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Sterol/steroid metabolism and absorption in a generalist and specialist caterpillar: Effects of dietary sterol/steroid structure, mixture and ratio

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ABSTRACT

Insects cannot synthesize sterols *de novo*, so they typically require a dietary source. Cholesterol is the dominant sterol in most insects, but because plants contain only small amounts of cholesterol, plant-feeding insects generate most of their cholesterol by metabolizing plant sterols. Plants almost always contain mixtures of different sterols, but some are not readily metabolized to cholesterol. Here we explore, in two separate experiments, how dietary phytosterols and phytosteroids, in different mixtures, ratios, and amounts, affect insect herbivore sterol/steroid metabolism and absorption; we use two caterpillars species – one a generalist (*Heliothis virescens*), the other a specialist (*Manduca sexta*). In our first experiment caterpillars were reared on two tobacco lines – one expressing a typical phytosterol profile, the other expressing high amounts/ratios of stanols and 3-ketosteroids. Caterpillars reared on the control tobacco contained mostly cholesterol, but those reared on the modified tobacco had reduced amounts of cholesterol, and lower total sterol/steroid body profiles. In our second experiment, caterpillars were reared on artificial diets containing known amounts of cholesterol, stigmasterol, cholestanol and/or cholestanone, either singly or in various combinations and ratios. Cholesterol and stigmasterol-reared moths were mostly cholesterol, while cholestanol-reared moths were mostly cholestanol. Moth tissue cholesterol concentration tended to decrease as the ratio of dietary cholestanol and/or cholestanone increased. In both moths cholestanone was metabolized into cholestanol and epicholestanol. Interestingly, *M. sexta* generated much more cholestanol than epicholestanol, while *H. virescens* did the opposite. Finally, total tissue steroid levels were significantly reduced in moths reared on diets containing very high levels of cholestanol. We discuss how dietary sterol/steroid structural differences are important with respect to sterol/steroid metabolism and uptake, including species-specific differences.

1. Introduction

Sterols serve two well-known functions for vertebrates and insects. First, they are important cellular membrane components that provide rigidity and adjust permeability. Second, they are essential precursors to steroid hormones. In insects, sterols are the required precursors to ecdysone, which drives many metabolic processes, including molting and metamorphosis. The bulk of sterols go towards structural purpose, because only a very low quantity is needed for metabolic purposes (Behmer and Nes, 2003; Lafont et al., 2005). The great majority of insects and vertebrates have cholesterol as their dominant body sterol, but unlike vertebrates, insects cannot cyclize squalene – they lack the required critical enzymes for *de novo* synthesis (Clayton, 1964; Niwa and Niwa, 2011). For most insects, sterols are acquired from their food.

Plants, however, generally contain only small amounts of cholesterol (Piironen et al., 2000). Instead, most contain a range of phytosterols (Fig. 1), which mainly differ from cholesterol by the presence of a C24 alkyl group (Behmer et al., 2011; Nes et al., 1977; Salt et al., 1991); in some instances, phytosterols can also contain a double bond at position C22 on the side-chain (e.g., stigmasterol), and possess a Δ7 double bond (e.g., spinasterol), rather than a Δ5 double bond, in the sterol nucleus (Behmer and Nes, 2003). Thus, plant-feeding insects generate most of their cholesterol by metabolizing phytosterols. Sterol metabolism has been examined in a number of different plant-feeding insects, and with the exception of grasshopper (Behmer and Elias, 2000), plant-feeding insects generally have little trouble converting common phytosterols (e.g., sitosterol, campesterol, and stigmasterol) into cholesterol (Svoboda,

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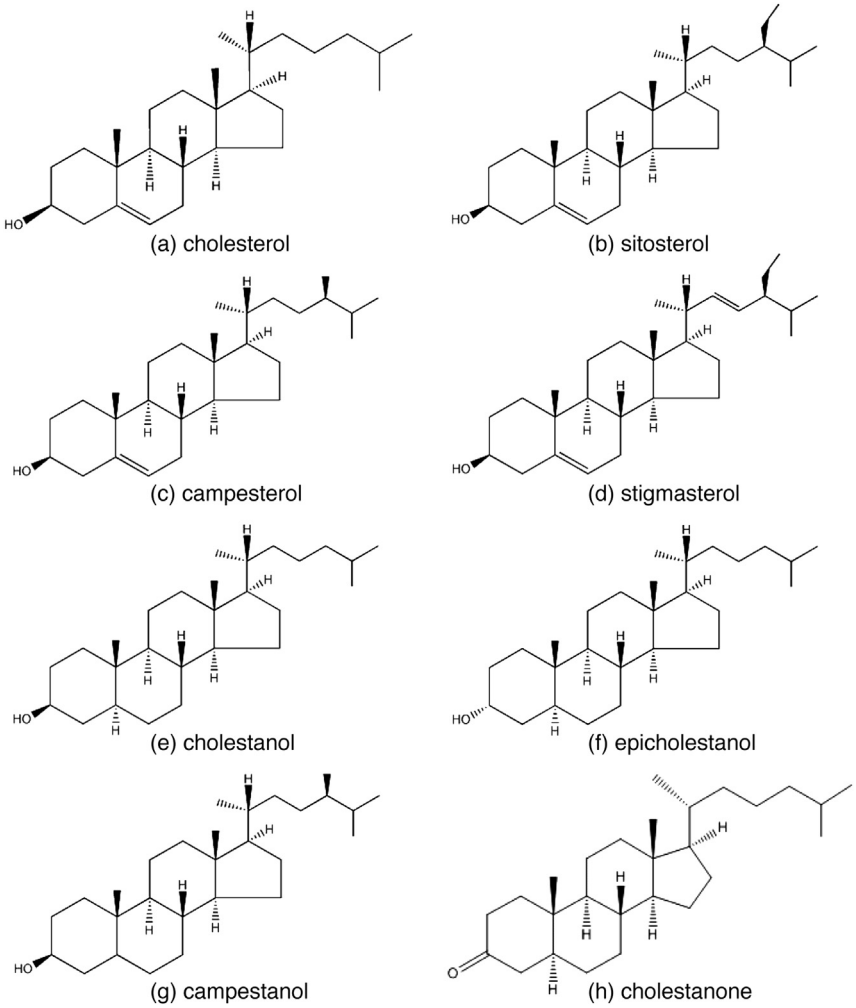

Fig. 1. Key sterols/steroids used in this study. Cholesterol (a) is the dominant sterol in most insects including those that feed on plants. Sitosterol (b), campesterol (c), and stigmasterol (d) are all common phytosterols that many caterpillars readily convert to cholesterol. Sitosterol and campesterol differ from cholesterol by having a C24 ethyl or methyl group, respectively. Stigmasterol differs from cholesterol by having a C24 ethyl group, plus a C22 double bond. Cholestanol (e), epicholestanol (f), and campestanol (g) are sterols lacking a Δ5 double bond. Epicholestanol is an isomer of cholestanol (it has a 3α-hydroxyl group instead of 3β-hydroxyl). Campestanol differs from cholestanol and epicholestanol by having a C24 methyl group. Cholestanone (h) is a keto-steroid. In contrast to cholesterol, this steroid has a C3 ketone instead of a C3 hydroxyl, and there is no Δ5 double bond in the sterol nucleus.

1999; Svoboda and Weirich, 1995). However, some structural features, particularly ones not found in typical phytosterols, may cause problem for some plant-feeding insects. In a recent study (Jing et al., 2012a), three caterpillar species (two generalists and one specialist) were reared on two tobacco plants expressing very different sterol profiles – one line contained normal plant sterols, the other contained normal sterols (24.9%) plus a high proportion of novel steroids (i.e., stanols (21.9%) and 3-ketosteroids (53.2%); see Fig. 1 for structural differences between sterols, stanols and 3-ketosteroids). Insect survival, growth, eclosion success and reproduction were reduced on the tobacco plants expressing high concentration of stanols and 3-ketosteroids, and it was suggested that this reduced performance was linked to an inability of these caterpillars to metabolize and use these novel steroids. Although cockroaches can convert cholestanol into Δ^7-5α-cholesten-3β-ol (albeit slowly (Clayton and Edwards, 1963)), and houseflies can convert cholestanone into cholestanol and epicholestanol (Dutky et al., 1967), no studies have examined the ability of plant-feeding insects to metabolize stanols and 3-ketosteroids.

The aim of the current study was to understand how dietary sterols/steroids, including different combinations and amounts, affected sterol metabolism and absorption in chewing insect herbivores. Two separate experiments, using two caterpillar species (one a generalist (*Heliothis virescens*), the other a specialist (*Manduca sexta*)), were conducted. In the first experiment caterpillars were reared from hatching to pupation on two genetically modified tobacco lines – one expressing a normal sterol profile, the other expressing a highly modified steroid profile (described above, see Heyer et al. 2004 for full details). In the second experiment, caterpillars were reared on artificial diets that contained sterols/steroids found in the modified tobacco line. Dietary sterols/steroids were studied singly, and in different combinations (including different ratios and absolute amounts). Our intent in this second experiment was to replicate, at a very basic level, the steroid profile of the modified tobacco.

2. Material and methods

2.1. Experimental insects

One generalist caterpillar, the tobacco budworm (*H. virescens* (Noctuidae)) and one specialist caterpillar, the tobacco hornworm

(*Manduca sexta* (Sphingidae)), were used in this experiment; both caterpillars readily feed on tobacco in nature. The *H. virescens* caterpillars originated from eggs purchased from Benzon Research Inc. (Carlisle, PA), while the *M. sexta* caterpillars originated from eggs purchased from Carolina Biological Supply Company (Burlington, NC). The eggs were incubated at 27 °C and neonates hatching within 6 h were used as a source for the start of the experiments.

2.2. Experimental plants with different sterol/steroid profiles

Two tobacco (*Nicotiana tabacum*) lines having different sterol/steroid profiles (Jing, 2011) were used in this experiment. The first line (modified tobacco) expressed the chloroplast-targeted 3-hydroxysteroid oxidase gene (pMON33814), from a bacterium, *Actinomyces* sp. A19249 – it shows an atypical sterol/steroid profile (a high percentage of stanols and 3-ketosteroids). The second line (control tobacco) was transformed with an empty vector, and shows a normal tobacco sterol profile (mostly stigmasterol and sitosterol). The two lines show no morphological differences (Corbin et al., 2001; Heyer et al., 2004). The plants and two caterpillar species were grown and reared using methods previously described (Jing et al., 2012a). Adults, immediately upon eclosion, were randomly collected and frozen at −20 °C, and then freeze-dried.

2.3. Artificial diets with different sterol/steroid profiles

Both caterpillar species were reared on an artificial diet developed by Ritter and Nes (1981), but with some modifications (Jing, 2011). In total, 11 unique diets were generated from various combination of 4 different sterols/steroids (see Fig. 1): (1) Cholest-5-en-3β-ol (cholesterol, ≥95%), (2) stigmasta-5,22E-dien-3β-ol (stigmasterol, ≥98%), (3) 5α-cholestan-3β-ol (cholestanol, 95%), and (4) 5α-cholestan-3-one (cholestanone, ≥98%); cholesterol and cholestanol were purchased from Sigma Chemical (St. Louis, MO, USA), and stigmasterol and cholestanone were purchased from Steraloids Inc. (Newport, RI, USA). Caterpillar performance on diets containing only cholestanol or cholestanone is poor relative to diets containing cholesterol or stigmasterol (Jing, 2011). Thus, we classify cholestanol and cholestanone as "bad" dietary steroids, and cholesterol and stigmasterol as "good" sterols (*sensu* Behmer and Grebenok, 1998).

The 11 different diets used in this study represent a range of single and mixed-sterol/steroid diets, with mixed diets containing a combination of "good" and "bad" sterols/steroids; the absolute amounts and ratios of sterol/steroid in each treatment, and shorthand code for each of our 11 diets, is shown in Table 1. Three of these diets were single-sterol diets, with cholesterol, stigmasterol and cholestanol added at a concentration of 1 mg/g dry mass respectively. Five diets contained two sterols/steroids (one "good" (stigmasterol), the other "bad" (cholestanol or cholestanone)). The first of these five diets contained stigmasterol (S) and cholestanol (A) in a 1:7 ratio (total concentration of 2 mg/g dry mass). The four remaining diets contained stigmasterol (S) and cholestanone (K) paired at various ratios and amounts. Three of these had S plus K at a total concentration of 2 mg/g dry mass, but in different ratios: 1:1, 1:3, and 1:7. The last of these five diets had S plus K in a 1:3 ratio, but with a total concentration of 4 mg/g dry mass). The final three diets contained 3 sterols/steroids: stigmasterol (S), cholestanol (A), and cholestanone (K). The ratio of A to K reflected the ratio in which they occur (1:2), relative to each other, in the modified tobacco line (Jing et al., 2012a). The three triple-sterol/steroid diets had the following "good" (S) to "bad" (A plus K) steroid ratios: 1:1, 1:3, and 1:7.

Table 1
Dietary sterol/steroid combinations. In total, 11 unique diets were generated using various combinations of 4 different sterols/steroids: cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K). The concentration of each sterol/steroid in each treatment is expressed as mg/g diet; treatment codes are shown in parentheses in each row.

Treatment	Sterols/steroids				
	Cholesterol	Stigmasterol	Cholestanol	Cholestanone	Total
Single steroids					
(C 1.0)	1.0	–	–	–	1.0
(S 1.0)	–	1.0	–	–	1.0
(A 1.0)	–	–	1.0	–	1.0
Two steroid mixtures					
(S 0.25 + A 1.75)	–	0.25	1.75	–	2.0
(S 1.0 + K 1.0)	–	1.0	–	1.0	2.0
(S 0.5 + K 1.5)	–	0.5	–	1.5	2.0
(S 0.25 + K 1.75)	–	0.25	–	1.75	2.0
(S 1.0 + K 3.0)	–	1.0	–	3.0	4.0
Three steroid mixtures					
(S 1.0 + A 0.33 + K 0.67)	–	1.0	0.33	0.67	2.0
(S 0.5 + A 0.5 + K 1.0)	–	0.5	0.5	1.0	2.0
(S 0.25 + A 0.58 + K 1.17)	–	0.25	0.58	1.17	2.0

The two caterpillar species were reared on these diets using protocols previously described (Jing, 2011). Adults, immediately upon eclosion, were randomly collected and frozen at −20 °C, and then freeze-dried for sterol/steroid analysis.

2.4. Sterol/steroid identification and quantification

2.4.1. H. virescens on tobacco plants

The freeze-dried *H. virescens* adults (3–5 randomly collected individuals) were homogenized and weighed. We extracted sterols/steroids in ethanol (100 ml/g samples) by shaking and sonicating samples for 3 consecutive 5-min bouts. To facilitate proper quantification of the sterols/steroids in each sample, 50 μg of cholestane (an internal standard) was added to each sample. Following a 12 h incubation period, 5 ml chloroform and 5 ml H_2O were added to each sample, mixed, and allowed to separate for 12 h. Following separation, the chloroform (lower layer) was removed and evaporated under nitrogen to a volume of 200 μl.

The method for the release of hydrolysable sterol esters has been described previously (Jing et al., 2012b). In summary, a basic reagent was used, and free sterols/steroids were extracted from the reaction reagent using H_2O-equilibrated hexane. Subsequently, the hexane fraction was backwashed to neutrality against 50% methanol/H_2O and concentrated to 50 μl for analysis by gas chromatography (GC-fid) and gas chromatography – mass spectroscopy (GC–MS).

Free sterols/steroids, and sterols/steroids freed following saponification were quantified by GC-fid by comparison with authentic standards. Identification of sterols/steroids by GC-fid was based on their relative retention times to standards on a DB-17 column (Agilent Technologies; this column had dimensions of 30 m, 0.25 mm diameter and 0.25 μm film thickness), using an Agilent 6890 Networked GC-fid outfitted with a 7683B autosampler. The system maintained a carrier gas flow rate of 1.3 ml per minute, inlet temperature of 280 °C, detector temperature of 290 °C and an oven ramp beginning at 80 °C ascending at 25 °C per minute to a temperature of 240 °C and ascending to a final temperature of 290 °C at a rate of 5 °C per minute and holding the final

temperature for 20 min. The elution pattern of the sterols/steroids on the GC-fid was in agreement with those reported previously by Heyer et al. (2004), and was confirmed by GC–MS (Agilent 5973) running the identical column, gas and temperature protocols as those described for the GC-fid. The Agilent 5973 mass selective detector maintained an ion source at 230 °C and quadrupole temperature of 150 °C.

2.4.2. H. virescens on diets

We used a slightly different sterol extraction and quantification techniques to analyze *H. virescens* moths reared on diets; these were more robust, economic, and sensitive (Jing et al., 2012b). Here each individual dry body was ground and weighed in a 1.5 ml VWR Eppendorf centrifuge tube, and sterols/steroids were extracted using a mixture of chloroform and methanol, with cholestane (5 μg) added as internal control. Next, the chloroform was removed and evaporated under nitrogen to a volume of 200 μl. The hydrolysable sterol esters in this solution were then released using the same base saponification method described above. All free sterols/steroids were derivatized to trimethylsilyl ether (TMS), and then analyzed by GC–MS (see system described above).

2.4.3. M. sexta on plants and diets

The sterol/steroid extraction and quantification for plant and diet reared *M. sexta* followed those used for diet reared *H. virescens*, with slight modification to account for *M. sexta* being larger compared to *H. virescens*. Here individual moths were transferred to a 50 ml VWR centrifuge tube that contained 8 ml chloroform, 8 ml methanol, 8 μg cholestane plus 15 glass beads. The sample was then shaken vigorously; only 0.70 ml for each sample was used for the analysis because of the high sensitivity of this method.

2.5. Statistical analysis

The steroid profiles for *H. virescens* reared on the two tobacco lines came from pooled samples (3–5 individuals), so only descriptive data are presented. For all other experiments, sterol/steroid analyses were performed on individuals; samples size for diet reared *H. virescens*, and diet reared *M. sexta*, were between 4 and 6, while sample size for plant reared *M. sexta* was 12. For the *M. sexta* plant experiment, a *t*-test was used for the comparisons of total steroid body content and total cholesterol body content on the two tobacco lines (normal and modified steroid profiles). For the diet experiments, total body steroid content and total cholesterol body content was analyzed by ANOVA; False Discovery Rate, which controls the expected proportion of falsely rejected hypotheses, was used for the adjustment in multiple comparison (Benjamini and Hochberg, 1995). All analyses were performed in SAS v. 9.2 (Cary, NC, USA).

3. Results

3.1. Body sterol/steroid composition of insects reared on plants

For *H. virescens*, individual dry mass was not recorded (3–5 individuals were pooled, and their combined total dry mass was recorded). This precluded any formal statistical analysis of body size, so instead we show the relative sterol/steroid amount of the combined sample (μg/g dry mass). For *M. sexta*, individual dry mass was recorded, and no difference was observed in adult dry mass between the two tobacco lines (*t*-test: $t_{22} = 1.33$, $P = 0.196$). Therefore, the relative steroid amount/individual (μg/g dry mass) was used in the analyses.

Total steroid levels were lower in insects reared on modified tobacco plants (Table 2). For *H. virescens*, moths from modified tobacco had a total steroid level about half (57.4%) that recovered from moths reared on the control tobacco. For *M. sexta*, the reduction in total steroid amount was less dramatic (86.7% in moths reared on modified tobacco, relative to moths reared on control tobacco), but this difference was significant (*t*-test: $t_{22} = 3.01$, $P = 0.006$).

Distinctly different sterol/steroid profiles were recovered from the two species reared on the two tobacco lines (Table 2). Only cholesterol and stigmasterol were recovered from *H. virescens* reared on the control tobacco line; these two sterols were also recovered from *H. virescens* reared on the modified tobacco lines, but these moths also contained cholestanol and a small amount of cholestanone. Cholesterol and campesterol (but not stigmasterol) were recovered in *M. sexta* reared on the control tobacco line; these two sterols, plus cholestanol and a tiny amount of cholestanone were recovered from *M. sexta* reared on the modified tobacco plants.

Cholesterol was the dominant sterol recovered from both *H. virescens* and *M. sexta*, regardless of whether they were reared on the control or modified tobacco plants. However, the cholesterol tissue profile (expressed as a % of the total sterol/steroid profile) differed between the two species. In *H. virescens*, control and modified tobacco reared insects had similar cholesterol profiles (Table 2). In contrast, the proportion of cholesterol recovered from *M. sexta* moths reared on the modified tobacco was significantly lower compared to those reared on the control tobacco (*t*-test: $t_{22} = 15.50$, $P < 0.001$, Table 2). Moreover, in *M. sexta*, the relative

Table 2
Sterol/steroid profiles of *H. virescens* and *M. sexta* reared on tobacco plants with normal (control) and modified sterol/steroid profiles. The *H. virescens* data represent a pooled sample of 3–5 individuals; the *M. sexta* data are from individuals (mean ± SEM; $n = 12$ for both the control and the modified plants).

Sterol type	Variable	*H. virescens*		*M. Sexta*	
		Control tobacco	Modified tobacco	Control tobacco	Modified tobacco
Total steroids	Relative amount (μg/g)	1658	951	4046 (191)[a]	3468 (150)
	Tissue profile (%)	100	100	100	100
Cholesterol	Relative amount (μg/g)	1445	715	3731 (179)[a]	1808 (126)
	Tissue profile (%)	87.2	75.2	92.2 (0.4)[a]	52.1 (2.6)
Stigmasterol	Relative amount (μg/g)	213	24	Not detected	Not detected
	Tissue profile (%)	12.8	2.6	–	–
Campesterol	Relative amount (μg/g)	Not detected	Not detected	315 (22)	99.3 (117)
	Tissue profile (%)	–	–	7.8 (0.4)	2.9 (0.5)
Cholestanol	Relative amount (μg/g)	Not detected	186	Not detected	1556 (124)
	Tissue profile (%)	–	19.5	–	44.9 (2.7)
Cholestanone	Relative amount (μg/g)	Not detected	26	Not detected	5 (3)
	Tissue profile (%)	–	2.7	–	0.2 (0.1)

[a] Indicates a higher value for the measure of the specific sterol type between two tobacco lines.

cholesterol amount was significantly reduced in moths from modified tobacco compared to moths from control tobacco (*t*-test: $t_{22} = 8.78$, $P < 0.001$). For *H. virescens*, the relative cholesterol level in moths from the modified tobacco was half compared to moths from the control tobacco (Table 2). Interestingly, *H. virescens* and *M. sexta* reared on the modified tobacco lines contained more cholestanol compared to cholestanone, even though the modified tobacco plants contained twice as much 3-ketosteroids as stanols.

3.2. Body sterol/steroid composition of insects reared on artificial diets

There was no difference in adult dry mass between different treatments (*H. virescens*: $F_{10,53} = 1.86$, $P = 0.073$; *M. sexta*: $F_{9,51} = 1.94$, $P = 0.067$), so the relative sterol/steroid amount was used for the analyses. Somewhat surprisingly, no *M. sexta* adults were collected on the stigmasterol diet (this was observed in two separate runs), so in place of adult sterol/steroid profiles we report the sterol/steroid profile of 3rd-instar larvae. This stigmasterol data is not included in our broader statistical analysis; we do, however, present these data for reference purposes.

The relative total sterol/steroid amount differed between treatments for both species (*H. virescens*: $F_{10,53} = 11.17$, $P < 0.001$; *M. sexta*: $F_{9,51} = 11.84$, $P < 0.001$). On the cholesterol- and stigmasterol-only treatments, total relative steroid levels were equally high for both species (Figs. 2 and 3). Perhaps most notable, though, was that the total relative steroid level was statistically lowest on the cholestanol-only treatment for both species, and that levels remained low for both species even when a small amount of dietary stigmasterol was present with cholestanol (Figs. 2 and 3). This was not the case when stigmasterol was paired with cholestanone; furthermore, the two species had different responses towards these treatments. For *H. virescens*, but not *M. sexta*, the total steroid level was significantly higher compared to the cholesterol-only treatment (Figs. 2 and 3). The two species also had different responses when stigmasterol, cholestanol and cholestanone were combined in the diet. For *H. virescens*, total steroid levels did not differ relative to the cholesterol-only treatment, but for *M. sexta* they tended to be lower compared to levels on the cholesterol-only treatment (Figs. 2 and 3).

There were many similarities in body sterol/steroid composition between two species (Tables 3 and 4). For example, cholesterol was always recovered, regardless of the sterol/steroid content of the diet. Additionally, cholestanol was recovered from insects fed cholestanol and/or cholestanone, but a higher percent of it was recovered from insects fed diets containing some cholestanol. Finally, epicholestanol was only found in insects fed diets containing cholestanone. But there were also differences in body sterol/steroid content between the two species, particularly on the cholestanone diets. In *H. virescens*, a high percentage of epicholestanol, relative to cholestanol, was recovered; in *M. sexta* this pattern was reversed. The two species also differed in their cholestanone profiles. In *H. virescens*, cholestanone was only recovered from moths reared on cholestanone-containing diets. In contrast, cholestanone was recovered from *M. sexta* fed cholestanol-containing diets that lacked cholestanone (i.e., treatment A, and treatment S 0.25 + A 1.75). Finally, stigmasterol was found in *H. virescens* when this sterol was mixed in the diet; it was not always recovered in *M. sexta* fed stigmasterol-containing diets.

For both species, cholesterol content (as a % of the total sterol/steroid profile) differed between treatments (*H. virescens*:

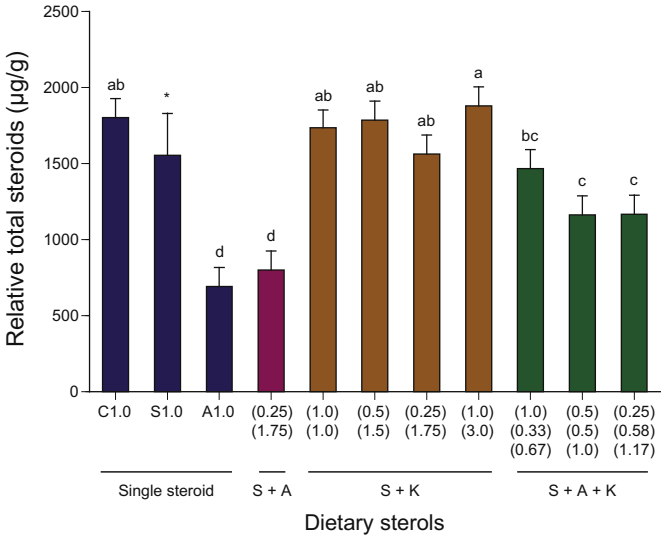

Fig. 3. Total body steroid profiles (mean ± SEM) of *M. sexta* adults that had been reared as larvae on diets containing one, two or three sterols/steroids, at various concentrations. Four different sterols/steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 11 diets shown in this figure. The first three treatments were single steroid diets, with each steroid at a concentration of 1 µg/g (dry mass). The next five treatments contained two sterols/steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the concentration (µg/g) of each sterol/steroid in the diet is shown in parentheses directly below the bars. The last three treatments represented diets with three sterols/steroids (stigmasterol, cholestanol and cholestanone), with the concentration of each sterol/steroid in the diet shown below each bar. Data were analyzed using ANOVA, and different letters above the bars indicate statistically significant differences among the treatments. There is an asterisk on the single steroid stigmasterol bar; these data were collected from 3rd stadium caterpillars, because no adults were produced on this treatment (in two separate experiments). These data are shown for reference only, and have not been included in the formal statistical analysis (see Table 3 for the number of samples used in each treatment).

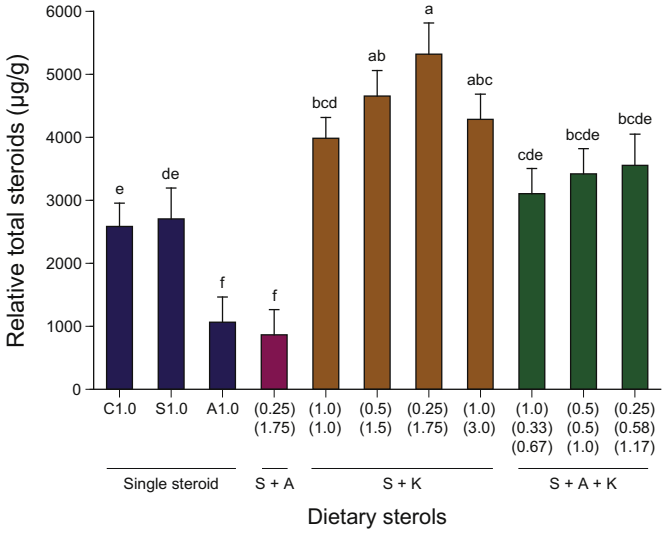

Fig. 2. Total body steroid profiles (mean ± SEM) of *H. virescens* adults that had been reared as larvae on diets containing one, two or three sterols/steroids, at various concentrations. Four different sterols/steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 11 diets shown in this figure. The first three treatments were single steroid diets, with each steroid at a concentration of 1 µg/g (dry mass). The next five treatments contained two sterols/steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the concentration (µg/g) of each sterol/steroid is shown in parentheses directly below the bars. The last three treatments represented diets with three sterols/steroids (stigmasterol, cholestanol and cholestanone), with the concentration of each sterol/steroid in the diet shown below each bar. Data were analyzed using ANOVA, and different letters above the bars indicate statistically significant differences among the treatments (see Table 2 for the number of samples used in each treatment).

Table 3
Body sterol profiles as a percent (mean ± SEM) for *H. virescens* reared to the adult stage on artificial diets containing one, two or three sterols/steroids. In total 4 different sterols/steroids were used: cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K). For each treatment, the sterol/steroid present in the diet, and its concentration (the number adjacent to a capital letter; mg/g), are presented (see Table 1 for more detail). Statistical comparisons were limited to insect cholesterol content; ANOVA was performed, and different uppercase letters indicate a significant difference between sterol treatments ($P < 0.05$).

Body sterol type (%) (sample size)	Sterol treatments										
	Single sterol diets			Double steroid diets					Triple steroid diets		
									S 1.0	S 0.5	S 0.25
				S 0.25	S 1.0	S 0.5	S 0.25	S 1.0	A 0.33	A 0.5	A 0.58
	C 1.0 (7)	S 1.0 (4)	A 1.0 (6)	A 1.75 (6)	K 1.0 (9)	K 1.5 (6)	K 1.75 (4)	K 3.0 (6)	K 0.67 (6)	K 1.0 (6)	K 1.17 (4)
Cholesterol (±SEM)	100^{A} (0)	96.9^{A} (1.4)	29.3^{E} (1.0)	43.8^{D} (1.6)	59.8^{B} (2.0)	40.3^{D} (2.2)	27.1^{E} (1.9)	48.9_{C} (4.4)	54.1^{BC} (2.3)	37.9^{D} (2.0)	24.9^{E} (1.2)
Stigmasterol (±SEM)	–	3.1 (1.3)	–	1.5 (0.7)	3.6 (0.7)	2.2 (0.6)	1.3 (0.7)	3.3 (0.7)	3.6 (0.7)	1.7 (0.6)	1.1 (0.4)
Cholestanol (±SEM)	–	–	70.7 (1.0)	55.7 (1.4)	5.0 (1.3)	7.2 (1.3)	13.3 (0.8)	4.1 (1.1)	10.7 (0.7)	23.3 (0.8)	23.6 (2.1)
Epicholestanol (±SEM)	–	–	–	–	31.2 (2.0)	49.5 (2.9)	57.3 (2.4)	42.7 (3.8)	33.8 (2.6)	36.1 (2.2)	49.3 (3.2)
Cholestanone (±SEM)	–	–	–	–	0.5 (0.2)	1.0 (0.2)	1.0 (0.2)	1.1 (0.2)	0.8 (0.2)	1.1 (0.3)	1.1 (0.1)

$F_{10,53} = 130.31$, $P < 0.001$; *M. sexta*: $F_{9,51} = 55.45$, $P < 0.001$). It was highest in insects fed the cholesterol- and stigmasterol-only diets (Tables 3 and 4), and equally lowest on the cholestanol-only treatment and triple sterol treatment with 12.5% stigmasterol (i.e., S 0.25 + A 0.58 + K 1.17); for *H. virescens* cholesterol levels were also equally low on the 1:7 S + K treatment. Cholesterol percentage was also more closely related to the sterol/steroid ratio, rather than the total amount in the diet. For example, cholesterol content (as a percent) was highest on diets with a 1:1 ratio of "good" (stigmasterol) to "bad" (cholestanol and/or cholestanone) sterol, intermediate on diets with a 1:3 ratio, and lowest on diets with a 1:7 ratio. In contrast, diets that had identical ratios, but different absolute amounts (i.e., S 0.5 + K 1.5 and S 1.0 + K 3.0), had similar cholesterol content (as a percent).

4. Discussion

More than 100 different sterols have been identified in plants, and individual plants always contain multiple types of sterols (Nes, 1977), so insect herbivores often have to simultaneously process multiple dietary phytosterols. In our control tobacco line, there were five different phytosterols (Jing et al., 2012a) – stigmasterol (~38%), campesterol (~28%), sitosterol (~14%), cholesterol (~11%), and isofucosterol (~9%) – yet cholesterol was the dominant tissue sterol recovered from both species. It is well documented that *M. sexta* dealkylates a number of C24 substituted phytosterols to cholesterol (Svoboda, 1968; Svoboda and Weirich, 1995), and our study confirms this; as for *H. virescens*, our study is the first to document that this economically important insect also readily metabolizes C24 substituted phytosterols to cholesterol.

Interestingly, both species reared on the control tobacco accumulated a small amount of unmetabolized phytosterol, and each species had a slightly different tissue sterol profile. This suggests that insect herbivores do not metabolize all absorbed phytosterols, and that the specificity of dealkylation enzymes to different phytosterols varies between insect herbivores. For instance, our plant data shows that *H. virescens*, compared to *M. sexta*, is more efficient at converting campesterol to cholesterol. Differences in sterol profiles between insect herbivore species is well documented (Behmer and Nes, 2003), but studies that examine similar species reared on the same host plant are likely to generate novel insights with respect to sterol metabolic capabilities in insect herbivores (e.g., Janson et al., 2009).

But what happens when our two moths are reared on plants that contain large quantities of atypical steroids? Atypical phytosteroids could affect insect herbivores in two non-mutually exclusive ways. First, they might inhibit absorption (Allayee et al., 2000; Berge et al., 2000). Second, after being absorbed, atypical phytosteroids might interfere with sterol metabolism. With respect to our insects, the first notable observation was that total tissue steroid concentration was significantly reduced in moths reared on the modified tobacco plants (a 43% and 14% reduction for *H. virescens* and *M. sexta*, respectively). This occurred even though the absolute amount of phytosterols in the modified and control tobacco plants was essentially equal. We suspect that a high concentration/absolute amount of dietary stanol might contribute to this phenomenon, as we also observed reduced body tissue concentrations in moths that had been reared on artificial diets containing a high concentration/absolute amount of cholestanol (i.e., the cholestanol-only and stigmasterol plus cholestanol (S 0.25 + A

Table 4
Body sterol profiles as a percent (mean ± SEM) for *M. sexta* reared to the adult stage on artificial diets containing one, two or three sterols/steroids. In total 4 different sterols/steroids were used: cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K). For each treatment, the sterol/steroid present in the diet, and its concentration (the number adjacent to a capital letter; mg/g), are presented (see Table 1 for more detail). Statistical comparisons were limited to insect cholesterol content; ANOVA was performed, and different uppercase letters indicate a significant difference between sterol treatments ($P < 0.05$). For the stigmasterol treatment, the sterol profile was obtained from 3rd instar larvae, so it was excluded from the cholesterol comparison.

Body sterol type (%)(sample size)	Sterol treatments										
	Single sterol diets			Double steroid diets					Triple steroid diets		
									S 1.0	S 0.5	S 0.25
				S 0.25	S 1.0	S 0.5	S 0.25	S 1.0	A 0.33	A 0.5	A 0.58
	C 1.0 (6)	S 1.0 (6)	A 1.0 (6)	A 1.75 (6)	K 1.0 (7)	K 1.5 (6)	K 1.75 (6)	K 3.0 (6)	K 0.67 (6)	K 1.0 (6)	K 1.17 (6)
Cholesterol (±SEM)	100^{A} (0)	72.5 (2.7)	24.9^{E} (1.3)	31.2^{D} (1.0)	42.9^{B} (1.4)	38.0^{C} (0.5)	30.1^{D} (1.2)	36.7^{C} (0.8)	42.9^{B} (1.7)	36.8^{C} (0.9)	26.1^{E} (1.6)
Stigmasterol (±SEM)	–	27.5 (2.7)	–	–	0.8 (0.6)	0.6 (0.5)	–	0.2 (0.2)	–	–	–
Cholestanol (±SEM)	–	–	72.4 (1.2)	64.9 (1.1)	48.4 (1.3)	51.6 (1.5)	56.1 (1.5)	52.6 (1.5)	51.1 (1.2)	53.4 (1.1)	63.1 (1.5)
Epicholestanol (±SEM)	–	–	–	–	3.8 (1.2)	7.9 (1.4)	9.6 (0.8)	7.7 (0.8)	3.8 (0.9)	7.1 (0.8)	6.7 (0.7)
Cholestanone (±SEM)	–	–	2.8 (0.6)	3.9 (0.8)	4.1 (0.8)	1.8 (0.3)	4.1 (0.9)	2.8 (0.6)	2.2 (0.3)	2.7 (0.4)	4.2 (0.8)

1.75) treatments). In mammals, dietary stanols are known to reduce body sterol content via ABC transporters (Allayee et al., 2000; Berge et al., 2000). During this process stanols can also block the absorption of dietary cholesterol by replacing or precipitating cholesterol in the intestine (Moreau et al., 2002). ABC transporters are known from insects (Ewart et al., 1998; Komoto et al., 2009; Tarr et al., 2009), including caterpillars (Jing et al., 2012b), but their role in regulating sterol uptake in insects is poorly documented. This is an area of study that warrants further investigation.

The second notable observation was that despite the modified tobacco plants having a very diverse phytosterol/phytosteroid profile, only four types of sterol/steroids were recovered in each moth. Cholesterol was the most abundant steroid recovered from both insects (comprising ~75% and ~52% of the total steroid pool for *H. virescens* and *M. sexta*, respectively) although levels were greatly reduced compared to insects reared on the control tobacco plants. Additionally, the relative amount (μg/g tissue) of cholesterol in moths reared on the modified tobacco was half compared to levels in moths reared on control tobacco. These results are particularly interesting considering the control and modified tobacco plants had very similar *absolute* amounts of phytosterols. We also observed significant quantities of cholestanol in both insects (~20% and ~45% of the total sterol profile, in *H. virescens* and *M. sexta*, respectively), and very low levels of cholestanone. Collectively our results suggest three things about caterpillars, and likely chewing insect herbivores more broadly. First, a high ratio of atypical sterols/steriods in a plant reduces insect cholesterol concentrations. Second, phytostanols and phytoketosteroids are readily dealkylated. Third, phytoketosteroids are readily metabolized to stanols.

Plant sterol profiles are complex and highly variable, so an artificial diet approach provides greater clarity with respect to understanding sterol/steroid metabolism, including how different types of dietary sterols interact to affect metabolism and absorption. The cholesterol-only treatment served as a reference with respect to relative tissue sterol levels control. We found that moths reared on the stigmasterol-only diets metabolized stigmasterol to cholesterol, and had total tissue sterol concentrations similar to cholesterol-reared moths. However, sterol analysis of insects from this treatment indicated that *H. virescens* was more efficient at converting stigmasterol into cholesterol. Interestingly, *Manduca* stigmasterol tissue levels were <1% on all the other treatments containing stigmasterol (and this was also the case for *Manduca* on the control tobacco plants). This suggests that phytosterol metabolism in insect herbivores can vary depending on the sterol profile of their foods.

Somewhat surprisingly we observed, in two separate runs, that *M. sexta* reared on the stigmasterol-only treatment exhibited arrested development in the 2nd or 3rd instar. Previous studies (e.g., Svoboda et al., 1995) have shown *Manduca* caterpillars had little problem pupating when reared on stigmasterol diets, but closer inspection of the diets used in these studies reveals substantial phytosterol (e.g., sitosterol, campesterol) contamination from a key dietary component (wheat germ). Interestingly, sterol analysis of the caterpillars from the Svoboda et al. (1995) study found unmetabolized stigmasterol made up 11.2% of the sterol profile. In our caterpillars, unmetabolized stigmasterol comprised ~28% of the total sterol profile. We suspect that the failure of our caterpillars to pupate on our stigmasterol-only diet might be tied to the incorporation of a high percentage of unmetabolized dietary phytosterols into cell membranes. Where this occurs, the packing properties in cell membranes might be altered (Carvalho et al., 2012), leading to 'leaky' cells (Stein, 1981). A similar mechanism has been invoked to explain why grasshoppers, which cannot dealkylate stigmasterol, fail to complete development when reared on stigmasterol diets (Behmer and Elias, 1999b; Behmer et al., 1999).

With respect to stanols, most insects that have been studied cannot introduce a double bond at the C5 position (Behmer and Nes, 2003); our results from the cholestanol-only treatment suggest this is also the case for *H. virescens* and *M. sexta* (both species had tissue sterol profiles dominated by cholestanol (>70%)). The rest of the tissue sterol profile in these moths was mostly cholesterol (~25% of the total tissue steroid profile), which we suspect originated from one of two sources: 1) parental cholesterol transferred to the egg (Costet et al., 1987; Kircher and Gray, 1978), and/or 2) cholesterol contamination in the diet (Jing, 2011). When a small amount of stigmasterol was present with cholestanol (i.e., the S 0.25 + A 1.75 treatment), both species had significantly elevated cholesterol concentrations compared to moths from the cholestanol-only treatment. This indicates that stanols do not completely block the uptake of ingested phytosterols. Furthermore, because very little stigmasterol was recovered from the S 0.25 + A 1.75 treatment, it suggests stanols do not impede the phytosterol dealkylation process.

The sterol/steroid profile of cholestanone-fed insects clearly showed that both species could convert cholestanone to cholestanol. Interestingly, we also recovered epicholestanol from cholestanone-fed insects, but because it was never found in the cholestanol-fed insects, it is clearly a cholestanone-specific metabolite. We suspect two midgut enzymes, 3α-reductase and 3β-reductase are at work here, both actively involved in converting 3-keto-ecdysone into 3-hydroxyl-ecdysone (Gilbert, 2004; Weirich et al., 1993). The known product catalyzed by 3α-reductase is epiecdysone, while the one catalyzed by 3β-reductase is ecdysone; these two products have stereo-structure differences similar to the isomers derived from cholestanone in the current experiment. Furthermore, the contrast in the composition of cholestanol and epicholestanol in our two insects (i.e., more epicholestanol in *H. virescens*, but more cholestanol in *M. sexta*) suggests that 3β-reductase activity is dominant in *M. sexta*, while 3α-reductase activity is dominant in *H. virescens*. Additional evidence supports this deduction. For example, cholestanone was detected in *M. sexta* on the diets containing cholestanol (e.g., the A 1.0 and S 0.25 + A 1.75 treatment), but cholestanone was not detected in *H. virescens*; furthermore, 3β-reductase activity, but not 3α-reductase activity, is reversible (Gilbert, 2004; Yang et al., 2010). On a technical note, we likely did not detect epicholestanol in *H. virescens* reared on modified tobacco plants because free sterols, rather than derivatized sterols, were examined during sterol/steroid analysis. Here, an interaction between the 3-OH and GC column can mask stereo-structure differences. For *M. sexta* moths reared on the modified tobacco plants, we might not have seen epi-cholestanol because the amount generated by caterpillars was too low for detection.

In contrast to moths reared on the stigmasterol plus cholestanol treatment (S 0.25 + A 1.75), moths reared on the stigmasterol plus cholestanone treatments showed total steroid levels that were either similar (*M. sexta*) or elevated (*H. virescens*) relative to moths from the cholesterol- and stigmasterol-only treatments. This indicates that dietary 3-ketosteroids, unlike stanols, do not hinder sterol/steroid uptake, or induce sterol efflux. Furthermore, given that 3-ketosteroids are mostly converted into stanols, a process that likely takes place in midgut cells following absorption (Behmer and Nes, 2003), our data suggests that stanols are most likely acting to reduce steroid levels during the absorption phase. We also observed that the cholesterol percentage decreased as the stigmasterol:cholestanone ratio in the diet decreased. This suggests that cholesterol content reflects the dietary stigmasterol:cholestanone ratio, not the absolute amount of stigmasterol in the diet. That cholesterol content is a function of the ratio of "good" to "bad" dietary sterol/steroid is now known from four insects (the grasshopper *Schistocerca americana* (Behmer and Elias, 2000), the caterpillar

Helicoverpa zea (Nes et al., 1997), and the two caterpillar species in the current study). This suggests that the ratio of "good" to "bad" sterol/steroid in the diet is broadly important for insect herbivores.

Many questions remain concerning sterol use in insects. For example, we observed that the relative total steroid amount in plant-reared *M. sexta* was more than doubled compared to plant-reared *H. virescens*. Questions related to differences in sterol tissue amounts between species have been previously raised, particularly as they relate to differences in size and feeding biology (Behmer and Nes, 2003), but little data currently exists regarding these issues. Our two species differ in their size (last instar *M. sexta* are much larger than are *H. virescens*), and feeding biology (*M. sexta* is a specialist, *H. virescens* is a generalist), so these two factors might contribute to the observed differences. Our data also suggest that dietary stanols can reduce sterol/steroid uptake and utilization, but we still know little about the extent to which dietary stanol concentrations and ratios affect this process, and/or the mechanisms by which it occurs. It would be particularly informative to measure gene expression and activity levels of ABC transporters in the presence of dietary stanols, and use RNAi techniques to examine the effects of blocking ABC transporter expression. We also observed that cholestanone was always maintained at a very low level in insects. The structure of cholestanone is similar to insect molting hormones (ecdysteroids) so perhaps rapid metabolism into stanol is a mechanism that limits interruption of normal physiological process. Our modified tobacco plants demonstrate that sterol profiles can be changed, and recent work investigating dietary sterol ratios suggests there is a species-specific good:bad dietary sterol ratio that negatively affects performance, through lowering survival, increasing development time, reducing body mass, and reducing reproduction (Behmer and Elias, 1999a, 2000; Costet et al., 1987; Dutky et al., 1967; Nes et al., 1997). Taken as a whole, our data suggests that manipulating plant sterol profiles offers real potential for controlling phytophagous insect pests, but with a caveat – its effectiveness will likely vary depending upon the species being targeted.

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