

POST-INGESTIVE FEEDBACKS AND ASSOCIATIVE LEARNING REGULATE THE INTAKE OF UNSUITABLE STEROLS IN A GENERALIST GRASSHOPPER

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Accepted 4 January; published on WWW 17 February 1999

Summary

Behavioural studies of the grasshopper *Schistocerca americana* were undertaken to identify the mechanisms that regulate the intake of dietary sterols. In the first experiment, grasshoppers were allowed to feed on spinach, a plant containing only unsuitable sterols; immediately after this first meal, a suitable or unsuitable sterol was injected into the haemolymph. Grasshoppers injected with unsuitable sterols had second meals on spinach that were significantly shorter than those of grasshoppers injected with suitable sterols, indicating that unsuitable dietary sterols are detected post-ingestively. In the second experiment, grasshoppers were fed food containing only unsuitable sterols and were then presented with glass-fibre

discs containing different concentrations of a suitable sterol or sucrose only (the control). The results suggest that grasshoppers do not use a direct feedback operating on mouthpart chemoreceptors to regulate their intake of suitable sterols. In the third experiment, grasshoppers were presented with artificial diets containing different sterols and flavours, and feeding was observed over a sequence of meals. The results from both the first and last experiments suggest a role for associative learning in regulating the intake of unsuitable sterols.

Key words: grasshopper, *Schistocerca gregaria*, associative learning, sterol, feeding, post-ingestive feedback.

Introduction

Grasshoppers employ a number of different mechanisms to evaluate the nutritional quality of their host plants and, in turn, can use this information to regulate feeding behaviour. Contact chemoreceptors on the maxillary and labial palps, tarsi and antennae, and chemoreceptors located on the inner face of the labrum, labium and hypopharynx, allow grasshoppers to evaluate host-plant quality directly (Chapman, 1995). For many grasshoppers, a period of feeding is often followed by a sustained period of non-feeding (Blaney et al., 1973; Simpson, 1982) during which post-ingestive feedbacks can be used to evaluate further the quality of the food (Lee and Bernays, 1990; Simpson and Raubenheimer, 1993). If successive meals are taken on the same plant, associative learning, either positive or negative, can develop (Lee and Bernays, 1988; Simpson and White, 1990; Bernays, 1993).

Sterols are essential nutrients for all insects, and the specific requirements for particular sterols are well known for a number of different species (Dadd, 1977; Fig. 1). Most phytophagous insects metabolize phytosterols to cholesterol, which is the dominant sterol found in cell membranes and serves as the precursor for the insect moulting hormone 20-hydroxyecdysone (Grieneisen, 1994). Sitosterol is the most common phytosterol found in plants and is readily metabolized to cholesterol in most phytophagous insects (Svoboda and Thompson, 1985; Ikekawa et al., 1993). For grasshoppers,

however, phytosterols that possess a double bond at position 7 (Δ^7), or at position 22 (Δ^{22}), cannot be metabolized to cholesterol; these sterols will not support normal growth and development (Dadd, 1960; Behmer, 1998).

Little is known about the relative importance of phytosterols as phagostimulants or as nutrients that might influence feeding either indirectly or through post-ingestive feedbacks. Sitosterol, a suitable phytosterol for most phytophagous insects, is a suggested biting stimulant at high concentrations for the silkworm *Bombyx mori* (Hamamura, 1970; but see Nayar and Fraenkel, 1962) and for the Colorado potato beetle *Leptinotarsa decemlineata* (Hsiao and Fraenkel, 1968). Results from long-term studies (18h), however, suggest that neither sitosterol nor cholesterol, which are both suitable sterols, stimulate feeding in the grasshopper *Locusta migratoria* (L.) (Cook, 1977). Grasshoppers, however, do appear to be capable of regulating feeding in response to unsuitable sterols. When the grasshopper *Schistocerca americana* was presented with spinach, a plant that contains only unsuitable phytosterols, sustained acceptance was only seen when high concentrations of utilizable sterols were added (Champagne and Bernays, 1991). That unsuitable sterols are the cause of the aversion response in *S. americana* was recently confirmed in experiments using artificial diets (Behmer and Elias, 1999a). Feeding behaviour was recorded on diets that were

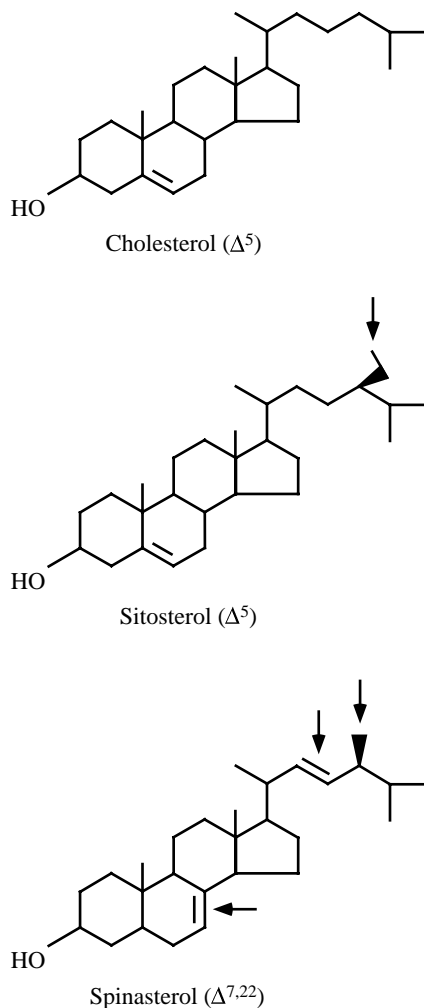


Fig. 1. Sterol structures of interest. Cholesterol is the dominant insect sterol. Sitosterol and spinasterol are phytosterols. Spinasterol is the dominant phytosterol in spinach and does not support normal growth and development in grasshoppers. Arrows on the phytosterols indicate structural differences from cholesterol.

nutritionally identical except for the dietary sterol and, as observed in the experiments in which the insects were fed on spinach, the acceptability of diets containing unsuitable sterols declined with experience. This aversion to unsuitable sterols, which develops over time, suggests that post-ingestive feedbacks are operating.

In the present study, behavioural studies of grasshopper feeding were conducted to identify the mechanisms that might regulate the intake of phytosterols. If the effect is indeed post-ingestive, there are two possibilities: (1) a direct feedback on mouthpart taste receptors that is mediated through the haemolymph, and/or (2) a learned association with some feature of the food. Direct nutrient feedbacks, which are known to influence food selection in the grasshopper *L. migratoria* (Simpson and Simpson, 1990; Simpson and Raubenheimer, 1993), are one possibility. For example, a shortage of carbohydrate or protein in the diet lowers the levels of sugar

and amino acids in the haemolymph, leading to increased sensitivity of mouthpart chemoreceptors to sugars and amino acids, respectively (Simpson et al., 1991; Simpson and Simpson, 1992; Zanotto et al., 1996). Associated with these peripheral changes are directly correlated compensatory changes in feeding behaviour and diet selection (Abisgold and Simpson, 1987; Simpson et al., 1991). For such a mechanism to operate, however, there must be gustatory responsiveness to the compounds in question (Simpson and Raubenheimer, 1993). Alternatively, grasshoppers may learn to associate dietary sterols with a specific taste or other food-related cue. For example, sixth-stadium nymphs of *S. americana* are capable of learning to associate the gustatory cues of an initially acceptable novel food with detrimental effects caused by plant secondary compounds injected directly into the haemolymph after feeding (Lee and Bernays, 1990); the learned association leads to a reduction in the acceptability of the food.

In our first experiment, we tested whether unsuitable sterols could be detected post-ingestively. Following a meal on spinach, either a suitable or an unsuitable dietary sterol was injected directly into the haemolymph, making sure not to puncture the alimentary tract, and the duration of the subsequent meal on the same food was measured. In our second experiment, grasshoppers were given food, overnight, that contained only unsuitable sterols; on the following morning, they were presented, in a no-choice assay, with a glass-fibre disc containing one of three different sitosterol (a suitable sterol) concentrations. If direct feedbacks are involved in the regulation of sterol intake, the shortage of appropriate sterols in the haemolymph may cause an increase in the responsiveness of mouthpart chemoreceptors to suitable sterols on the glass-fibre discs. In the last experiment, the importance of gustatory cues to the development of aversion learning was quantified using artificial diets containing different combinations of dietary sterols and flavours.

Materials and methods

Experimental insect

Schistocerca americana (Drury) is a polyphagous grasshopper occurring throughout the southeastern 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). Insects were from a laboratory colony reared in Bioquip cages (30 cm×30 cm×30 cm) and fed on a diet of Romaine lettuce, 7- to 10-day-old wheat seedling and wheat bran. They were maintained under standard laboratory conditions with a 16h:8h L:D photoperiod and radiant heat to maintain the temperature at 24–35 °C during the light phase and at 19–22 °C during the dark phase. The radiant heat was supplied by a 150 W incandescent bulb during the photophase and allowed the grasshoppers to regulate their body temperature. During the scotophase, the air temperature fell to 19–22 °C. Grasshoppers were removed daily as they moulted to the sixth stadium.

To test for the detection of unsuitable sterols via post-ingestive feedbacks (experiment 1)

The goal of this experiment was to quantify the effect of different injected sterols on the acceptability of spinach, as indicated by the amounts ingested, in meals, before and after injections. Newly moulted sixth-stadium grasshoppers were transferred to 51 acrylic tubs and fed Romaine lettuce and wheat bran for 3 days; during this time, they were maintained under standard laboratory conditions. On the morning of day 4, grasshoppers were transferred to an observation room held at 31–33 °C and removed individually to plastic boxes (21.5 cm×11 cm×3.5 cm with two screened ventilation holes).

Two separate sets of injection experiments were conducted. First, 'soybean sitosterol' (suitable)-injected insects were compared with insects injected with clean mineral oil; this test served as a control relative to the treatments where sterols were added to the mineral oil. Second, 'soybean sitosterol'-injected insects were compared with spinach sterol (all unsuitable)-injected insects. Sterols are insoluble in aqueous solutions and are only transported through the haemolymph by lipophorin, a generalized lipid-binding protein; the mineral oil served as a non-toxic delivery system for the sterols and should be readily bound by lipophorin.

In all the experiments that follow, insects were observed in a way that minimized disturbance from each other as well as from the observers. Boxes were placed side by side and illuminated from above with a single 15 W fluorescent lamp, and partitions were placed between boxes to eliminate any possible visual interaction between grasshoppers. All the feeding behaviour was recorded from a distance of approximately 0.6 m; care was taken not to disturb the grasshoppers with sudden movements during the observation period. The following behaviour patterns were recorded on a laptop computer using the software package The Observer 3.0 (Noldus Information Technology, Inc.): (1) palpating the food/substratum, (2) biting the food/substratum, (3) feeding, (4) on the food/substratum but not palpating, biting or feeding, and (5) off the food/substratum.

Starting at approximately 08:30 h on day 4, each insect was provided with cut seedling wheat standing in a vial of water and observed until the end of the first meal; giving the grasshoppers a first meal on wheat standardized them nutritionally and ensured that they were in a similar state of readiness to feed when presented with the next food item. For all experiments, meals were defined as the sum of all bouts of feeding from the first contact until a feeding/contact gap of more than 5 min, excluding intra-meal pauses. The initiation of a meal began after the insect approached the food and then sampled it, either by palpating the surface or by taking a sample bite. It was considered to have terminated if the grasshopper was off the food and had not contacted it for more than 5 min (Simpson et al., 1995). If palpation or biting occurred but was not followed by ingestion within 5 min, a 'meal' length of 0 min was recorded.

Following the end of the first meal, the wheat was replaced with a leaf of spinach standing in a vial of water. As soon as

the first meal on spinach had been completed, mineral oil, containing one of three sterol treatments, was injected from a micrometer syringe into the insects. The three injection treatments were (1) no sterols, (2) 'soybean sitosterol' and (3) spinach sterols. The needle was inserted through an abdominal intersegmental membrane, taking care not to penetrate the alimentary tract, and 4.5 µl of solution was injected into each grasshopper (for grasshoppers receiving sterols, the dosage was 15 µg of sterol per injection). The sterols had been added to the mineral oil in a small volume of ether and then sonicated for approximately 10 min; an equal volume of ether was also added to the clean mineral oil. Each mineral oil solution was then put on a hot plate and placed under a fumehood to drive off the ether. The spinach sterols used in this and the following experiment were extracted from store-bought spinach, *Spinacia oleracea*, using methods previously described (Heupel, 1989), and were quantified using gas chromatography. After the injection, the insects were given a fresh leaf of spinach and observations continued until they had completed another meal.

If the aversion response to spinach develops because of post-ingestive effects from unsuitable sterols, feeding duration would be expected to decrease dramatically following the injection of spinach sterols. Comparisons of meal lengths among the different injection treatments were made using the nonparametric Mann–Whitney *U*-test. For the two different experiments, each treatment was replicated a minimum of 14 times.

To test for a direct feedback on mouthpart chemoreceptors (experiment 2)

This experiment tested whether direct nutrient feedbacks on mouthpart chemoreceptors might regulate sterol intake. Grasshoppers, both male and female, were transferred to 51 acrylic tubs as they moulted to the sixth stadium and were given high-quality food (Romaine lettuce and wheat bran) for 2 days; this treatment standardized their nutrient status. On day 3, grasshoppers were individually transferred to plastic boxes (21.5 cm long × 11 cm wide × 3.5 cm deep with screened ventilation holes at each end) that contained 2–3 leaves of spinach in a vial of water. This pretreatment deprived them of suitable sterols for a period of approximately 18 h. During these 3 days, grasshoppers were maintained under standard laboratory conditions. On the morning of day 4, at approximately 08:00 h, the grasshoppers were transferred to an observation room held at 31–33 °C.

For observations, individuals were removed to new plastic boxes (11 cm×11 cm×3.5 cm with screened ventilation holes on each end) that contained a single glass-fibre disc (Whatman GF/A) treated with 0.25 % sucrose (the control) or 0.25 % sucrose plus 1 % or 10 % sitosterol (all concentrations expressed with respect to the dry mass of the disc). Sitosterol, purchased from Sigma Chemical (St Louis, MO, USA), was derived from soybean and was a mixture of 60 % sitosterol, 27 % campesterol (Δ^5) and 13 % dihydrobrassicasterol (Δ^5), all of which are suitable phytosterols for *S. americana* (Dadd,

1960). Throughout the rest of this study, we refer to this mixture as 'soybean sitosterol'. The 'soybean sitosterol' was dissolved in chloroform and applied to the discs in a volume of 100 µl using an automated Pipetman (this volume completely saturated a disc); the sucrose control discs were also treated with chloroform. Discs were mounted on inverted push-pins so that grasshoppers could easily access the disc edge and feed; the push-pins were secured to the arena floor using Sticky Tack.

All grasshoppers were observed continuously throughout their first meal. As in the previous experiment, a gap of non-feeding/off the food lasting more than 5 min was used as the criterion for the end of a meal. For all grasshoppers, the length of the first feeding bout of a meal, the total length of the first meal and the number of contacts made during the first meal were compared using the nonparametric Kruskal–Wallis test. At least 12 replicates were made for each treatment. If a direct nutrient feedback on mouthpart chemoreceptors is involved, it would be expected that sterol-deprived individuals should respond more positively to the discs with 'soybean sitosterol' compared with sterol-deprived individuals presented with sucrose only discs.

To test for post-ingestive feedbacks and associative learning (experiment 3)

If the grasshoppers cannot directly taste sterols, learning to avoid unsuitable sterols may depend on their ability to associate some independent character of the food, such as the taste or flavour, with the unsuitable sterols. This experiment was designed to determine the impact of food taste on the development of learned aversions to unsuitable sterols that are not directly tasted.

On the second day of the sixth stadium, female grasshoppers that had previously fed on Romaine lettuce and wheat bran were individually transferred to plastic boxes (11 cm × 11 cm × 3 cm with two screened ventilation holes). These grasshoppers were then fed for 2 days on a 7% protein/7% digestible carbohydrate artificial diet (Simpson and Abisgold, 1985) that contained 'soybean' sitosterol (0.2% dry mass). The diet was suspended in a 1% agar solution in a dry:wet ratio of 1:4 and presented to individual grasshoppers as small cubes. If grasshoppers did not eat the artificial diet freely, as determined by the production of faecal pellets at the end of the 2 day period, they were not used in observations. During this period, grasshoppers were maintained in a Percival growth chamber on a 16 h:8 h L:D photoperiod at 32 °C during the light phase and at 28 °C during the dark phase. They were given fresh diet cubes twice daily. Grasshoppers were moved on the morning of day 4 from the Percival growth chamber to an observation room held at 31–33 °C.

Observations of insect feeding were initiated at 08:00 h on each occasion, approximately 2 h after lights on. Feeding for all grasshoppers was first recorded on a fresh cube of 'soybean sitosterol' diet (meal 1), which was the same as that used during the previous 2 days; this was to establish that each insect was in a similar state of readiness to feed when presented with

the next type of food (meal 2). After each grasshopper had stopped feeding for 5 min, the 'soybean sitosterol' diet cube was removed and replaced with a new cube of diet containing spinach sterols (0.2% dry mass); all other ingredients remained identical. The lengths of the next two meals (meals 2 and 3), which were both on the diet with spinach sterol, were recorded. Five minutes after the end of meal 3, the spinach sterol diet was removed and replaced with one of four fresh diet cubes. These were (A) a spinach sterol diet (the control), (B) a 'soybean sitosterol' diet, (C) a spinach sterol diet with the chemical marker coumarin, or (D) a 'soybean sitosterol' diet with the chemical marker coumarin. The sterol concentration in each diet was 0.2% dry mass. Coumarin was added to the diet at a dry mass concentration of 0.5%; at this concentration, it is tasted by grasshoppers and is, in fact, deterrent relative to diets lacking coumarin (Kruskal–Wallis test: d.f.=4, H -value=25.787, $P < 0.01$; Fig. 2). The lengths of the next two meals (meals 4 and 5) were then recorded. A representation of the full experimental design is shown in Fig. 3.

For each treatment, the time spent feeding (s) for meals 2–5 was analyzed using a 2 × 2 repeated-measures analysis of variance (ANOVA) test with sterol (suitable or unsuitable) and flavour (none or coumarin) as the main effects; the analysis was performed using the software package SPSS 7.5. All data were log-transformed to meet the underlying assumptions required for ANOVA procedures. At least 14 replicates were obtained for each treatment.

Results

Detection of unsuitable sterols via post-ingestive feedbacks (experiment 1)

Two separate injection experiments were performed. The first experiment compared feeding responses on spinach following the injection of clean mineral oil or 'soybean sitosterol' (all suitable sterols). The second experiment compared the effects of injecting different phytosterols (either suitable or unsuitable) on feeding responses to spinach.

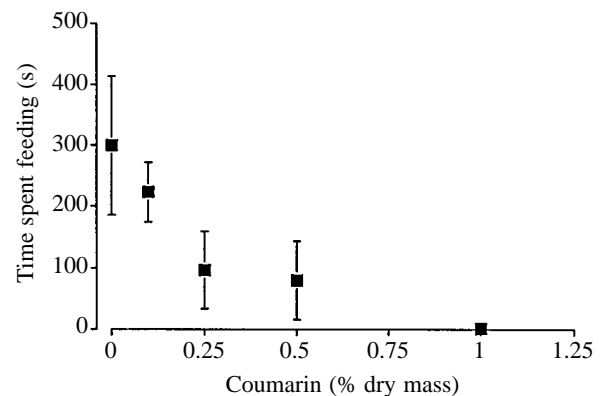
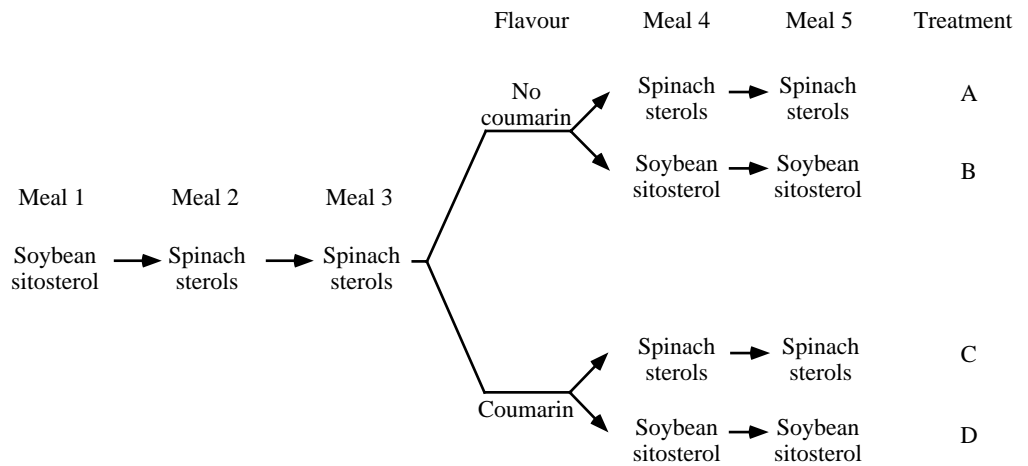


Fig. 2. Meal durations of *Schistocerca americana* in response to different concentrations of coumarin. Three- and four-day-old grasshoppers were used, and each treatment had a minimum of 10 replicates. Data are presented as medians ± M.A.D.

Fig. 3. The experimental protocol used to determine the impact of food taste and dietary sterol on the development of the learned aversion to spinach sterols. All grasshoppers received the same diet sequence for the first three meals. At meal 4, the spinach sterol diet was replaced with a new diet that contained one of four sterol/flavour combinations. The 'novel' flavour used in this experiment was coumarin. Letter codes, shown in the far right column, are assigned to each treatment and are used in the text to refer to the different diet sequences.



As assessed using the Mann–Whitney *U*-test (MWU), the length of the initial pretest meal on wheat (meal 1) did not differ between injection groups for either the first (MWU, $Z=-1.562$, $P=0.118$) or the second (MWU, $Z=-0.569$, $P=0.569$) experiment. Similarly, there was no difference in the length of the first meal on spinach (meal 2) in either the first (MWU, $Z=-0.873$, $P=0.383$) or the second (MWU, $Z=-0.759$, $P=0.448$) experiment.

Following the second meal (spinach meal 1), individuals received their injections. In the first experiment, there was no difference in the length of the third meal (spinach meal 2) between grasshoppers injected with clean mineral oil or 'soybean sitosterol' (MWU, $Z=-0.184$, $P=0.854$; Fig. 4A). In the second experiment, however, the third meal (spinach meal 2) was greatly reduced in insects injected with the spinach sterol compared with those injected with 'soybean sitosterol' (MWU, $Z=-4.102$, $P<0.01$; Fig. 4B). Grasshoppers injected with spinach sterols had median meal lengths of only 3.3 ± 1.8 s compared with 82.3 ± 24.0 s (medians \pm median absolute deviation, M.A.D.) for grasshoppers given the 'soybean sitosterol' injection.

A direct feedback on mouthpart chemoreceptors (experiment 2)

Sixth-stadium *S. americana* nymphs, pretreated with spinach, were fed on glass-fibre discs soaked with 0.25%

sucrose with or without various concentrations of added 'soybean sitosterol'. The median length of the first feeding bout of a meal, the median length of the first meal and the median number of contacts on the control disc and discs with 1% or 10% 'soybean sitosterol' did not differ significantly from one another (Table 1).

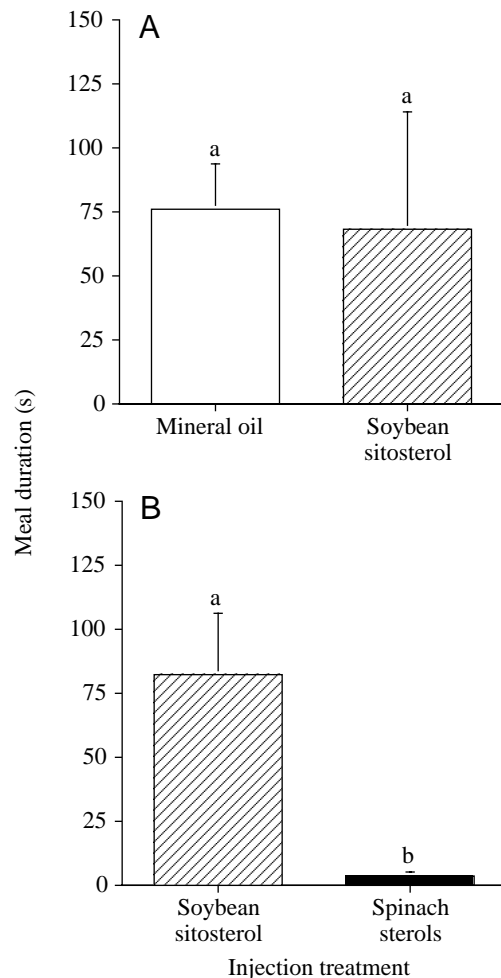


Fig. 4. Length of time spent feeding on spinach after different sterols had been injected into the haemolymph of *Schistocerca americana*. Prior to the injections, grasshoppers were given a meal of wheat, which was then followed by a meal of spinach (the data from these meals are reported in the Results). (A) A comparison of spinach meal durations of grasshoppers injected with either clean mineral oil (open column) or 'soybean' sitosterol (hatched column). (B) A comparison of spinach meal durations of grasshoppers injected with either 'soybean' sitosterol (hatched column) or spinach sterols (filled column). Meal lengths are presented as medians with M.A.D., and different letters above the columns indicate significant differences between the two injection treatments within a figure part.

Table 1. Feeding behaviour of *Schistocerca americana* in response to different concentrations of 'soybean' sitosterol applied to glass-fibre disks

Feeding behaviour	Sucrose control	Sitosterol		Kruskal–Wallis test	
		1 %	10 %	d.f.	<i>P</i> -value
First bout length (s)	83.8±58.0	60.0±46.1	102.7±53.1	2	0.510
First meal length (s)	223.0±130.5	201.2±102.8	217.9±97.3	2	0.913
Number of contacts during the first meal	3.0±1.0	3.0±2.0	2.5±1.5	2	0.969

All disks contained 0.25 % dry mass of sucrose.
Prior to the test, grasshoppers were fed spinach for 18 h.
Values are presented as medians (±M.A.D.) and were analyzed using the nonparametric Kruskal–Wallis test.

Post-ingestive feedbacks and associative learning (experiment 3)

As assessed using the Kruskal–Wallis test (KW), there was no significant difference in the length of the first meal ('soybean sitosterol') among the four treatment groups (KW, d.f.=3, *H*-value=2.018, *P*=0.569). The length of the next meal (meal 2), which was the first meal on the spinach sterol diet, also did not vary significantly among the four treatment groups (KW, d.f.=3, *H*-value=2.340, *P*=0.505). Since there were no significant differences in meal lengths within the first or second meal, data for both meal 1 and meal 2, respectively, were pooled; the first and second meal lengths were then compared to test whether there was an immediate aversion to the spinach sterol diet. There was no significant difference in meal lengths between the standardizing 'soybean sitosterol' diet and the first meal on the spinach sterol diet (Wilcoxon's Signed Rank Test, *Z*=-0.520, *P*=0.603); the median meal length on the 'soybean sitosterol' diet was 6.0±2.8 min, while the median meal length on the first spinach sterol diet was 6.4±2.4 min (medians ± M.A.D.).

Since there was no immediate reduction in feeding duration on the first spinach sterol diet (meal 2) compared with the 'soybean sitosterol' standardizing diet (meal 1), we proceeded to analyze the pattern of feeding across meals 2–5, using the first meal on the spinach sterol diet as the starting point. Neither dietary sterol nor flavour significantly affected the meal duration when this variable was averaged over meals 2–5 (Table 2). Similarly, no significant interaction between sterol type and flavour was observed. The repeated-measure analysis did, however, detect a significant difference in meal duration among the last four meals. Feeding duration decreased from meal 2 to meal 3, but then increased over the last two meals; polynomial contrasts indicated a significant quadratic trend. More interestingly, however, significant interactions of meal number with both flavour and sterol type were observed (Table 2). First, grasshoppers given diets containing coumarin increased their feeding duration at meal 4 compared with those maintained on diets that had a similar taste to that in meals 2 and 3 (Fig. 5A,B); the difference in meal duration was, however, less dramatic in meal 5 compared with meal 4. Additionally, grasshoppers that were switched to diets containing sitosterol at meals 4 and 5 showed increased feeding

Table 2. Results from the repeated-measures ANOVA test measuring time spent feeding over four successive meals

Source	d.f.	Sum of squares	<i>F</i> -value	<i>P</i> -value
Sterol	1	2.177	3.201	0.079
Flavour	1	1.999	2.938	0.092
Sterol × Flavour	1	0.992	1.459	0.232
Subject (group)	61	41.496		
Meal number	3	21.287	18.207	**
Contrast (linear)	1	5.964	15.303	**
Contrast (quadratic)	1	11.633	29.852	**
Meal number × Sterol	3	3.337	2.854	*
Meal number × Flavour	3	4.062	3.474	*
Meal number × Sterol × Flavour	3	0.733	0.627	0.598
Meal number × Subject (group)	181	71.317		

In all four treatments, the first two meals were on an artificial diet that contained spinach sterols; the next two meals were on artificial diets that contained different sterols and/or flavours depending on the treatment (see Fig. 3 for a review of the experimental protocol).
Coumarin was added to the diet at meal 4 to give it a 'novel' flavour.
P*<0.05; *P*<0.01.

compared with grasshoppers maintained on diets with spinach sterols (Fig. 5A,B). The difference in meal duration was greatest at the fifth meal, where grasshoppers feeding on diets with sitosterol, regardless of flavour treatment, had meals that were, on average, 71 s longer than those of grasshoppers fed diets with spinasterol.

Discussion

The results from this study suggest that unsuitable phytoosterols, but not suitable ones, play a role in the regulation of grasshopper feeding behaviour and that they exert their effects post-ingestively. Grasshoppers injected with spinach sterols had significantly shorter meal lengths and more rejections of their second spinach meal (13 out of 18 individuals took meals lasting less than 5 s) than did

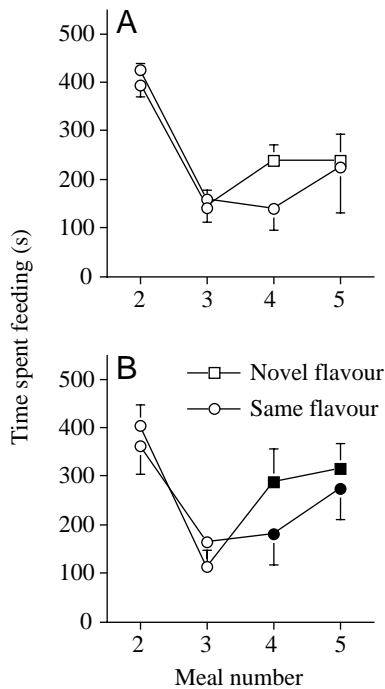


Fig. 5. Meal durations of *Schistocerca americana* over four successive meals on artificial diet containing different combinations of sterols/flavours. Meal lengths are expressed as the mean time (in seconds) spent feeding during each meal; values are means \pm S.E.M.; statistical results are presented in the text. Meals 2 and 3, in A and B, were on diets containing spinach sterols (open circles). (A) Feeding responses of grasshoppers maintained on diets of spinasterol; at meals 4 and 5, however, coumarin was added to one of the diets to give it a novel taste (open squares). (B) Feeding responses of grasshoppers switched to diets containing 'soybean' sitosterol at meals 4 and 5 (filled circles and squares); additionally, coumarin was added to one of these diets to give it a novel taste (filled squares). See Fig. 3 for a description of the experimental protocol.

grasshoppers injected with either 'soybean sitosterol' or clean mineral oil. Studies with different grasshopper species indicate that unsuitable phytosterols exhibit toxic effects and that these effects are manifested in performance measures after one or two stadia (Behmer, 1998). It would therefore be adaptive for grasshoppers to respond to the presence of unsuitable phytosterols in their food.

The results from the second experiment suggest that a direct sterol feedback, *via* mouthpart taste receptors, is unlikely. None of the behavioural variables measured in the second experiment showed any significant differences between the 'soybean sitosterol' discs and the sucrose control discs. If grasshoppers were using a direct feedback to detect suitable phytosterols, it might have been expected that giving them unsuitable ones overnight would increase the sensitivity of gustatory receptors on the mouth and palps and thus increase their propensity to feed on the sitosterol-treated discs (Simpson and Simpson, 1990). This lack of a positive response to suitable phytosterols is consistent with the previous studies of Cook (1977). Similarly, Champagne and Bernays (1991) found that, when

discs containing suitable sterols but no sucrose were presented to spinach-fed grasshoppers, the discs did not appear to be recognized as food. They also showed that cholesterol, when combined with a low concentration of sucrose, did not significantly affect acceptability. This led them to suggest that a synergistic interaction between the sterols and sucrose was unlikely and that chemoreception did not play a role in the initial recognition of usable sterols. In the same study, however, they present data indicating that spinach-fed grasshoppers preferentially fed on discs containing suitable sterols with 5% sucrose compared with sucrose only controls. It needs to be stated, though, that the preference was typically shown when suitable sterol concentrations were very high (i.e. 10%, which is never observed in plants) and that the interpretation of their data may be somewhat tenuous because in some instances inappropriate statistical analyses were employed and there were discrepancies in the presentation of statistical results.

It also appears that grasshoppers are unable to taste unsuitable phytosterols directly. In our last experiment, there was no immediate decrease in meal length when grasshoppers were given artificial diets with spinach sterols following meals on 'soybean sitosterol' diets. In addition, Champagne and Bernays (1991) found that pretreating grasshoppers with spinach does not affect the acceptability of unsuitable sterols; meal durations on glass-fibre discs with sucrose and stigmasterol (an unsuitable sterol) were not shorter than those on sucrose control discs. Currently, most of the evidence suggests that sterols (both suitable and unsuitable) are not tasted directly by grasshoppers, particularly when they are presented singly. Electrophysiological studies, however, will be required to resolve this question unequivocally.

The injection experiments suggest that associative learning may be playing a role in the aversion response towards unsuitable phytosterols. In associative learning, experience results in an animal being able to link a stimulus having no specific meaning (i.e. it is neutral) with some meaningful positive or negative effect (Bernays, 1995). For the grasshoppers in the injection experiments, unsuitable phytosterols would have been the meaningful effect, whereas the neutral stimulus that came to be associated with them was some property of the spinach leaf, probably taste since previous work has shown spinach to have strong gustatory cues (Lee and Bernays, 1988). In a similar experiment, also using *S. americana*, associative learning was implicated as the primary mechanism explaining food aversion behaviour (Lee and Bernays, 1990). When grasshoppers were given a meal on spinach and then injected with noxious plant secondary compounds, the duration of the following spinach meal decreased significantly compared with the first meal. If, however, the grasshoppers were given broccoli following the injection, the meal duration on broccoli compared with that of the first spinach meal did not decrease significantly. It was suggested that grasshoppers in this experiment learned to associate the taste of spinach with the noxious effects that followed the injection of the plant secondary compounds. It is possible that grasshoppers in the current experiment were

temporarily 'sickened' by the injection of unsuitable sterols and that this may have reduced the length of the subsequent meal on spinach. Grasshoppers receiving the injection of unsuitable sterols, however, behaved similarly to grasshoppers from the other injection treatments except with regard to feeding behaviour. The immediate rejection of foods following the injection of unsuitable sterols suggests, to us, that a rapid learned association between the presence of unsuitable sterols and the taste of spinach is the most likely explanation for the strong aversion response.

That taste is a key component in the development of the aversion response to unsuitable phytoosterols is seen in the third set of experiments. Across all the different treatment combinations, grasshoppers developed an aversion response to the artificial diet containing spinach sterols after only a single meal; this result is consistent with other studies examining behaviour in response to unsuitable phytoosterols in both plants (Champagne and Bernays, 1991) and artificial diets (Behmer and Elias, 1999a). When the taste, or flavour, of the diet was modified at meal 4 by adding coumarin, however, feeding immediately increased compared with feeding on diets where the taste remained similar to that of meals 2 and 3. This increased feeding occurred even though coumarin at this concentration is normally a feeding deterrent (see Fig. 2). Under the guise of associative learning, we suggest that the decreased feeding duration observed from meal 2 to meal 3 was because the unsuitable spinach sterols came to be associated with the taste of the artificial diet. We suggest that the presence of a novel flavour (coumarin) in the diet at meal 4 disrupts the link between the meaningful stimulus (the unsuitable phytoosterols) and the conditioned stimulus (the old taste of the diet). That feeding increased significantly even when spinach sterols remained (no meal number \times sterol \times flavour interaction) suggests that the novel flavour is being used by the insects to signal that a new food has been encountered. Novelty has been suggested as an important aspect of food acceptability in the highly polyphagous grasshopper *Taeniopoda eques* (Bernays et al., 1992; Raubenheimer and Bernays, 1993).

Finally, dietary sterol can influence feeding independently of flavour. The significant meal number \times sterol interaction indicates that, when unsuitable sterols in the diet are replaced with suitable ones, meal duration increases with time. If the presence of unsuitable sterols in the haemolymph suppresses feeding, as the current data from the injection experiments suggest, feeding on diets containing suitable phytoosterols after having fed on diets with spinach (unsuitable) sterols should reduce the concentration of unsuitable sterols in the haemolymph; this dilution effect would act to dampen any negative feedback that would otherwise suppress feeding. It is interesting to note that feeding in meals 4 and 5 never recovered fully to the levels observed in meals 1 and 2, regardless of the sterol/flavour combination; this finding is consistent with the idea that a carry-over of unsuitable phytoosterols in the haemolymph from the first two meals could have a negative impact on feeding in later meals. It would be

interesting to measure how long this effect might manifest itself and to what extent it represents the toxicity of the ingested unsuitable sterols.

The results from this study also raise some interesting questions that warrant discussion and perhaps further investigation. First, which sterols are absorbed and how quickly are they absorbed following ingestion? Previous studies have demonstrated that the midgut is the main site of absorption in most phytophagous insects (Joshi and Agarwal, 1977), but it has been proposed that grasshoppers may not absorb unsuitable phytoosterols (Dadd, 1960). Recent results from long-term studies using *S. americana*, however, indicate that unsuitable phytoosterols are absorbed without being metabolized (Behmer, 1998). With regard to absorption times, previous work with the grasshopper *Melanoplus sanguinipes* (Champagne, 1990) and the caterpillar *Helicoverpa zea* (Kuthiala and Ritter, 1988) has suggested that it takes 3 h for suitable dietary sterols to be taken up across the midgut and detected in the haemolymph. The aversion response to the unsuitable spinach sterols in the current experiments, however, developed after only one meal; this suggests that sterol absorption may occur more rapidly than has been previously reported. A second interesting question is how are unsuitable phytoosterols detected once they reach the haemolymph? One possibility is that a nutrient deficit may develop, producing an imbalance of neurotransmitters in the central nervous system (CNS) (Cohen et al., 1988). It seems unlikely, however, that a substantial sterol deficit could develop after a single meal. A second possibility is that grasshoppers may simply be responding to the general toxic effects that unsuitable sterols exhibit following absorption. A third, and perhaps more likely, possibility is that unsuitable phytoosterols released into the haemolymph are detected internally and/or act directly on some unspecified region of the CNS.

Regulating the intake of unsuitable phytoosterols, but not of suitable ones, may not be altogether surprising considering the sterol requirements of grasshoppers and the distribution of sterols in plants. First, grasshoppers have a dietary sterol requirement of approximately 0.05% dry mass (Dadd, 1960; Behmer, 1998), while most plants have total phytosterol concentrations ranging from 0.05 to 0.1% dry mass (Nes and McKean, 1977), these often consisting of largely suitable sterols (Nes et al., 1977; Patterson, 1994). It is likely, therefore, that grasshoppers may obtain their minimum sterol requirement by chance alone. Interestingly, it does not appear that exceeding the minimum requirement enhances survival and/or performance (Behmer and Elias, 1999b); perhaps once dietary sterols have been allocated for structural purposes there is no further benefit to ingesting larger quantities. Further work, however, is required to examine whether additional quantities enhance fecundity. In contrast, regulating the intake of unsuitable phytoosterols would reduce the likelihood of deleterious effects associated with the accumulation of unsuitable phytoosterols in the body. When *S. americana* were reared on diets containing mixtures of suitable and unsuitable sterols, they failed to complete development (Behmer and

Elias, 1998b); this occurred even when suitable phytosterols were present at concentrations that alone would support normal growth. The results from the present study, when combined with results from other studies, strongly suggest that the role of unsuitable phytosterols as a potential factor influencing host-plant affiliations may have been underemphasized, particularly in those phytophagous insects, such as grasshoppers, that have limited sterol metabolic capabilities.

We thank D. Byrne, R. F. Chapman, S. Simpson, M. Singer (Arizona) and an anonymous reviewer for thoughtful criticisms and suggestions on the manuscript. R. Grebenok assisted greatly in the collection of the spinach sterols. 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.

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