashion. An additional point of interest relates to the non-polar nature of sterols. In contrast to sugars and some amino acids that are soluble in the aqueous haemolymph, sterols must be transported through the haemolymph by lipophorin (Blacklock & Ryan, 1994). Much remains unknown about the fate of absorbed sterols, but lipophorin may be playing a role in the regulation of this feeding behaviour.

Grasshopper feeding is a complex behaviour, but many of the causal factors that interact to regulate this behaviour have been identified (reviewed by Simpson, 1995). These include the developmental and nutritional state of the insect, the prevailing environmental conditions, and the nature and availability of food. In the current study, these causal factors were controlled, as much as possible, by using grasshoppers that were: the same sex and age, reared and observed under identical laboratory conditions, preconditioned to feed on artificial diets that were nutritionally identical within an experiment except for sterol content, and provided food *ad libitum* so that individuals could regulate intake rates in a natural manner. Any changes in feeding behaviour among the

different treatments therefore would be correlated with the sterol composition of the diet.

The functional significance of acquired rejection to unsuitable phytosterols, whether it be through aversion learning or a more direct mechanism, is evident considering that phytosterols with Δ^7 - or Δ^{22} -bonds cannot support normal growth and development in grasshoppers (Dadd, 1960; Behmer, 1998). In the laboratory, the failure to regulate the intake of unsuitable sterols can have extreme consequences for grasshoppers (Behmer, 1998). The accumulation of unsuitable sterols in the bodies of *S. americana* results in high mortality, even if suitable sterols are present at concentrations that alone would allow complete development. In the field, grasshoppers often rest on or near their foods (Chambers *et al.*, 1996) and aversion learning may be an effective strategy to regulate the intake of unsuitable phytosterols. This habit of developing a short-term fidelity to a particular resource increases the likelihood that learned aversion can develop over a series of meals on a single food type, whereupon rejection and movement away may follow if the food is unsuitable (Bernays, 1993).

Acknowledgements

We gratefully acknowledge E. A. Bernays and D. Galbraith for logistic support. We thank E. A. Bernays, R. F. Chapman, A. Chesler, R. Issacs and M. Singer (Arizona) for thoughtful criticisms and suggestions on initial versions of the manuscript. This work was supported by grants from Sigma Xi and the Orthopterists' Society awarded to S.T.B. Support was also provided through the Interdisciplinary Training Group on Plant–Insect Interactions (NSF BIR-9220332) at the University of Arizona. An NIH undergraduate research training grant from the University of Arizona (T32 A107475) and additional funds from the Interdisciplinary Training Group on Plant Insect Interactions provided partial support for D.O.E.

References

- Abacus Concepts, Inc. (1989) *SuperANOVA*: Accessible General Linear Modeling. Abacus Concepts, Inc., Berkeley, CA, U.S.A.
- Abisgold, J.D. & Simpson, S.J. (1987) The physiology of compensation by locusts for changes in dietary protein. *The Journal of Experimental Biology*, **129**, 329–346.
- Abisgold, J.D. & Simpson, S.J. (1988) The effect of dietary protein levels and hemolymph composition on the sensitivity of the maxillary palp chemoreceptors of locusts. *The Journal of Experimental Biology*, **135**, 215–229.
- Behmer, S. (1998) *Phytosterols as neglected nutrients for grasshoppers*. Unpublished PhD dissertation, University of Arizona, U.S.A.
- Bernays, E.A. (1993) Aversion learning and feeding. *Insect Learning: Ecological and Evolutionary Perspectives* (ed. by D. R. Papaj and A. C. Lewis), pp. 1–17. Chapman and Hall Ltd, London.
- Bernays, E.A. (1995) Effects of experience on feeding. *Regulatory Mechanisms in Insect Feeding* (ed. by R. F. Chapman and G. deBoer), pp. 279–306. Chapman and Hall Ltd, London.
- Blacklock, B.J. & Ryan, R.O. (1994) Hemolymph lipid transport. *Insect Biochemistry and Molecular Biology*, **24**, 855–873.
- Chambers, P., Sword, G., Angel, J.E., Behmer, S. & Bernays, E.A. (1996) Foraging by generalist grasshoppers: two different strategies. *Animal Behaviour*, **52**, 155–165.
- Champagne, D.E. & Bernays, E.A. (1991) Phytosterol unsuitability as a factor mediating food aversion learning in the grasshopper *Schistocerca americana*. *Physiological Entomology*, **16**, 391–400.
- Clayton, R.B. (1964) The utilization of sterols by insects. *Journal of Lipid Research*, **5**, 3–19.
- Cook, A.G. (1977) Nutrient chemicals as phagostimulants for *Locusta migratoria*. *Ecological Entomology*, **2**, 113–121.
- Dadd, R.H. (1960) The nutritional requirements of locusts. II. Utilization of sterols. *Journal of Insect Physiology*, **5**, 161–168.
- Friedman, S., Waldbauer, G.P., Eertmoed, J.E., Naeem, M. & Ghent, A.W. (1991) Blood trehalose levels have a role in the control of dietary self-selection by *Heliothis zea* larvae. *Journal of Insect Physiology*, **37**, 919–928.
- Garg, V.K., Douglas, T.J. & Paleg, L.G. (1987) Presence of unusually high levels of cholesterol in the shoot-apices of flowering plants. *The Metabolism, Structure and Function of Plant Lipids* (ed. by P. K. Stumpf, J. B. Mudd and W. D. Nes), pp. 83–89. Plenum Press, New York.
- Grebenok, R.J., Perry, V.R. & Adler, J.H. (1991) Occurrence and levels of ecdysteroids in spinach. *Lipids*, **26**, 666–668.
- Grieneisen, M.L. (1994) Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. *Insect Biochemistry and Molecular Biology*, **24**, 115–132.
- Hamamura, Y. (1970) The substances that control the feeding behaviour and the growth of the silkworm *Bombyx mori* (L.). *Control of Insect Behavior by Natural Products* (ed. by D. L. Wood, R. M. Silverstein and M. Nakajima), pp. 55–80. Academic Press, New York.
- Harvey, A.W. (1981) A reclassification of the *Schistocerca americana* complex (Orthoptera: Acrididae). *Acrida*, **10**, 61–77.
- Heupel, R.C. (1989) Isolation and characterization of sterols. *Analysis of Sterols and other Biologically Significant Steroids* (ed. by W. D. Nes and E. T. Parish), pp. 1–31. Academic Press, New York.
- Huang, H., Johanning, G.L. & O'Dell, B.L. (1986) Phenolic acid content of food plants and possible nutritional implications. *Journal of Agriculture Food Chemistry*, **34**, 48–51.
- Ikekawa, N., Morisaki, M. & Fujimoto, Y. (1993) Sterol metabolism in insects: dealkylation of phytosterol to cholesterol. *Accounts of Chemical Research*, **26**, 139–146.
- Kuitert, L.C. & Connin, R.V. (1952) Biology of the American grasshopper in the southeastern United States. *Florida Entomologist*, **35**, 22–33.
- Lee, J.C. & Bernays, E.A. (1988) Declining acceptability of a food plant for the polyphagous grasshopper *Schistocerca americana*: the role of food aversion learning. *Physiological Entomology*, **13**, 291–301.
- Libert, B. & Franceschi, V.R. (1987) Oxalate in crop plants. *Journal of Agriculture Food Chemistry*, **35**, 926–938.
- Nes, W.R. & McKean, M.L. (1977) *Biochemistry of Steroids and other Isopentenoids*. University Park Press, Baltimore, U.S.A.
- Raubenheimer, D. & Bernays, E.A. (1993) Patterns of feeding in the polyphagous grasshopper *Taeniopoda eques*: a field study. *Animal Behaviour*, **45**, 153–167.
- Salt, T.A., Xu, S., Patterson, G.W. & Adler, J.H. (1991) Diversity of sterol biosynthetic capacity in the Caryophyllidae. *Lipids*, **26**, 604–613.
- Simpson, S.J. (1995) Regulation of a meal: chewing insects. *Regulatory Mechanisms in Insect Feeding* (ed. by R. F. Chapman and G. deBoer), pp. 137–156. Chapman and Hall Ltd, London.

- Simpson, S.J. & Abisgold, J.D. (1985) Compensation by locusts for changes in dietary nutrients: behavioural mechanisms. *Physiological Entomology*, **10**, 443–452.
- Simpson, S.J. & Simpson, C.L. (1992) Mechanisms controlling modulation by amino acids of gustatory responsiveness in the locust. *The Journal of Experimental Biology*, **168**, 269–287.
- Svoboda, J.A. & Thompson, M.J. (1985) Steroids. *Comprehensive*

Insect Physiology, Biochemistry and Pharmacology, Vol. 4 (ed. by G. A. Kerkut and L. I. Gilbert), pp. 137–175. Pergamon Press, Elmsford, New York.

Zar, J.H. (1996) *Biostatistical Analysis*, 3rd edn. Prentice Hall, New Jersey.

Accepted 1 June 1998