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Diet micronutrient balance matters: How the ratio of dietary sterols/steroids affects development, growth and reproduction in two lepidopteran insects

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ABSTRACT

Insects lack the ability to synthesize sterols *de novo* so they acquire this essential nutrient from their food. Cholesterol is the dominant sterol found in most insects, but in plant vegetative tissue it makes up only a small fraction of the total sterol profile. Instead, plants mostly contain phytosterols; plant-feeding insects generate the majority of their cholesterol by metabolizing phytosterols. However, not all phytosterols are readily converted to cholesterol, and some are even deleterious when ingested above a threshold level. In a recent study we showed that caterpillars reared on tobacco accumulating novel sterols/steroids exhibited reduced performance, even when suitable sterols were present. In the current study we examined how the dominant sterols (cholesterol and stigmasterol) and steroids (cholestanol and cholestanone) typical of the modified tobacco plants affected two insect herbivores (*Heliothis virescens* and *Helicoverpa zea*). The sterols/steroids were incorporated into synthetic diets singly, as well as in various combinations, ratios and amounts. For each insect species, a range of performance values was recorded for two generations, with the eggs from the 1st-generation adults as the source of neonates for the 2nd-generation. Performance on the novel steroids (cholestanol and cholestanone) was extremely poor compared to suitable sterols (cholesterol and stigmasterol). Additionally, performance tended to decrease as the ratio of the novel dietary steroids increased. We discuss how the balance of different dietary sterols/steroids affected our two caterpillar species, relate this back to recent studies on sterol/steroid metabolism in these two species, and consider the potential application of sterol/steroid modification in crops.

1. Introduction

Insects, like all eukaryotes, use sterols for three key purposes. First, they are structural components in cell membranes, where they modulate and regulate permeability, thermomechanics, lateral diffusion, and protein function (Mouritsen and Zuckermann, 2004). Second, they are critical precursors for many hormones, especially those regulating growth and development (Grieneisen, 1994). Third, sterols are signaling molecules driving developmental processes (Incardona and Eaton, 2000; Porter et al., 1996). However, unlike most eukaryotes, arthropods (including insects) cannot make their own cholesterol (Svoboda and Feldlaufer, 1991); they lack the enzyme squalene synthase, which is needed to synthesize squalene, an essential intermediate in the sterol biosynthetic pathway leading to cholesterol (Klowden, 2007). Insects therefore require a dietary source of sterol. Cholesterol (Fig. 1a) supports growth for the large majority of insects (Behmer and Nes, 2003), and like all sterols its defining characteristics are a tetracyclic ring structure, a hydroxyl group at the C3 position, and a side chain branching off the D ring at the C17 position. Cholesterol is the most common sterol recovered in animal tissue, including insects (Svoboda, 1999; Svoboda and Thompson, 1987). In plant vegetative tissues, however, cholesterol comprises only a small portion of the plant sterol profile (Lusby, 1994; Nes, 1977; Svoboda et al., 1995). Thus, the large majority of the cholesterol recovered from the tissue of plant-feeding insects is the product of phytosterol metabolism (Behmer and Nes, 2003; Jing et al., 2013; Robbins et al., 1971).

Hundreds of different phytosterols have been identified in plants, and individual plants always contain a mixture of phytosterols (Nes, 1977; Piironen et al., 2000). Thus, insect herbivores ingest a mixture of phytosterols when they feed, and this is

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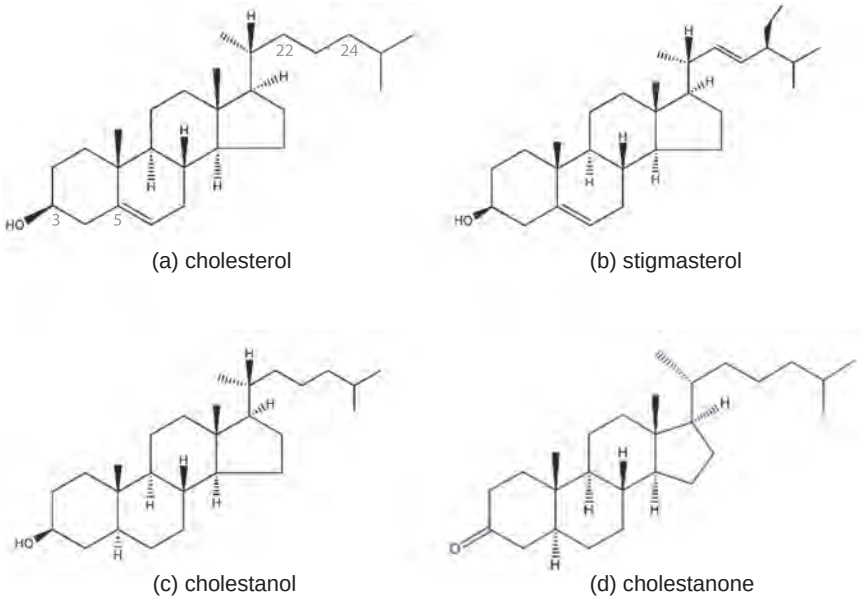

Fig. 1. Sterols of interest in this study. Cholesterol (a) is the most common sterol found in insects. Stigmasterol (b) is a common phytosterol that differs structurally from cholesterol. It has an ethyl group at C24, plus a C22 double-bond. Cholestanol (c) is identical to cholesterol, except that it lacks a double bond at C5 in the B-ring. Cholestanone (d) is a keto-steroid (it has a ketone instead of a hydroxyl at C3), and like cholestanol, it lacks a double bond at C5 in the B-ring.

especially true for generalist insect herbivores that have diets consisting of multiple different plant species (Behmer and Elias, 2000). Sitosterol and stigmasterol (Fig. 1b) are two common phytosterols recovered in plant vegetative tissue (the dominant phytosterol is a function of plant taxonomy (Nes, 1977)). Most plant-feeding insects readily convert sitosterol to cholesterol (Behmer and Elias, 1999a,b;, 2000; Svoboda, 1999) by removing the C24 alkyl group (Jing et al., 2013), but not all insect herbivores (e.g., grasshoppers (Behmer et al., 1999)) can metabolize stigmasterol to cholesterol; for grasshoppers the double bond at C22 blocks dealkylation (sitosterol lacks this double bond). As a consequence, grasshoppers fed a diet with stigmasterol as the sole dietary sterol fail to complete development (Behmer and Elias, 2000; Behmer et al., 1999). However, grasshoppers also fail to complete development when the dietary ratio of stigmasterol exceeds a threshold (Behmer and Elias, 2000). This suggests that the balance of "good" (e.g., cholesterol) to "bad" (e.g., stigmasterol in the case of grasshoppers) sterol in an insect herbivore's diet can have very meaningful consequences.

In contrast to grasshoppers, most caterpillars can metabolize stigmasterol to cholesterol (Jing et al., 2012a,b, 2013; Short et al., 1996; Svoboda, 1999; Svoboda et al., 1988). However, we have recently shown that caterpillars reared on tobacco that contains a mixture of stigmasterol and a high percentage of atypical steroids (e.g. stanols (e.g., Fig. 1c) and ketosteroids (e.g., Fig. 1d)) suffered reduced survival, growth and reproduction (Jing et al., 2012a); these negative effects were even more pronounced in the subsequent generation. Steroid profiles of the tobacco plants used in this study were modified by expressing, in the chloroplasts, a bacterial gene (*choM*) encoding 3-hydroxysteroid oxidase (Corbin et al., 2001; Heyer et al., 2004). This gene generates a ChoM protein, which has insecticidal activity against the cotton weevil (Greenplate et al., 1995; Purcell et al., 1993). However, the mechanism by which this enzyme alters plant phytosterol profiles, or its affect on other aspects of tobacco physiology, is unknown. Additionally, its insecticidal effect on lepidopterans is poorly documented.

In this study we reared two different noctuid caterpillar species (*Heliothis virescens* and *Helicoverpa zea*) on artificial diets containing some of the dietary phytosterols and phytosteroids identified in tobacco plants that expressed 3-hydroxysteroid oxidase. Using an artificial diet approach allowed us to study the effects of these different sterols/steroids independent of effects (direct or indirect) associated with the 3-hydroxysteroid oxidase enzyme. Four different sterols/steroids were investigated (cholesterol, stigmasterol, cholestanol, and cholestanone), and in total 12 different diets were constructed (4 contained single sterols, the other 8 contained various sterol mixtures, in different ratios and amounts). Testing different ratios allowed us to determine the threshold level of "bad" sterol that is needed to negatively affect caterpillar/moth performance. For both species, neonates were tracked through larval and pupal development; those that eclosed were then mated. We measured survival, larval development, pupation success, pupal mass, pupal development, eclosion success, egg production and egg viability. We then used the eggs from the mated pairs as a source of neonates to repeat the experiment. This approach allowed us to examine how the sterol history of mated pairs affected offspring performance. We predicted that for both species there was a threshold ratio of "good" to "bad" dietary sterol that must be reached before performance would be negatively affected, but that once it was reached, performance would increasingly worsen as this ratio decreased. We also predicted that negative effects would be stronger in the second generation, because maternal cholesterol allocation to eggs used as the source of neonates for the 2nd generation would be reduced, relative to 1st generation neonates. We discuss our results at the organismal level, and in light of recent studies on sterol/steroid metabolism. We conclude by considering the potential of modifying plant sterol/steroid profiles, and the extent to which profiles would need to be modified, to control insect herbivore pests.

2. Materials and methods

2.1. Insects

Two generalist noctuid caterpillars, corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*), were used in this experiment. Eggs of both species were purchased from Benzon Research Inc (Carlisle, PA). The eggs were incubated at 27 °C, and

neonates (<6 h old) were used as a source for the experiments. Individual neonates were randomly selected and transferred onto different experimental diets (described below) using a small paintbrush.

2.2. Experiment design

An artificial diet originally developed for *H. zea* (Ritter and Nes, 1981a) was used. However, because this diet did not support our insect colony beyond one generation, and insect performance expressed as larval development, pupal mass and reproduction was greatly reduced (unpublished data), some modifications were made, including the addition of sterol-extracted torula yeast, sterol-extracted non-fat dry milk, a vitamin mix, and sorbic acid (Jing, 2011).

In total, 4 different sterols/steroids were used (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), while stigmasterol and cholestanone were purchased from Steraloids Inc. (Newport, RI, USA). Because performance was significantly reduced on the modified tobacco plants containing high concentrations of cholestanol and cholestanone, we categorize these two sterols/steroids as "bad"; in contrast, cholesterol and stigmasterol promote strong performance, so these two sterols are categorized as "good". We have used this shorthand description in previous papers (Behmer and Elias, 1999a,b, 2000; Behmer and Grebenok, 1998; Jing et al., 2013, 2012b). The sterol profiles of the 12 different diets, including the amounts of each sterol in each diet (mg/g), are shown in Table 1. The first four diets were single-sterol/steroid diets, with each sterol/steroid added at a concentration of 1 mg/g dry mass (Table 1a). The next five diets (Table 1b) contained stigmasterol (a "good" sterol) paired with cholestanol or cholestanone (both considered "bad" sterols/steroids). The final three diets contained stigmasterol, cholestanol and cholestanone, in various amounts and ratios (Table 1c). The ratios of "good" sterol to "bad" sterol/steroid used in this study covered a range of possibilities, including the ratio reported for the modified tobacco lines (Jing et al., 2012a). In the triple sterol/steroid diets, we used a 2:1 cholestanone:cholestanol ratio because this is the ratio found in the modified tobacco lines (Jing et al., 2012a).

Insects were reared on these diets for two generations. Upon hatching neonates were transferred individually to small rearing chambers (1 oz. plastic condiment cups (Fabri-Kal)) containing diet (for each treatment, $n = 60$). Each cup had a lid punctured with small holes to allow air movement. All the caterpillars were then transferred to a Percival incubator (Model # I66VLC8, Percival Scientific, Inc) set at 27 °C with a 14:10 light:dark cycle. We examined each container every three days to record development and mortality. When caterpillars reached the last instar, they were observed daily to accurately record pupation time. The sex and mass of each individual was recorded 3 days after pupation. Upon eclosion, 10 mating pairs (1 female and 2 males) were established in 32 oz. plastic deli cups (11.5 cm tall); egg production and egg viability were scored using methods outlined in (Jing et al., 2012a). Occasionally mating pairs did not separate following copulation; these individuals were not included in the statistical analysis.

To determine whether there was any paternal dietary sterol/steroid effect, a second generation was reared on the same paternal diet (we did this for both species). The rearing procedure and data collection followed that used in the first generation. Six random neonates, produced from each of the 10 maternal pairs, were used as the starting material for the second generation ($n = 60$ individuals per sterol treatment); these neonates were reared on the same diet as their parents.

2.3. Statistical analysis

Larval development was analyzed by non-parametric survival analysis; dead larvae were censored. A Log-rank test was used in our analysis because it tends to be more sensitive, relative to a Wilcoxon test, to distributional difference late in time (Martinez and Naranjo, 2010); this was the case for our two lepidopteran species. Similar methods were used for pupal development. Larval survival and adult survival (i.e., pupation and eclosion) followed binomial distributions, so were analyzed using Likelihood Ratio Chi-Square statistics. Pupal mass, egg production and egg viability was analyzed by ANOVA; no difference was observed in pupal mass between males and females. For all analysis, we set the type I error rate at 0.05. Additionally, the False Discovery Rate, which controls the expected proportion of falsely rejected hypotheses, was used to make adjustments where multiple comparisons were performed (Benjamini and Hochberg, 1995). All statistical analyses were performed using SAS v. 9.2 (Cary, NC, USA).

3. Results

The effects of the different dietary sterol treatments on two larval traits (days to pupation and survival to pupation), three

Table 1
Dietary sterol/steroid combinations in experimental foods, including concentrations (mg/g dry mass) and ratios. Four different sterols/steroids – cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K) – were used to construct 12 unique diets. For the triple sterol/steroid treatments, the "bad" sterol/steroid portion was a 1:2 mix of cholestanol and cholestanone.

Diet treatments	Sterol/steroid					
	Cholesterol (C) (mg/g)	Stigmasterol (S) (mg/g)	Cholestanol (A) (mg/g)	Cholestanone (K) (mg/g)	Dietary ratio of "good" to "bad" sterol/steroid	Total (mg/g)
(1a) Single sterol/steroid	1.0	–	–	–	–	1.0
	–	1.0	–	–	–	1.0
	–	–	1.0	–	–	1.0
	–	–	–	1.0	–	1.0
(1b) Double sterol/steroid	–	0.25	1.75	–	1:7	2.0
	–	1.0	–	1.0	1:1	2.0
	–	0.5	–	1.5	1:3	2.0
	–	0.25	–	1.75	1:7	2.0
	–	1.0	–	3.0	1:3	4.0
(1c) Triple sterol/steroid	–	1.0	0.33	0.67	1:1	2.0
	–	0.5	0.5	1.0	1:3	2.0
	–	0.25	0.58	1.17	1:7	2.0

Table 2
Statistical analysis for *H. virescens* and *H. zea* larval and pupal performance, plus reproduction, between the 12 diet treatments (see Table 1). Analyses were conducted for each generation separately. The statistical method used for each variable is reported in the methods.

	H. virescens		*H. zea*	
Variable	First generation	Second generation	First generation	Second generation
Larval development (days)	$\chi^2_{10} = 307.9$ ($P < 0.001$)	$\chi^2_{10} = 124.0$ ($P < 0.001$)	$\chi^2_{11} = 252.3$ ($P < 0.001$)	$\chi^2_{10} = 99.8$ ($P < 0.001$)
Survival to pupation (%)	$\chi^2_{11} = 406.0$ ($P < 0.001$)	$\chi^2_{10} = 136.6$ ($P < 0.001$)	$\chi^2_{11} = 155.4$ ($P < 0.001$)	$\chi^2_{11} = 52.2$ ($P < 0.001$)
Pupal mass (mg)	$F_{10,594} = 10.5$ ($P < 0.001$)	$F_{10,407} = 7.4$ ($P < 0.001$)	$F_{11,579} = 11.3$ ($P < 0.001$)	$F_{10,439} = 13.7$ ($P < 0.001$)
Pupal development (days)	$\chi^2_{10} = 50.3$ ($P < 0.001$)	$\chi^2_{9} = 51.7$ ($P < 0.001$)	$\chi^2_{11} = 92.5$ ($P < 0.001$)	$\chi^2_{10} = 37.3$ ($P < 0.001$)
Survival to eclosion (%)	$\chi^2_{11} = 335.9$ ($P < 0.001$)	$\chi^2_{10} = 140.6$ ($P < 0.001$)	$\chi^2_{11} = 151.8$ ($P < 0.001$)	$\chi^2_{11} = 51.9$ ($P < 0.001$)
Egg production (number/female)	$F_{10,101} = 6.8$ ($P < 0.001$)	$F_{8,80} = 3.0$ ($P = 0.006$)	$F_{11,85} = 6.2$ ($P = 0.003$)	$F_{10,66} = 3.0$ ($P = 0.003$)
Egg viability (% of eggs hatching)	$F_{10,101} = 5.4$ ($P < 0.001$)	$F_{8,80} = 3.0$ ($P = 0.006$)	$F_{11,85} = 2.3$ ($P = 0.015$)	$F_{10,66} = 4.9$ ($P < 0.001$)

pupal traits (pupal mass, days from pupation to eclosion, and survival to eclosion), and two adult traits (egg production and egg viability) were analyzed statistically. For each caterpillar, and for each generation, significant treatment effects were observed for each physiological trait (Table 2). The outcomes of these analyses are outlined below. We start with a comparison of the single sterol/steroid treatments, transit to inspection of the double sterol/steroid treatments, and conclude with coverage of the triple sterol/steroid treatments.

3.1. Single-sterol/steroid treatment

There were three particularly notable results from the single sterol treatments. First, for both *H. virescens* and *H. zea*, there were no dramatic differences in larval, pupal, or reproductive performance on the cholesterol and stigmasterol treatments (Figs. 2–7). Second, the effects of cholestanol on 1st-generation *H. virescens* (Figs. 2 and 4) and *H. zea* (Figs. 3 and 5) larval and pupal performance did not differ dramatically compared to the cholesterol and stigmasterol treatments. Two exceptions to this were that *H. virescens* larvae had slightly increased larval development, and pupae were slightly smaller. However, cholestanol did affect 1st-generation reproduction in both *H. virescens* (Fig. 6) and *H. zea* (Fig. 7) – egg production and egg viability were both significantly reduced (Figs. 6 and 7). In the 2nd-generation, only 5% of the *H. virescens* caterpillars from these eggs reached the pupal stage, and all failed to successfully eclose. Slightly more than half of the 2nd-generation *H. zea* caterpillars pupated on the cholestanol diet, but only half of these eclosed (and they were smaller relative to 2nd-generation pupae from the cholesterol and stigmasterol treatments). Those that did eclose produced eggs (in numbers similar to cholesterol and stigmasterol reared moths), but none of these eggs were viable. Third, cholestanone was a very poor steroid for both insects. All 1st-generation *H. virescens* caterpillars reared on this diet died before pupating, while all 2nd-generation *H. zea* caterpillars died before pupating. In the first generation, cholestanone-reared *H. zea* caterpillars showed extended larval development, and decreased pupation success relative to the cholesterol and stigmasterol diets (Fig. 3). The caterpillars that did pupate were smaller and had very low eclosion success (Fig. 5); females that eclosed and mated produced small egg batches with low viability (Fig. 7).

3.2. Double sterol/steroid mixture treatment

In these treatments stigmasterol was mixed with cholestanol in a 1:7 ratio, or with cholestanone in one of four different combinations (see Table 1). Performance on these treatments was compared to the cholesterol and stigmasterol treatments.

We first start with the single diet that mixed stigmasterol and cholestanol (the S 0.25 + A 1.75 treatment). For *H. virescens*, in both generations, performance on this diet was similar to that on the cholesterol-only and stigmasterol-only treatments (Figs. 2, 4 and 6). The 1st-generation performance of *H. zea* was also similar on these three diets (Figs. 3a, 5 and 7), with the exception of pupal development, which was slightly extended (Fig. 3b). However, 2nd-generation *H. zea* reared on the S 0.25 + A 1.75 treatment showed greatly reduced performance relative to caterpillars reared on the cholesterol-only and stigmasterol-only treatments. Larval development was longer (Fig. 3a), pupal mass was lower (Fig. 5a) and eclosion success was reduced (Fig. 5b). The most notable effect was related to egg viability, which was virtually zero (Fig. 7b).

Next we present results on diets containing stigmasterol and cholestanone. Generally, overall performance relative to the cholesterol-only and stigmasterol-only treatments was greatly reduced on diets with a 1:7 ratio, weakly reduced on diets with a 1:3 ratio, and unaffected when the ratio was 1:1 (Figs. 2–7). On the 1:7 diets, both species showed extended larval development, plus reduced pupation and eclosion success (in both generations); *H. zea* showed reduced mass in the 2nd-generation. With respect to reproduction, *H. virescens* showed reduced egg production and egg viability in both generations, while 2nd-generation *H. zea* showed reduced egg viability. For both species 2nd-generation egg viability was near zero (Figs. 6b and 7b). When diets contained S + K in a 1:3 ratio, fewer differences were observed relative to the cholesterol-only and stigmasterol-only treatments. Furthermore, these differences were not consistent between the species. For *H. virescens*, negative effects were only seen in the 2nd-generation, and only when the total sterol/steroid concentrations were high (the S 1.0 + K 3.0 treatment). Here pupal mass (Fig. 4a), eclosion (Fig. 4c), egg production (Fig. 6a) and egg viability (Fig. 6b) were all reduced. For *H. zea*, negative performance was observed in the 1st-generation, but never in the 2nd-generation. In the 1st-generation larval duration was slightly extended regardless of total concentration; on the diet with a lower concentration, 1st-generation pupation and eclosion success was reduced relative to the cholesterol-only and stigmasterol-only treatments.

3.3. Triple-sterols/steroids mixture treatment

Here three sterols/steroids were paired – one "good" sterol (stigmasterol) plus two "bad" sterols/steroids (cholestanol and cholestanone). We again examined three "good" to "bad" ratios (as above), with the "bad" component being a 2:1 mixture of

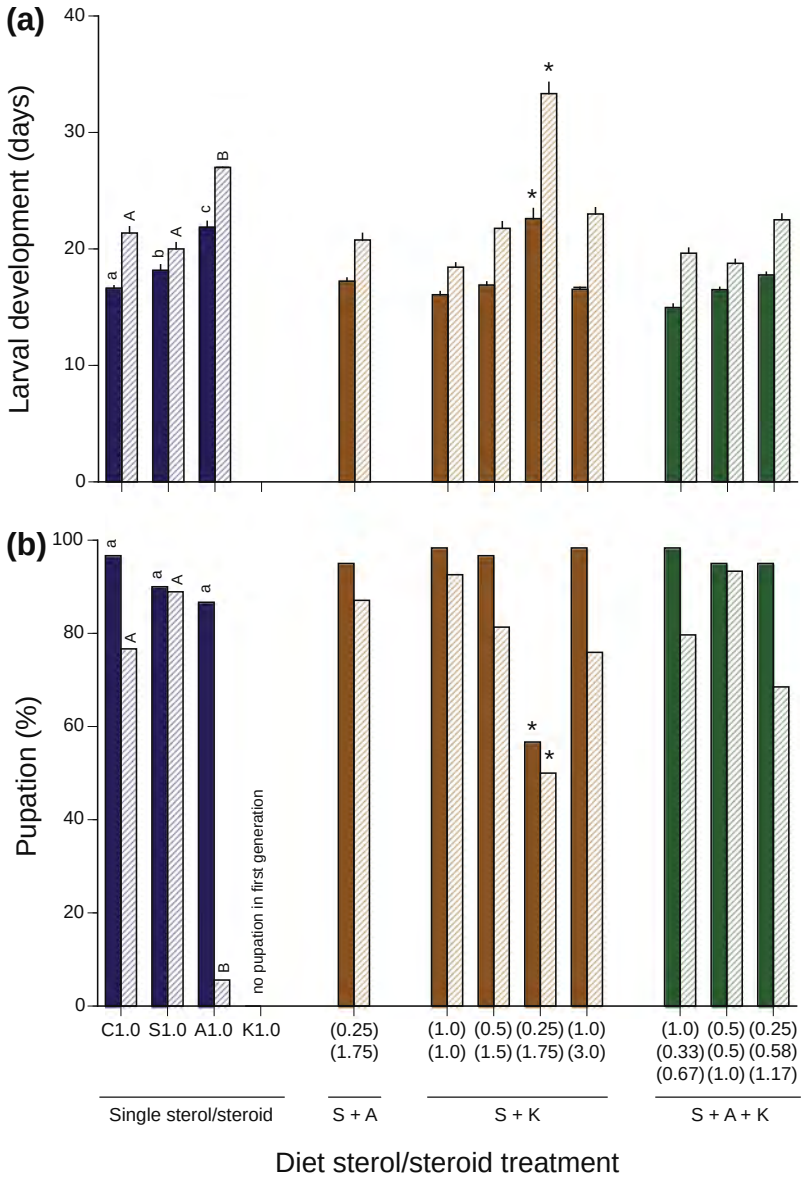

Fig. 2. Larval development time (a) and pupation (b) of *H. virescens* on diets containing different dietary sterols/steroids. Data [means ± SE for (a), and as a percent for (b)] are shown for two generations (1st-generation = solid bars, 2nd-generation = stippled bars). Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 12 diets represented on this figure. The first four treatments were single steroid diets (blue bars), with each steroid at a concentration of 1 μg/g (dry mass). The next five treatments (orange bars) represent diets with two steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the last three treatments (green bars) represent diets with three steroids (stigmasterol, cholestanol and cholestanone). The concentration of each steroid, in each diet with multiple sterols, is shown in parentheses below each pair of bars. Different lower case letters above bars indicate statistically significant differences in the 1st generation between the single sterol treatments; different upper case letters above the single sterol diets indicate statistically significant differences in the 2nd generation. An asterisk above a diet containing multiple sterols represents a statistically significant difference relative to either the cholesterol (C1.0) or stigmasterol (S1.0) treatments (for that particular generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cholestanol to cholestanone. For both *H. virescens* and *H. zea*, larval and pupal performance on the 1:1 and 1:3 mixtures was similar to that on the choleseterol-only and stigmasterol-only treatments. In contrast, the two species exhibited different responses on the 1:7 mixture; *H. virescens* was relatively unaffected, in both generations, while 2nd-generation *H. zea* showed extended development (Fig. 3b), had smaller body size at pupation (Fig. 5a), and suffered from reduced pupation (Fig. 3b) and reduced eclosion (Fig. 5c) success. With respect to egg production, two of these diets affected *H. virescens* egg viability in the 1st generation, but not the 2nd generation. In contrast, *H. zea* suffered from reduced egg viability only in the 2nd generation.

4. Discussion

There was little difference in survival, growth and reproduction for *H. virescens* and *H. zea* reared on diets containing cholesterol or stigmasterol, which isn't surprising given that biochemical studies have shown both species efficiently metabolize stigmasterol to cholesterol (Jing, 2011; Jing et al., 2012b). Most lepidopteran studies that have compared cholesterol and stigmasterol utilization showed similar performance responses (reviewed by Behmer and Nes, 2003), likely based on the ability of most caterpillars to readily metabolize stigmasterol to cholesterol (Ritter and Nes, 1981b; Svoboda et al., 1969, 1988; Svoboda and Weirich, 1995). However,

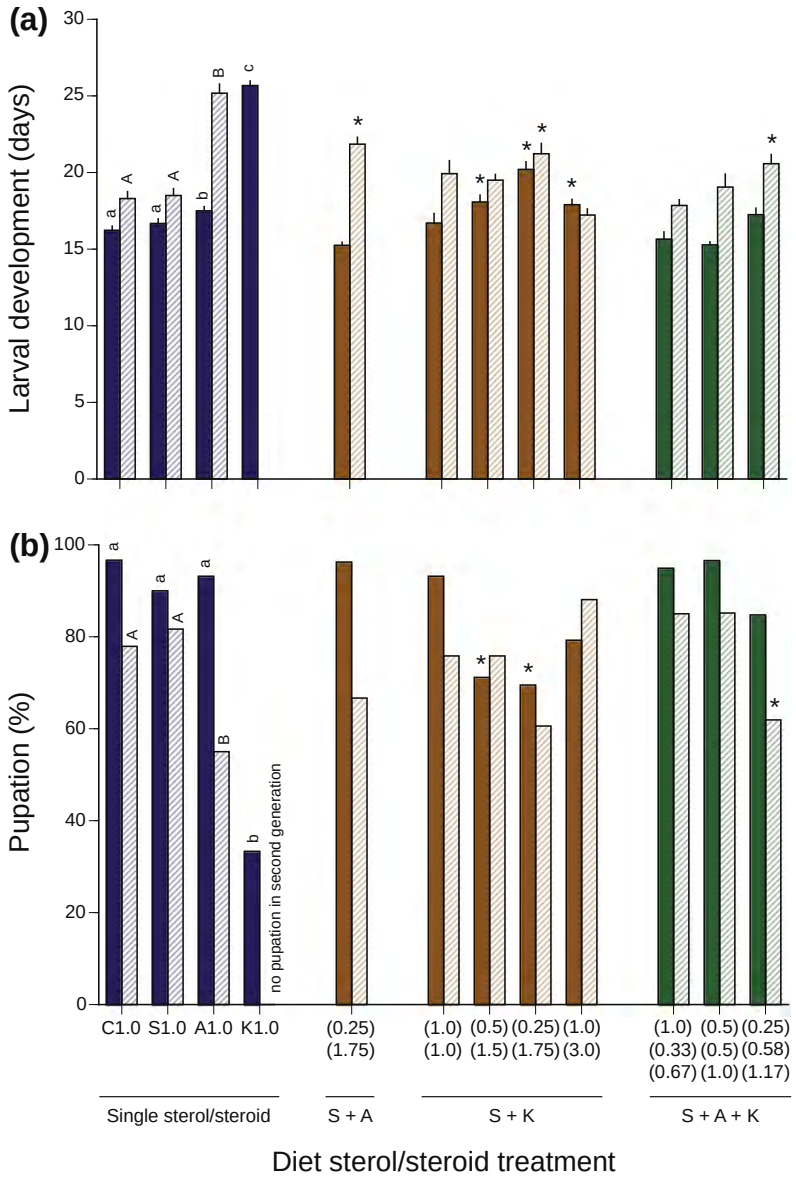

Fig. 3. Larval development time (a) and pupation success (b) of *H. zea* on diets containing different dietary sterols/steroids. Data [means ± SE for (a), and as a percent for (b)] are shown for two generations (1st-generation = solid bars, 2nd-generation = stippled bars). Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 12 diets represented on this figure. The first four treatments were single steroid diets (blue bars), with each steroid at a concentration of 1 μg/g (dry mass). The next five treatments (orange bars) represent diets with two steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the last three treatments (green bars) represent diets with three steroids (stigmasterol, cholestanol and cholestanone). The concentration of each steroid, in each diet with multiple sterols, is shown in parentheses below each pair of bars. Different lower case letters above bars indicate statistically significant differences in the 1st generation between the single sterol treatments; different upper case letters above the single sterol diets indicate statistically significant differences in the 2nd generation. An asterisk above a diet containing multiple sterols represents a statistically significant difference relative to either the cholesterol (C1.0) or stigmasterol (S1.0) treatments (for that particular generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

some insect herbivores do poorly when fed stigmasterol diets. This is true for grasshoppers (Behmer and Elias, 1999a, 2000) which cannot metabolize stigmasterol to cholesterol (Behmer et al., 1999). Likewise, the pea aphid (Bouvaine et al., 2012) and green peach aphid (Bouvaine et al., 2014), show depressed reproduction (but no effect on juvenile growth or adult longevity) when reared on stigmasterol-only diets.

Cholestanol differs from cholesterol in one specific way – it lacks a C5 double bond (Fig. 1). However, because cholestanol has stereochemistry that is similar to cholesterol, membranes containing cholestanol can function like those containing cholesterol. This likely explains why in many insects (e.g., beetles, cockroaches, flies) cholestanol supports good juvenile growth (reviewed by Behmer and Nes, 2003), as long as a minimum supply of cholesterol is available to serve as the required precursor for steroid hormones (Clark and Bloch, 1959; Clayton, 1964; Dutky et al., 1967). For our two species cholestanol supports relatively normal growth and developmental (through to eclosion) in the 1st generation, but it negatively affects egg production and viability. We know from previous work that our two species cannot generate cholesterol from cholestanol (Jing, 2011; Jing et al., 2013; Jing et al., 2012b), so we suspect that reduced reproductive performance is linked to a deficiency in cholesterol as the essential precursor for steroid hormones, which drives important physiological events at multiple time points during embryo development (Costet et al., 1987). Interestingly, 2nd-generation *H. zea* neonates from eggs of 1st-generation moths performed

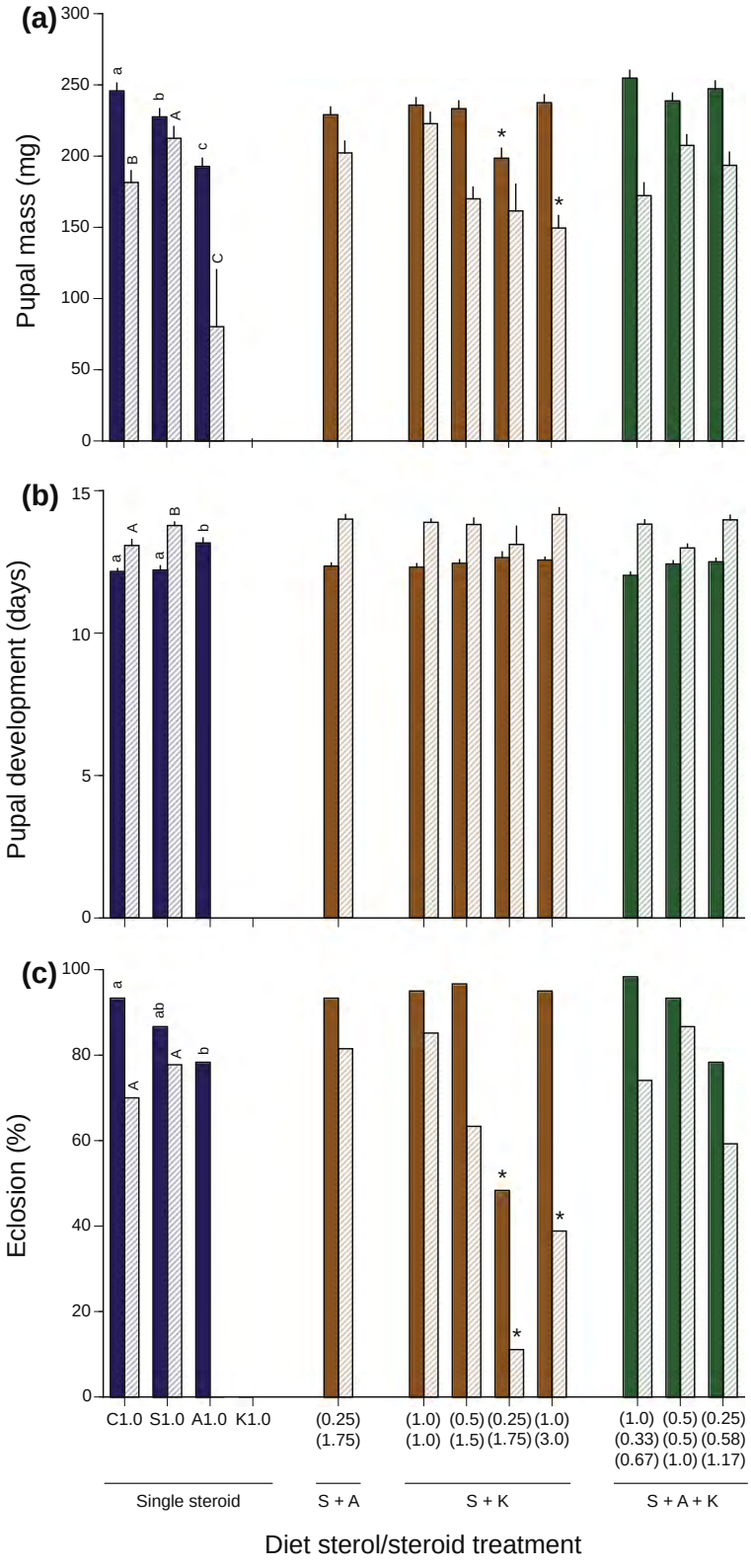

Fig. 4. Pupal mass (a), pupal developmental time (b), and eclosion success (c) of *H. virescens* on diets containing different dietary sterols/steroids. Data [means ± SE for (a) and (b), and as a percent for (c)] are shown for two generations (1st-generation = solid bars, 2nd-generation = stippled bars). Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 12 diets represented on this figure. The first four treatments were single steroid diets (blue bars), with each steroid at a concentration of 1 μg/g (dry mass). The next five treatments (orange bars) represent diets with two steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the last three treatments (green bars) represent diets with three steroids (stigmasterol, cholestanol and cholestanone). The concentration of each steroid, in each diet with multiple sterols, is shown in parentheses below each pair of bars. Different lower case letters above bars indicate statistically significant differences in the 1st generation between the single sterol treatments; different upper case letters above the single sterol diets indicate statistically significant differences in the 2nd generation. An asterisk above a diet containing multiple sterols represents a statistically significant difference relative to either the cholesterol (C1.0) or stigmasterol (S1.0) treatments (for that particular generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

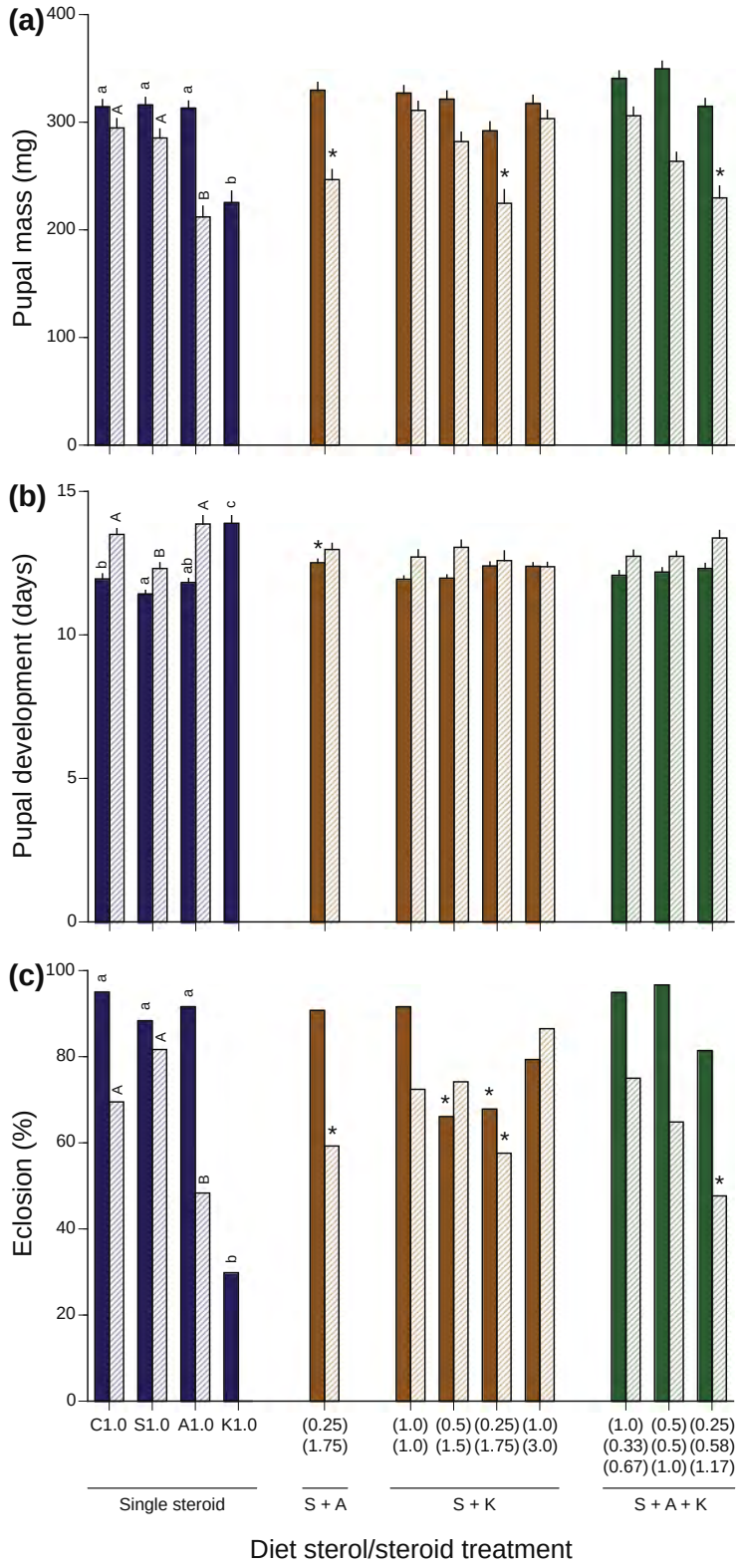

Fig. 5. Pupal mass (a), pupal developmental time (b), and eclosion success (c) of *H. zea* on diets containing different dietary sterols/steroids. Data [means ± SE for (a) and (b), and as a percent for (c)] are shown for two generations (1st-generation = solid bars, 2nd-generation = stippled bars). Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 12 diets represented on this figure. The first four treatments were single steroid diets (blue bars), with each steroid at a concentration of 1 μg/g (dry mass). The next five treatments (orange bars) represent diets with two steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the last three treatments (green bars) represent diets with three steroids (stigmasterol, cholestanol and cholestanone). The concentration of each steroid, in each diet with multiple sterols, is shown in parentheses below each pair of bars. Different lower case letters above bars indicate statistically significant differences in the 1st generation between the single sterol treatments; different upper case letters above the single sterol diets indicate statistically significant differences in the 2nd generation. An asterisk above a diet containing multiple sterols represents a statistically significant difference relative to either the cholesterol (C1.0) or stigmasterol (S1.0) treatments (for that particular generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

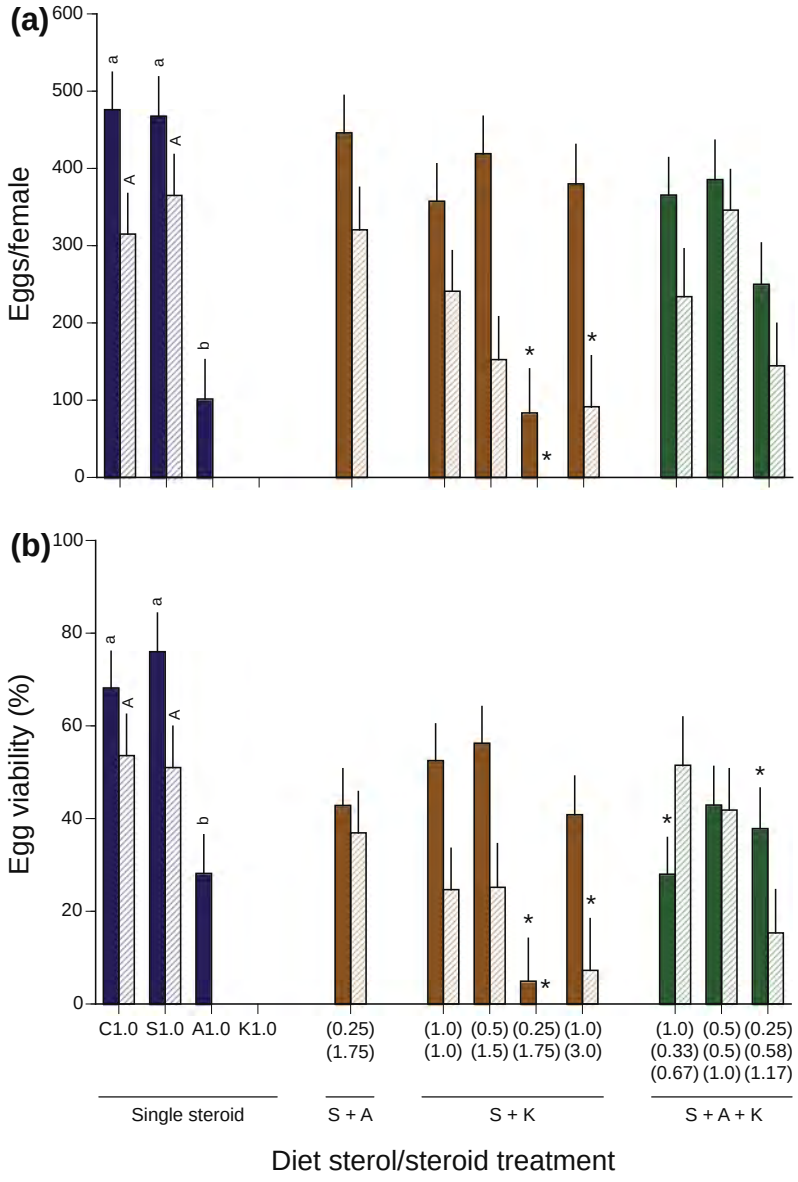

Fig. 6. Egg production (a) and egg viability (b) of *H. virescens* on diets containing different dietary sterols/steroids. Data, presented as means (±SEM), are shown for two generations (1st-generation = solid bars, 2nd-generation = stippled bars). Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 12 diets represented on this figure. The first four treatments were single steroid diets (blue bars), with each steroid at a concentration of 1 μg/g (dry mass). The next five treatments (orange bars) represent diets with two steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the last three treatments (green bars) represent diets with three steroids (stigmasterol, cholestanol and cholestanone). The concentration of each steroid, in each diet with multiple sterols, is shown in parentheses below each pair of bars. Different lower case letters above bars indicate statistically significant differences in the 1st generation between the single sterol treatments; different upper case letters above the single sterol diets indicate statistically significant differences in the 2nd generation. An asterisk above a diet containing multiple sterols represents a statistically significant difference relative to either the cholesterol (C1.0) or stigmasterol (S1.0) treatments (for that particular generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

slightly better than did similarly reared *H. virescens* neonates (30% and 5% eclosion, respectively). This suggests that closely related species can vary in their responses to dietary sterols. However, the requirement for cholesterol for steroid production eventually catches up with *H. zea*, as egg viability effectively drops to zero in the 2nd generation.

Cholestanone, relative to cholestanol, differs in one specific way – it has a ketone, rather than a hydroxyl, at the C3 position. But cholestanone as a dietary steroid has extreme negative effects for both species. In the 1st generation, all *H. virescens* neonates failed to eclose, while only 30% of the 1st-geneation *H. zea* neonates eclosed; in the 2nd generation, all *H. zea* neonates failed to pupate. But why were the negative affects so much stronger for *H. virescens*? One explanation is tied to differential metabolism of cholestanone to epicholestanol (a 3α-hydroxyl isomer) and cholestanol (a 3β-hydroxyl isomer) (Jing et al., 2013; Jing et al., 2012b). Specifically, when *H. virescens* metabolizes cholestanone, it produces more epicholestanol than cholestanol; the opposite is observed in *H. zea*. As a membrane insert, a 3α-hydroxyl isomer (epicholestanol,) does not function as well as a 3β-hydroxyl isomer (cholestanol) (Demel and De Kruyff, 1976) leading to membrane instability. It is also the case that both species retain significant amounts of cholestanone (Jing et al., 2013, 2012b), which could be problematic in two ways. First, insertion of cholestanone into cell membranes might affect membrane dynamics. Second, because cholestanone is structurally similar to 3-dehydroecdysone

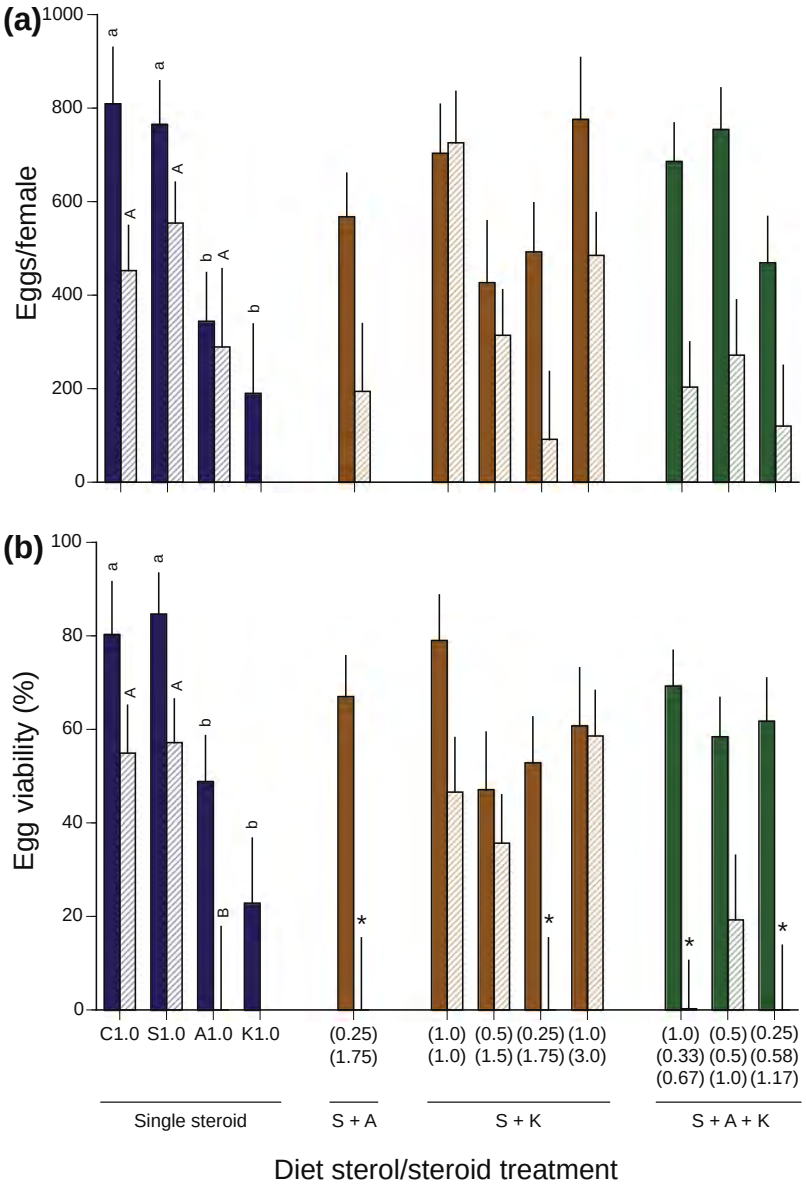

Fig. 7. Egg production (a) and egg viability (b) of *H. zea* on diets containing different dietary sterols/steroids. Data, presented as means (±SEM), are shown for two generations (1st-generation = solid bars, 2nd-generation = stippled bars). Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestanone (K)] were used to make the 12 diets represented on this figure. The first four treatments were single steroid diets (blue bars), with each steroid at a concentration of 1 μg/g (dry mass). The next five treatments (orange bars) represent diets with two steroids (stigmasterol paired with cholestanol, or stigmasterol paired with cholestanone); the last three treatments (green bars) represent diets with three steroids (stigmasterol, cholestanol and cholestanone). The concentration of each steroid, in each diet with multiple sterols, is shown in parentheses below each pair of bars. Different lower case letters above bars indicate statistically significant differences in the 1st generation between the single sterol treatments; different upper case letters above the single sterol diets indicate statistically significant differences in the 2nd generation. An asterisk above a diet containing multiple sterols represents a statistically significant difference relative to either the cholesterol (C1.0) or stigmasterol (S1.0) treatments (for that particular generation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Gilbert, 2004), it might disrupt physiological processes by acting as a hormone mimic. Dietary cholestanone certainly impacts gene regulation in *H. zea* (Jing et al., 2012b), particularly genes linked to immune responses. In reality, poor caterpillar performance on the cholestanone-only diets is likely the outcome of a combination of the aforementioned factors.

Plants, including our transgenic tobacco plants (Heyer et al., 2004), always contain a mixture of sterols (Benveniste, 2002, 2004) – so the likelihood of generating plants that contain only bad sterols/steroids (e.g., cholestanol or cholestanone) is low. Importantly, many studies have shown that sterols/steroids are passively absorbed by insects (Behmer et al., 1999; Jing et al., 2012a,b, 2013). A key question, therefore, is what proportion of a plant's sterol profile must be "bad" for insect herbivore performance to be negatively affected? In the grasshopper *Schistocerca americana* a 1:1 ratio of "good" (cholesterol) to "bad" (sitgmasterol) dietary sterol significantly reduced survival to the adult stage (Behmer and Elias, 1999a). In contrast, our two lepidopteran species reared on a 1:1 diet (stigmasterol + cholestanone) performed similarly to those reared on cholesterol- and stigmasterol-only diets. We suspect that this equivalent performance is tied to insect tissue sterol profiles. Jing et al. (2013) showed that *H. virescens* reared on diets with a 1:1 stigmasterol:cholestanone ratio had relatively high cholesterol levels (~60%); the remaining profile being a mixture of stigmasterol (4%), cholestanol (5%) and epicholestanol (31%). However, performance in our current study

steadily worsened as the ratio of cholestanone in the diet increased. Insects are known to incorporate non-cholesterol sterols into their cells (Behmer and Nes, 2003), but we suspect that all insects must maintain a threshold level of cholesterol. The causal link between cholesterol levels and reduced performance is suggested by comparing results in the current study with the sterol profile data. For example, Jing et al. (2013) showed that cholesterol levels in *H. virescens* dropped to 40% and 27% on diets with 1:3 and 1:7 stigmasterol:cholestanone ratios, respectively. Cholesterol is also an important component of eggs, where it serves both structural and metabolic roles (Costet et al., 1987); its concentration in eggs/embryos of Orthoptera and Lepidoptera is 10–100 times of that in larvae or pupae (Lafont et al., 2005). Ecdysteroids are actively involved in vitellogenesis and the uptake of yolk protein (Swevers et al., 2005), and deficiencies can result in embryonic lethality (Gilbert, 2004). Our results suggest that diets with high ratios of "bad" dietary sterols/steroids have significant negative effects on reproduction, especially egg viability.

Two other key findings emerged from the treatments pairing stigmasterol with cholestanone. First, *H. virescens*, compared to *H. zea*, was more negatively affected as the ratio of cholestanone in the diet increased (especially eclosion success). This likely reflects differences in how the two species metabolize cholestanone, and again suggests the superiority of incorporating a 3β-hydroxyl isomer (cholestanol), rather than a 3α-hydroxyl isomer (epicholestanol), into cell membranes. It also again suggests that responses to plant sterol profiles can vary between even closely related insect herbivore species. Second, patterns of larval, pupal, and reproductive performance were comparable for insects reared on diets with similar ratios (1:3), but different absolute amounts of dietary sterols/steroids (the S + K treatments, with total sterol/steroid concentrations of 2 mg/g and 4 mg/g, respectively). Previous studies with grasshoppers (Behmer and Elias, 1999a, 2000) have shown that the ratio of dietary sterol/steroid, not the absolute amount, is the critical issue because sterol/steroid uptake is not selective (Behmer and Elias, 2000). Jing et al. (2013) has reported similar sterol/steroid uptake findings for *H. virescens*.

When cholestanol was paired with stigmasterol, *H. virescens* performed better than *H. zea*. This clearly indicates the type of "bad" sterol in the diet matters, and reemphasizes the point that different species can respond to sterol mixtures in very different ways. But why is *H. zea* so negatively affected by dietary cholestanol, especially considering *H. zea* seems to perform fine with high relative concentrations of cholestanol (from the metabolism of cholestanone) in its tissues (Jing et al., 2012b)? Previous work has shown that dietary cholestanol reduces total sterol levels in our two species (Jing et al., 2013, 2012b); stanols (as a class) do this by inhibiting sterol absorption (Allayee et al., 2000; Berge et al., 2000). Perhaps stanols inhibit sterol absorption in *H. zea* to a greater extent? In terms of performance, the most pronounced effect was seen on *H. zea* egg viability in the 2nd generation, suggesting the need of a metabolic source of cholesterol for the production of ecdysteroids that drive egg development. On a related note, multiple studies have suggested that cholestanol can act as a sparing sterol when dietary cholesterol concentrations are low (reviewed by Behmer and Nes, 2003). However, no previous study has examined the effectiveness of cholestanol as a sparing sterol over multiple generations. Our data suggest that it has mixed effects as a sparing sterol, particularly over the longer term, and especially related to reproduction.

Finally, we tested the effects of a three sterol/steroid mixture, which represents the profile observed in transgenic tobacco plants that negatively affect lepidopteran growth (Jing et al., 2012a). In the current study, we did not observe any major differences in larval or pupal development for *H. virescens* on the three different ratios (1:1, 1:3, and 1:7), but pupation success, pupal mass, and eclosion success for *H. zea* were negatively impacted in the 2nd generation on the 1:7 treatment (S [0.25] + A [0.58] + K [1.17]). This pattern is similar to that observed for *H. zea* on the stigmasterol plus cholestanol treatment (1:7), and suggests that for *H. zea* high relative cholestanol dietary concentrations are problematic. The effect of dietary cholestanol on *H. zea* is also seen in the egg viability data (plus there is a pattern of reduced egg production, although not statistically significant).

In summary, our results demonstrate that: (1) stanols and ketosteroids (the dominant phytosterol/steroids identified in the modified tobacco that we used in an earlier study (Jing et al., 2012a)) have strong negative effects on lepidopteran performance; (2) lepidopteran performance tends to decrease as the ratio of unsuitable sterols increases; and (3) there is variation between our two species in their response to different phytosterol/phytosteroid treatments. The inability of insect herbivores to make sterols, coupled with constraints on their ability to use particular types of sterols/steroids, suggests modifying plant sterol profiles of important agricultural plants could be used as a novel approach to manage insect herbivores, including hemipteran insects (Behmer et al., 2011, 2013; Janson et al., 2009). Importantly, our study also confirms it is not necessary to remove all "good" phytosterols from plants. Instead, the critical issue is having the ratio of atypical sterols/steroids exceed a particular threshold.

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