



# 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 stigmaterol) 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 sterols (cholestanol and cholestanone) was extremely poor compared to suitable sterols (cholesterol and stigmaterol). Additionally, performance tended to decrease as the ratio of the novel dietary sterols 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.

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## 1. Introduction

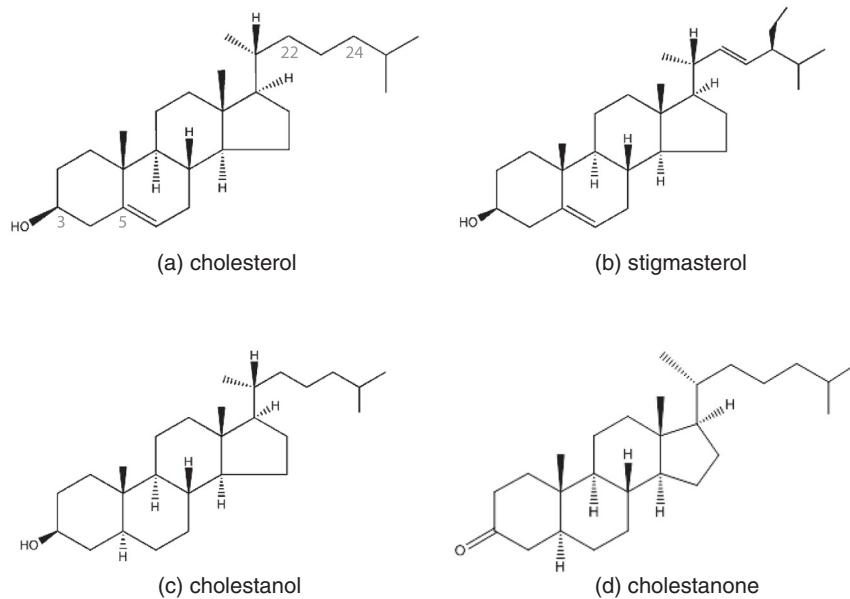
Insects, like all eukaryotes, use sterols for three key purposes. First, they are structural components in cell membranes, where they modulate and regulate permeability, thermomechanics, lateral diffusion, and protein function (Mouritsen and Zuckermann, 2004). Second, they are critical precursors for 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 sterols (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

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