Functional Ecology

British Ecological Society

Functional Ecology 2011, **25**, 484–491 doi: 10.1111/j.1365-2435.2010.01810.x

Plant sterols and host plant suitability for a phloem-feeding insect

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Summary

1. Arthropods, including insects, are unique among animals in that they cannot synthesize sterols, including cholesterol, *de novo*. Some phytophagous insects (e.g. caterpillars, grasshoppers) generate tissue cholesterol by metabolizing plant phytosterols. Currently, little is known about sterols in plant phloem sap, and their significance to the nutritional physiology and ecology of phloem-feeding insects.

2. The sterol profiles of leaves from two plant species, Chinese cabbage and tobacco, were dominated by the phytosterols sitosterol and stigmasterol, respectively. In contrast, the principle sterol in the phloem sap of both plants was cholesterol, which is traditionally considered an animal sterol. Cholesterol was also the most abundant sterol in the carcass and honeydew of *Myzus persicae* aphids feeding on these plants.

3. The effect of sterol structure on *M. persicae* was investigated using modified tobacco plants that contained high levels of atypical steroids, specifically ketone-steroids. Aphids reared on the modified tobacco plants had a high atypical steroid content, severely reduced reproduction and high mortality.

4. Our data indicate that the total sterol composition of plants is not necessarily representative of the sterol profile available to phloem-feeding insects, and that the sterol utilization patterns of phloem-feeding insects may differ from chewing insect herbivores utilizing the same plant. Atypical steroids are naturally at insufficient concentrations for significant deleterious effects on insect herbivores, and possible reasons why plants apparently do not use them as defensive compounds are considered.

Key-words: nutritional ecology, sterol nutrition, cholesterol, phloem sap, aphid, *Myzus persicae*, Hemiptera

Introduction

Sterols pose interesting but relatively little-studied problems for the nutritional ecology of herbivores. They are essential components of cell membranes, and serve as hormone precursors and signalling molecules in both plants and animals (Behmer & Nes 2003). However, their profiles in plants and animals are very different. Cholesterol (Fig. 1a) is usually the dominant animal sterol. Plants also often contain a small amount of cholesterol, but their dominant sterols are phytosterols, which are variants of cholesterol. Cholesterol and phytosterols share the same basic tetracyclic ring structure and a side chain at C17, but the side chain in phytosterols is alkylated at C24 with a methyl or ethyl substituent (e.g. sitosterol, Fig. 1b) and some phytosterols, e.g. stigmasterol, also have double bonds (Fig. 1c). Sterol composition varies among plants, most notably at the family level (e.g. Nes *et al*. 1977; Salt *et al*. 1991).

A further issue shaping the ecology of sterol nutrition in herbivores is that the nutritional significance of phytosterols to animals differs fundamentally between arthropods, including insects, and other animal groups because arthropods, uniquely among animals, are unable to synthesize sterols *de novo* (Grieneisen 1994; Behmer & Nes 2003). Most insects are, consequently, dependent on an exogenous source of sterols. Exceptionally, some insects (e.g. certain planthoppers and beetles) derive sterols from fungal endosymbionts (Behmer & Nes 2003); bacterial symbionts are not capable of

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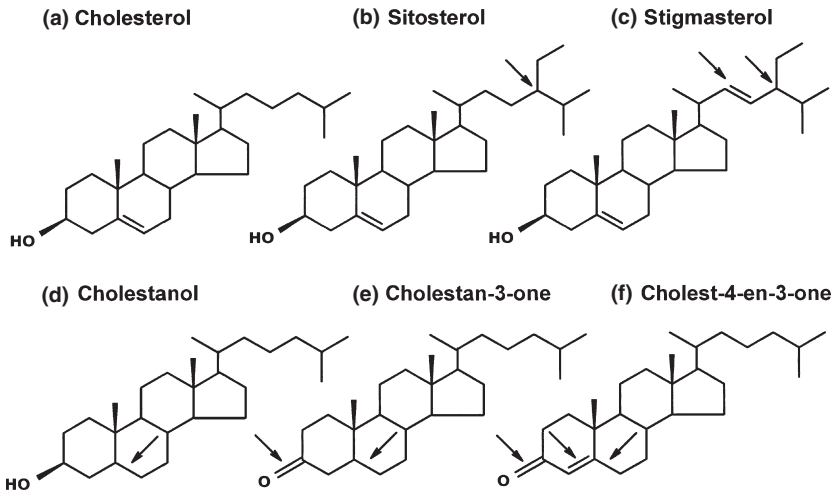

Fig. 1. Sterols and sterol metabolites of insects and plants. Cholesterol (a) is the common sterol in most insects, but is generally not found in plants in large quantities. Instead, plants contain cholesterol analogues (the arrows on each structure indicate differences from cholesterol). Sitosterol (b) and stigmasterol (c), two common phytosterols, each contains an ethyl group on the side-chain (at the C24-position). Stigmasterol also contains a double bond at the C22-position. The three steroids in the bottom row are all present in our modified tobacco plants. Cholestanol (d) is similar to cholesterol but it lacks a double bond at the C5-position (it is saturated). Cholestan-3-one (e) and Cholest-4-en-3-one (f) both have a ketone at the C3-position, and both lack a double bond at the C5-position. They differ in that Cholest-4-en-3-one has a double bond at the C4-position.

sterol synthesis (Douglas 2009). Herbivorous insects, such as caterpillars and grasshoppers (both of which have tissue sterol composition dominated by cholesterol), can subsist on plant diets with negligible cholesterol content because they can dealkylate phytosterols, such as sitosterol and stigmasterol, to produce cholesterol (Nes *et al.* 1997; Behmer, Elias & Grebenok 1999). Some insects can also use phytosterols in place of cholesterol in membranes, thereby achieving dietary sparing of their cholesterol requirement, although this capacity varies among species (Behmer & Nes 2003). Vertebrates, by contrast, have no dietary requirement for sterols, although they can supplement cholesterol of metabolic origin by assimilating ingested cholesterol from animal-derived dietary components. Most of the phytosterols in plant material ingested by vertebrates is voided without modification (Ostlund 2002, 2007).

Plants additionally contain small quantities of two non-sterol classes of steroids : stanols (Fig. 1d), which lack double bonds in the sterol nucleus (they are considered atypical), and 3-ketone-steroids in which the hydroxyl group at C3 is replaced by a ketone group (Fig. 1e); some ketone-steroids additionally contain a double bond in the sterol nucleus (e.g. Fig. 1f). Animals appear to be uniformly unable to introduce a double bond into the 5-position in the B-ring, but the functional consequence of this metabolic constraint differs between vertebrates and insects. The net absorption of dietary stanols by mammals, including humans is low (reviewed by Ostlund 2002). Additionally, stanols (particularly sitostanol) interfere with the uptake of Δ^5-sterols, including cholesterol, from the digestive tract in mammals (Katan *et al.* 2003). At levels naturally occurring in plant material, inhibition is trivial, but research on human volunteers indicates that a daily dose of 2 g stanols (40–100 times the unsupplemented intake) reduces uptake of dietary cholesterol from the intestine by 30–40% and reduces the LDL-cholesterol levels in serum by up to 20% (Katan *et al.* 2003; Mcgowan & Proulx 2009). Stanol supplements and stanol-fortified foods are currently sold for their reputed serum cholesterol-reducing effects.

In contrast to mammals, insects readily absorb and incorporate atypical steroids into their tissues (reviewed by Behmer & Nes 2003), and dietary sterol/steroid uptake in insects is non-selective (Behmer, Elias & Grebenok 1999). Therefore, the uptake of a given sterol/steroid increases with its dietary concentration. Some insects (e.g. *Drosophila melanogaster*, dermestid beetles and cockroaches) can tolerate dietary stanols as long as a small quantity of dietary cholesterol is available for the production of ecdysteroid, the common insect molting hormone (Clark & Bloch 1959a,b; Kircher & Gray 1978). Other insects, particularly insect herbivores [e.g. the caterpillar *Helicoverpa zea* (Nes *et al.* 1997)], are deleteriously affected by dietary stanols.

These key differences in the nutritional ecology of sterols and stanols between vertebrates and insect herbivores raise the possibility that plants supplemented with atypical steroids could confer resistance to major insect crop pests while being harmless to vertebrates and even providing health benefits for consumers. There are, however, several fundamental lacunae in our understanding. In particular, the fate of ketone-steroids in animals is not well understood; we are unaware of any published study on the capacity of animals to reduce the C3-ketone to a hydroxyl group. Additionally, all the research on the nutritional ecology of sterol utilization by herbivorous insects has been conducted on chewing taxa, especially lepidopteran caterpillars and orthopteroids. Little is known about the sterol nutrition of plant sap feeding insects, despite their importance in terrestrial food webs and as agricultural

pests (Dixon 1998; Van Emden & Harrington 2007). Phloem-feeding aphids have some limited capacity to dealkylate the phytosterol sitosterol (Campbell & Nes 1983), but their response to atypical steroids has not been investigated.

This study is the first analysis of the nutritional ecology of sterol utilization by plant sap-feeding insects. The specific objectives were: (i) to identify the dominant sterols available in phloem sap; and (ii) to determine the impact of plants with high atypical steroid levels on aphids. The experiments were conducted on the green peach aphid, *Myzus persicae*, and made use of Chinese cabbage and tobacco plants including a genetically modified tobacco line that produces high levels of atypical steroids.

Materials and methods

PLANTS AND APHIDS

The plants were pre-flowering Chinese cabbage (*Brassica rapa*) cv. Wingbok, and tobacco (*Nicotiana tabacum*) cv. Samsum. The genetically modified tobacco plants were transformed with the vector pMON20931, which carries the mature 3-hydroxysteroid oxidase gene (*choM*) of *Actinomyces* sp. A19249, translationally fused at the N terminus to the chloroplast-targeting peptide sequence CTP1 and under transcriptional control of the Arabidopsis SSU promoter. The control tobacco plants bore the empty vector (Corbin *et al.* 2001).

The green peach aphid *M. persicae* (Sulzer) clone HRI was derived from a single parthenogenetic female on a *Brassica oleracea* crop at Warwick Horticultural Research International, UK in October 2006, and maintained on Chinese cabbage. All plant and aphid cultures were raised at 20 °C with 18L : 6D at 500 μmol m^{-2} s^{-1} photosynthetically active radiation.

SAMPLE COLLECTION

Samples for sterol analysis were obtained from the top fully expanded leaf of plants and aphid colonies (mixed age) that had been feeding on this leaf for 3–7 days. Phloem sap samples were obtained by the EDTA exudation technique of King & Zeevaart (1974), following a protocol (Karley, Douglas & Parker 2002) that minimizes nonspecific metabolite leakage from plant cells. Briefly, the leaf was excised and inserted immediately into 0·2 mL 5 mM EDTA solution, pH 7·5. The samples were incubated for 90 min in the dark in a sealed chamber equilibrated at 25 °C with a dish of saturated KH_2PO_4 to maintain high humidity. The EDTA samples were frozen at −20 °C prior to analysis. Aphids were restricted to mesh-covered clip-on leaf cages (internal diameter 2·5 cm), lined with tinfoil and attached to the abaxial surface of the leaf. The honeydew produced over 2 days was washed off in 100 μL water and frozen at −20 °C. Prior to preparation for sterol extraction all leaf samples and aphid carcass samples were dried at 50 °C for 3 days.

APHID PERFORMANCE ANALYSES

Two experimental designs were adopted using tobacco plants at the 10-leaf developmental stage. The control and modified tobacco plants are phenotypically indistinguishable (Heyer *et al.* 2004), and so the identity of every plant as either control or modified was confirmed from the sterol profile of a test leaf.

The first experiment was designed to assess the suitability of tobacco plants for larviposition and larval performance of *M. persicae*. Fifteen adult apterae from the routine culture on Chinese cabbage plants were individually administered to the abaxial surface of leaves (one aphid per plant) in a clip-on cage, and checked daily for up to 7 days. All but one larva were removed, and the remaining offspring was monitored until it reached adulthood or died.

The second experiment investigated the response of aphids at the population level on the control and modified tobacco plants. Single leaves of Chinese cabbage plants bearing a dense aphid infestation (100–200 aphids) were excised and draped over the top fully expanded leaf of control ($n = 3$) and modified plants ($n = 4$) for 5–6 h, and the movement of aphids to the test plants was assessed. Each plant was bagged (to isolate it) and 7 days later the number of live and dead aphids on each plant was quantified. The percentage of live aphids was scored as a conservative estimate of aphid survivorship, recognizing that some dead aphids might have fallen from the plant to the substratum and been excluded from the analysis.

STEROL ANALYSIS

Plant tissue, phloem exudates and aphid samples were disrupted with 30 mL 95% ethanol and 8, #5 glass beads (Sigma, St Louis, MO, USA) in a modified paint shaker, shaking at a maximum rate for at least 5 min. Subsequently, the samples and ethanol extracts of honeydew were incubated in the dark at room temperature for at least 24 h. All samples were extracted three times with 95% ethanol followed by three extractions with chloroform. Extracts from each sample were combined and evaporated to dryness under nitrogen. The dried residue was resuspended in three volumes of 70% methanol : water and three volumes of methanol : water equilibrated hexane, with the sterols partitioning into the methanol : water-equilibrated hexane. Following evaporation of the hexane fraction to dryness under nitrogen, sterols were resuspended in a 1 mL volume of hexane and concentrated via normal phase high-pressure liquid chromatography (HPLC) performed on an Econosil SI, 5 micron, 250 mm × 4·6 mm column (Alltech, Nicholasville, KY, USA) and eluted with hexane/isopropyl alcohol (9 : 1, 1 mL min^{-1}) at 38 °C, using a Waters 717 Autosampler, 510 pump and 486 tunable wavelength detector, set at 205 nm. The sterols were concentrated by the injection of five 0·1 mL aliquots from each 1 mL sample with repeated common collection. Sterols coeluting with known standards were collected for further processing by gas chromatography-mass spectroscopy (GC-MS), with sterols reported in this study eluting within the first 18 min of injection on the HPLC. The GC-MS was accomplished using an Agilent 6850N GC coupled with a 5973 mass selective detector (Technologies, Inc., Santa Clara, CA, USA). The GC-MS was equipped with a fused capillary EC-5 column (30 m) (Alltech) with a 0·25 mm internal diameter and 0·25 μm film thickness. The running conditions were: inlet 260 °C, transfer line 280 °C, column 80 °C (1 min), ramp at 10 °C min^{-1} to 300 °C, 300 °C (20 min), with helium (1·5 mL min^{-1}) as carrier gas. The Agilent 5973 mass selective detector maintained an ion source at 250 °C and quadrupole at 180 °C. Fractions containing sterols obtained from the HPLC were evaporated to dryness and resuspended in 1 mL of anhydrous ethyl ether with repeated sonication. The ether fraction was evaporated to a 50-μL volume under nitrogen and a 2-μL volume was removed for injection on the GC-MS. All sterols isolated and examined by the above procedure were without conjugation and no conjugated sterols were examined in this study. Sterols were examined on the GC-MS without derivatization, and were identified and quantified by GC-MS

using Selected Ion Monitoring protocols for each steroid identified including (selected ions examined): cholesterol (386, 353, 301, 275), campesterol (400, 385, 315, 255), stigmasterol (412, 351, 299, 271, 255), sitosterol (414, 399, 396, 303, 275, 255), cholest-4-en-3-one (384, 342, 229, 124), cholestan-3-one (386, 231), campestan-3-one (400, 314, 231), sitostan-3-one (414, 231) and stigmast-4,22-diene-3-one (410, 367, 298, 271) (Rahier & Benveniste 1987). Quantification of specific sterols was based upon standard curves made to cholesterol, cholestan-3-one, cholest-4-en-3-one and cholestanol, with a limit of detection of the various steroids equalling 50 nanograms of material. No internal standard was used in this examination due to the small sample sizes examined. Authentic sterol standards were purchased commercially (Sigma and Steraloids).

STATISTICAL ANALYSIS

The sterol composition data (from leaves, phloem exudates, aphids and aphid honeydew) were not normally distributed, so where comparisons were made nonparametric tests were employed. However, because medians generally do not sum to 100, we present all the sterol composition data (Table 1) as adjusted medians. Here we normalized each tissue type, for each plant, by dividing the reported median value of a particular sterol type in a tissue by the sum of all sterol types in that tissue type. A sample calculation is given in Appendix S1 (Supporting information), and the raw data used to calculate the absolute medians and adjusted medians for each sterol for each tissue type, for each plant, is shown in Tables S1–S5 (Supporting information). The aphid performance data were normally distributed with homogenous variances, and were analysed by parametric tests (logistic regression and *t*-tests).

Results

LEAF AND PHLOEM STEROL PROFILES

The most abundant sterols in leaves of Chinese cabbage and control tobacco plants were phytosterols. Sitosterol (74%) was the dominant sterol in Chinese cabbage, while stigmasterol (54%) was the dominant sterol in tobacco (Table 1a and 1b, respectively). By contrast, the most abundant sterol in phloem exudates was cholesterol (40% in Chinese cabbage and 96% in control tobacco plants).

The total sterol profile of the modified tobacco plants bearing the 3-hydroxysteroid oxidase gene included atypical steroids; and these compounds were also detected in the phloem exudates (Table 1c). Overall, the atypical steroids accounted for 58% and 88% of total sterols in leaf extracts and phloem exudates, respectively. Most of this was 3-ketone-steriod, especially the cholesterol-derivative, cholest-4-en-3-one. Stanols were undetectable or barely detectable in both whole leaf and phloem exudate samples (Tables S1 and S2, Supporting information).

STEROL PROFILES OF APHIDS AND THEIR HONEYDEW

Cholesterol was the most abundant sterol in *M. persicae* aphids, and the % cholesterol content of the aphids did not vary significantly across the three rearing plants (Kruskal–Wallis test: d.f. = 2, $\chi^2 = 0{\cdot}67$, $P = 0{\cdot}715$). Atypical steroids were undetectable in the aphids on Chinese cabbage and control tobacco plants, but accounted for 31% of the sterols in aphids on the modified tobacco plants (Table 1; Tables S3 and S5, Supporting information).

Cholesterol was the most abundant sterol in the honeydew of aphids on Chinese cabbage and control tobacco plants (59% and 63%, respectively), but it was generally absent from the honeydew of aphids feeding on the modified tobacco plants (Table 1; Tables S4 and S5, Supporting information). Instead, atypical steroids dominated the sterol profile of honeydew collected from aphids feeding on modified tobacco (86%), with cholestan-3-one as by far the most abundant (58%).

APHID PERFORMANCE ON TOBACCO PLANTS

Myzus persicae formed robust cultures on the control tobacco plants and, if left unchecked, severely stunted plant growth [see Fig. S1 (Supporting information); control and modified plants grow at comparable rates and are phenotypically indistinguishable in the absence of aphids (Heyer *et al.* 2004)]. In contrast, aphids could not be sustained on the modified tobacco plants, which were generally aphid-free within 7–10 days of the initial infestation [Fig. S1 (Supporting information); in the absence of aphids the controls and modified plants exhibited similar growth, and this growth was similar to modified tobacco plants infested with aphids].

Two experimental designs investigated the basis of this striking difference in aphid performance on the two plant types. For the first experiment, adult aphids were caged individually to leaves of tobacco plants (see methods). Significantly more aphids produced offspring on the control plants than on the modified plants (logistic regression: $\chi^2 = 15{\cdot}64$, $P < 0{\cdot}001$; Fig. 2a). In total, 9/14 (64%) of the first larvae deposited by adult aphids on the control plants lived to the adult stage and reproduced, while all four of the offspring produced on the modified plants died as larvae.

Although the poor performance of the aphids on the modified plants was consistent with our routine observations (Fig. S1, Supporting information), the results could have been confounded by deleterious effects of clip-cages on plant physiology (Craft-Brandner & Chu 1999) or by constraining aphids to poor feeding sites on the modified plants. To circumvent these difficulties we let aphids select their preferred feeding sites freely in the second experimental design. When leaves of Chinese cabbage bearing dense colonies of *M. persicae* were draped over the target leaf of control or modified tobacco plants, the great majority of the aphids moved to the tobacco plant (in contrast, most aphids remained on excised Chinese cabbage leaves incubated in isolation). When the plants were re-examined 7 days later, the modified plants were found to bear many dead aphids. Expressed as % of the total number of aphids on the plants, the difference in numbers of dead aphids on the control and modified plants (Fig. 2b) was significantly different (one-tailed *t*-test: $t_5 = 3{\cdot}10$, $P = 0{\cdot}014$).

Table 1. Free-sterol profiles of plant leaf tissue, phloem exudates, and the carcass and honeydew of feeding aphids

	Sterol content [adjusted median % (w/w) of total in each tissue][†]								
	Phytosterols				Atypical steroids				
	Cholesterol	Campesterol	Sitosterol	Stigmasterol	Cholest-4-en-3-one	Cholestan-3-one	Campestan-3-one	Sitostan-3-one	Stigmast-4, 22-diene-3-ol
(a) Chinese cabbage									
Leaf tissue (4)	*	–	**74**	26	–	–	–	–	–
Phloem exudates (4)	**40**	–	32	28	–	–	–	–	–
Aphid carcass (4)	**50**	–	*	**50**	–	–	–	–	–
Honeydew (4)	**59**	–	11	30	–	–	–	–	–
(b) Tobacco (control)									
Leaf tissue (4)	15	15	16	**54**	–	–	–	–	–
Phloem exudates (3)	**96**	1	*	1	1	–	–	–	–
Aphid carcass (4)	**62**	5	31	2	–	–	–	–	–
Honeydew (4)	**63**	*	*	37	–	–	–	–	–
(c) Tobacco (modified)									
Leaf tissue (10)	13	14	1	3	**31**	12	11	8	4
Phloem exudates (9)	12	1	*	*	**76**	1	9	*	2
Aphid carcass (9)	**41**	28	*	*	–	31	–	*	*
Honeydew (6)	*	*	*	14	19	**58**	*	9	*

*Sterol/steroid was present in at least one sample, but that the median score for this particular tissue type was < 1%. The procedure for obtaining the median values is displayed in Supporting information.

[†]The most abundant sterol in each tissue is indicated in bold.

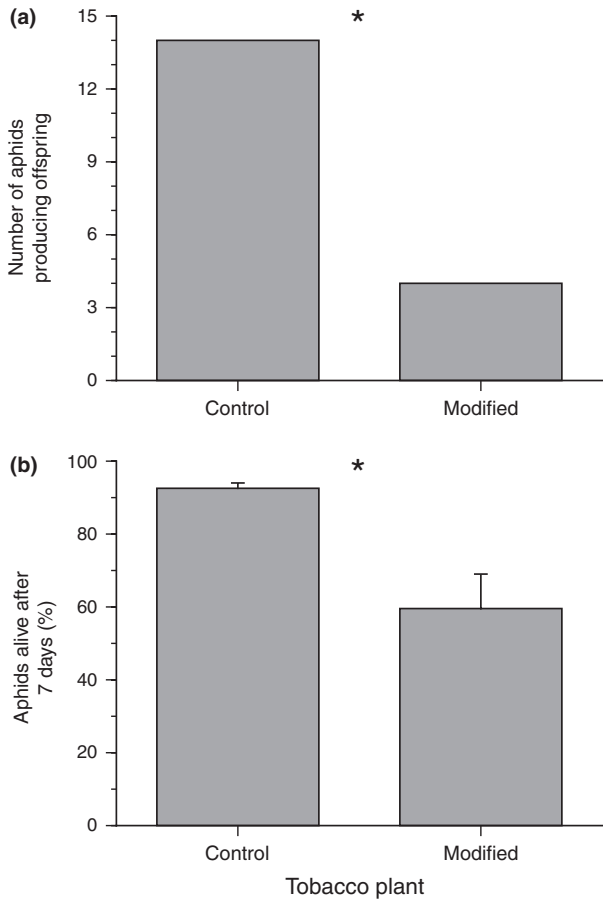

Fig. 2. Survival and reproduction of the green peach aphid *Myzus persicae* on control and modified tobacco plants. (a) percent of adult aphids that produced offspring ($n = 15$ for both treatments). *Statistically significant difference ($P < 0{\cdot}05$). (b) Percent survival (± SEM) of a mixed-age cohort of aphids after 7 days.

Discussion

Organic metabolites assimilated during photosynthesis are transported via the phloem sap in plant sieve elements (Van Bel, Ehlers & Knoblauch 2002; Kehr 2006). Phloem is also an important mediator of whole-plant communication (Ruiz-Medrano, Xoconostle-Cazares & Lucas 2001), with phytohormones (Baker 2000), proteins (Pearce *et al.* 1991) and RNAs (Jorgensen 2002) being examples of molecules involved in information transfer. Nevertheless, little effort has been invested in documenting phloem sterol composition, even though dietary sterols are crucial for the nutritional ecology of phloem-feeding insect herbivores (Behmer & Nes 2003). Our study indicates, somewhat surprisingly, that cholesterol is the dominant free sterol found in the phloem of plants from two phylogenetically different dicot plants, Chinese cabbage in the family Brassicaceae, and tobacco in the family Solanaceae.

The basis for the conclusion that cholesterol can be the dominant phloem-mobile sterol in plants requires consideration of methodological issues. Plant phloem sap is a challenging food source to study because it is restricted to plant sieve tubes (ca. 20 μm dia) that display rapid calcium-dependent sealing on damage. Sufficient material for detection of low abundance components, e.g. sterols, can be obtained by exudation into EDTA solution, which chelates Ca^{2+} ions and inhibits sealing (Knoblauch *et al.* 2001; Furch *et al.* 2007). Various studies have confirmed that EDTA exudates are generally representative of the composition, but not total concentration, of metabolites in phloem sap (King & Zeevaart 1974; Weibull, Ronquist & Brishammar 1990). Nevertheless, contamination of the exudates by contents of other plant cells can occur in some EDTA exudation protocols (discussed in Gaupels, Knauer & Van Bel 2008), resulting in convergence between the composition of the EDTA exudates and total plant tissue. Two lines of evidence indicate that this potential problem was minimal for our analyses. The first comes from the striking difference in the sterol composition of total leaf tissue and phloem exudates. Cholesterol accounted for nearly half and 90% of the sterols in the phloem sap of Chinese cabbage and the control tobacco plants, respectively, but just 4–16% of the leaf tissue, which was dominated by phytosterols, sitosterol and stigmasterol, respectively, confirming published studies (Nes *et al.* 1977). The second line of evidence comes from the similarity between the sterol composition of the phloem exudates and honeydew. Honeydew is aphid egesta from the alimentary tract, and it is dominated by compounds that have been ingested but not assimilated by the aphid. Thus, honeydew composition is a rough proxy for phloem sap composition. The concordance between the sterols in the EDTA exudates and the honeydew of aphids feeding on Chinese cabbage and control tobacco plants (Table 1) provides the strongest confirmation of the interpretation that cholesterol is the dominant sterol in the phloem sap of the plants tested in this study.

One interesting discrepancy in these comparisons relates to the aphids feeding on transgenic tobacco, the phloem exudates of which are dominated by the ketone-steroid cholest-4-en-3-one. This compound was also recovered from the honeydew, but undetectable in the aphid carcass. Both the aphid and aphid honeydew contained the related compound cholestan-3-one at one order of magnitude higher % content than in phloem sap, raising the possibility that cholest-4-en-3-one is metabolized to cholestan-3-one in the aphid gut, and that only the latter is transported into the aphid.

The implication of our results is that phloem-feeding and chewing phytophagous insects can encounter different profiles of sterols, even on the same plant, and that cholesterol, traditionally interpreted as an animal sterol, can be abundant in plant sieve elements. Our data also suggest that phloem-feeding insects utilizing the plant species that we have studied may have only a limited need to both dealkylate phytosterols and use phytosterols for sparing their cholesterol requirement. This interpretation is consistent with the relatively poor capacity of *M. persicae* to dealkylate sitosterol to cholesterol (Campbell & Nes 1983). Aphid species may, however, differ in their capacity for sterol dealkylation. Similarly, and as for other phloem nutrients (Douglas 2003), the sterol profile of plant phloem sap is likely to vary with plant age, environmental conditions and plant species. The reported

high phytosterol content in the phloem sap of rice plants (Shigematsu *et al*. 1982) illustrates among-plant species variation; and the dramatic difference between the sterol composition of two aphid species of *Uroleucon* feeding on *Solidago altissima* is indicative of substantial variation in one or both of sterol content within an individual plant and sterol utilization among related aphids species (Janson *et al*. 2009). These data suggest that sterols need to be considered as a factor shaping the ecology of aphids and other phloem-feeding insects. Specifically, plant resources, at every scale from the position of a leaf on a single plant, to plant species range, may be partitioned among aphid species according to compatibility between the plant sterol content and aphid sterol utilization traits (including requirement for dietary cholesterol, and capacity to utilize or dealkylate specific phytosterols). The importance of plant sterol profiles in the plant affiliation of chewing herbivorous insects has been demonstrated previously (Costet *et al*. 1987; Behmer & Grebenok 1998). This study raises the possibility that sterol nutrition is a generally important determinant of the nutritional ecology and plant utilization patterns of multiple feeding guilds of insect herbivores (Behmer & Elias 1999, 2000; Behmer & Nes 2003).

It has been established previously that some chewing insect herbivores perform very poorly on diets containing stanols (Nes *et al*. 1997), but as mentioned in the introduction the impact of ketone-steroids on insects has not been investigated. The demonstration in the current study that the aphid *M. persicae* has extremely low survival rates and reproductive output on tobacco plants containing phloem-mobile ketone-steroids suggests that these compounds are deleterious for phloem-feeding insects. The mode of action of atypical steroids in the insects remains to be established. As for stanols in mammals (see Introduction), ketone-steroids may interfere with cholesterol uptake from the insect gut lumen; alternatively, or additionally, they may become inserted into cell membranes and disrupt membrane function. The deleterious effect of the various atypical steroids on the performance of both an aphid (this study) and chewing herbivorous insects is unlikely to be of ecological significance because the natural levels of these steroids in both total tissues and phloem sap of plants is generally low. The data in Table 1 illustrate this point. Nonetheless, two issues arise. First, fortification of plants with atypical steroids (including both stanols and steroid-ketones), as currently used in functional foods for humans, has potential to control pest aphids; our current research is testing the impact of atypical steroids on other phloem-feeding insects, including whiteflies, leafhoppers and planthoppers. The second issue is why atypical steroids are apparently not used as a defense strategy against herbivorous insects, especially as they appear to have no deleterious consequences for the plant. Possible solutions to this puzzle may include evolutionary constraints, negative selective effects of atypical steroids on plants under certain conditions, or the rapid evolution of insect tolerance to atypical steroids. However, we cannot exclude the possibility that some plants yet to be studied have substantial protective atypical steroid levels. It is also the case that in addition to free sterols, plants sometimes contain acylated sterols, glycosylated sterols and/or acylated glycosylated sterols (Moreau, Whitaker & Hicks 2002; Janson *et al*. 2009); we currently know very little about the ability of insect herbivores (chewing or sucking) to use conjugated sterols. Further research on the evolution and functional ecology of steroid synthesis by plants and steroid utilization by phytophagous insects will be crucial to both our understanding of insect-plant relations and the use of modified plant steroids in the control of insect crop pests.

Acknowledgements

We thank Steaphan Hazell (University of Birmingham, UK) who provided the aphid, Marine Pascall (University of York, UK) for excellent technical assistance, Professor Robert Turgeon (Cornell University, USA) for advice and discussion, and Monsanto Inc. for the use of control and modified tobacco. This research was supported by a Travel Grant from the International Programs Office at Texas A&M University (to STB), a NRI-CSREES-USDA grant (to STB and RJG), and the Sarkaria Institute of Insect Physiology and Toxicology (AED).

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Received 28 April 2010; accepted 12 October 2010
Handling Editor: Hefin Jones

Supporting information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Explanation and sample calculation for adjusted medians in Table 1.

Figure S1. Control and modified tobacco responses to aphid infestations.

Table S1. Sterol profiles of control and modified tobacco leaves (raw data).

Table S2. Sterol profiles of control and modified tobacco exudate (raw data).

Table S3. Sterol profiles of aphid carcasses from control and modified tobacco (raw data).

Table S4. Sterol profiles of aphid honeydew from control and modified tobacco (raw data).

Table S5. Sterol profiles of leaf, exudate, aphid carcass and aphid honeydew from cabbage plants (raw data).

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