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Plant sterols and host plant suitability for generalist and specialist caterpillars

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ABSTRACT

Insects, unlike plants and vertebrates, lack the ability to biosynthesize sterols. Cholesterol is typically the most common sterol found in plant-feeding insects, but it is rarely found in plants above trace levels, so plant-feeding insects must produce the cholesterol they need by metabolizing the sterols found in the plants they eat. Plant-feeding insects are, however, often limited in terms of which sterols can be converted to cholesterol. In the current study we used a transgenic tobacco plant line that displays high levels of atypical plant steroids, specifically stanols and ketone-steroids, to explore how novel steroid structural features affect performance in three economically important caterpillars (*Heliothis virescens*, *Spodoptera exigua*, and *Manduca sexta*). For each species we measured pupation success, larval development, pupal mass, pupal development, and eclosion success. For the two generalists species (*H. virescens* and *S. exigua*) we also measured egg production and egg viability. We then used these eggs to replicate the experiment, so that we could examine the effect of parental steroid dietary history on survival, growth and reproduction of 2nd-generation individuals. Significant negative effects of novel steroids on larval and pupal performance were observed for each caterpillar in the first generation, although these were often subtle, and were not consistent between the three species. In the second generation, larval survival estimated by 'pupation number/plant' on the tobacco plants with novel steroids was significantly reduced, while eclosion success was significantly lower for *H. virescens*. With respect to adult reproduction (i.e. egg production and egg viability) there were no observed differences in the first generation, but novel steroids significantly negatively impacted reproduction in the second generation. The findings from this study, when integrated into a simple population growth model, demonstrate the potential in using plants with modified steroids as a novel approach to manage populations of economically important caterpillar species.

1. Introduction

Sterols serve two key physiological functions in insects (Behmer and Nes, 2003; Grieneisen, 1994). First they provide a structural role in cellular membrane, stabilizing the phospholipid bilayer. Second, they are essential precursors for several physiologically active metabolites, i.e. ecdysteroids. Additionally, studies reveal that cholesterol is actively involved in hedgehog signaling which controls cell proliferation and differentiation (Briscoe and Therond, 2005; Porter et al., 1996). Insects, however, cannot synthesize their own sterols (as plants and mammals do), and therefore must acquire sterols from their diets. Cholesterol (Fig. 1a) is the most common sterol found in insects (Behmer and Nes, 2003), but in plants cholesterol typically occurs at low concentrations (Piironen et al., 2000). The two most common and abundant plant sterols are sitosterol (Fig. 1b) and stigmasterol (Fig. 1c). Plant sterols typically differ from cholesterol in that they have a methyl or ethyl group at the C24 position and/or a double bond at the C22 position. Some plant sterols also have a C7 double bond (e.g., spinasterol) rather than a C5 double bond (as found in cholesterol, sitosterol and stigmasterol). With the exception of grasshoppers, which are severely limited in their phytosterol use (Behmer and Elias, 1999; Behmer et al., 1999), most chewing herbivorous insects that have been studied can convert most phytosterols with C5 double bonds into cholesterol (Behmer and Nes, 2003; Feldlaufer and Svoboda, 1988; Svoboda, 1999).

Artificial diets have been an critical tool for studying sterol use in insects (Dadd, 1957, 1960; Kodicek and Levinson, 1960; Monroe, 1959; Noland, 1954), providing, among other things, information on how variation in sterol structure affects insect performance, what amounts are needed to support growth and development, and the extent to which insects can metabolize different types of dietary sterols (reviewed in Behmer and Nes (2003)). In contrast, relatively few studies have attempted to manipulate dietary sterols in real foods, especially in plants. Costet et al., (1987, 1989) using the systemic fungicide fenpropimorph, modified the sterol profile in wheat (replacing typical wheat sterols with 9β,

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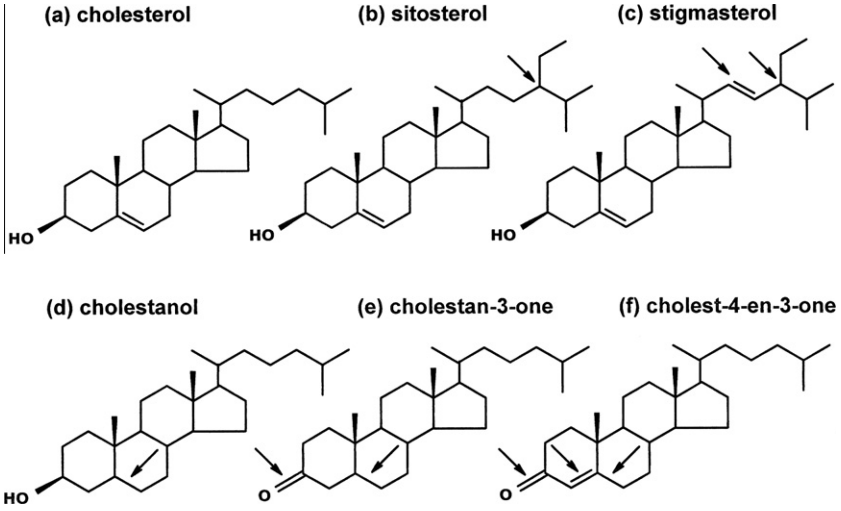

Fig. 1. Cholesterol and five typical sterols/steroids in our modified plants. Cholesterol (a), the most common sterol in insects, is seldom found in plants. Two phytosterols, sitosterol (b) and stigmasterol (c), are common sterols in plants. Both sitosterol and stigmasterol have a C24 ethyl group; stigmasterol also has a C22 double bond. The modified tobacco plants contain these three sterols, plus stanols and ketone-steroids. Cholestanol (d) is a stanol; stanols lack double bonds in the sterol nucleus. Cholestan-3-one (e), and cholest-4-en-3-one (f), are ketone-steroids. Ketone-steroids have a C3 ketone instead of a C3 hydroxyl, and sometimes have double bonds in the sterol nucleus (e.g., cholest-4-en-3-one).

19-cyclopropylsterols (up to 95% of total sterols)). They fed this fenpropimorph-treated wheat to grasshoppers, and found that it altered nymphal development, decreased ecdysteroid biosynthesis in adult females, and led to arrested embryonic development. More recently, Behmer et al. (2011) used genetically modified tobacco plants (Corbin et al., 2001) that express high levels of two non-sterol classes of steroids – stanols and 3-ketone-steroids – to explore how phytosteroid structures affect aphids. Stanols (e.g., cholestanol (Fig. 1d)), lack double bonds in the sterol nucleus. The defining feature for a ketone-steroid is a ketone at the C3-position (e.g., cholestan-3-one (Fig. 1e)); in some instances ketone-steroids contain a double bond in the sterol nucleus (e.g., cholest-4-en-3-one). Behmer et al. (2011) showed these atypical sterols occurred in the vegetative tissue and phloem, and that green peach aphids (*Myzus persicae*) reared on plants expressing high levels of these atypical steroids suffered reduced survival, growth and fecundity.

In the current study we use the same genetically modified tobacco lines described above to explore how plant steroid profile affects three caterpillar species. Two of these species, *Heliothis virescens* and *Spodoptera exigua*, are generalists that feed on a broad range of host plants, including tobacco. The third species, *Manduca sexta*, is a specialist on solanaceous plants. For each species we reared caterpillars from hatching to eclosion, and measured a range of larval and pupal traits (e.g., survival, mass gain, development time). For the two generalists we also collected data on reproduction. We then used the eggs from these mated adults as a source for neonates to explore parental dietary steroid effects. Finally, we employed a simple population model (Behmer and Grebenok, 1998), using the data generated from our generalist caterpillars, to estimate the effect of modified phytosteroid profiles at the insect population level.

2. Materials and methods

2.1. Insects

Three caterpillar species that have been recorded as feeding on tobacco were used for this study. Two of these, *H. virescens* (Noctuidae) and *S. exigua* (Noctuidae), are generalists that feed on a wide range of plants. The third species, *M. sexta* (Sphingidae), specializes on plants in the Solanaceae, most notably tomato and tobacco. The *H. virescens* and *S. exigua* caterpillars used in these experiments came from eggs purchased from Benzon Research Inc. (Carlisle, PA); the *M. sexta* caterpillars came from eggs purchased from Carolina Biological Supply Company (Burlington, NC). The hatchlings from these eggs were used as the source for the first generation caterpillars.

2.2. Tobacco plants

Two tobacco (*Nicotiana tabacum*) lines were used (Corbin et al., 2001). The first expressed the chloroplast-targeted 3-hydroxysteroid oxidase gene (pMON33814), from a bacteria, *Actinomyces* sp. A19249. The second, a control tobacco plant, was transformed with a control vector. These two tobacco lines show no morphological differences (Corbin et al., 2001; Heyer et al., 2004). Tobacco plants were grown in potting soil (Metro-Mix 366, Sun Gro Horticulture Canada CM Ltd.) under standard greenhouse conditions at the Borlaug Center at Texas A&M University. Plants were germinated in propagation trays and once established, individual seedlings were transferred to 1-gallon pots. After the plants reached the 5-leaf stage, one top leaf was cut from each plant so that it could be confirmed as having either normal or modified sterols, following methods described in Heyer et al. (2004). Each leaf was individually freeze-dried, pulverized and extracted in chloroform for 24 h. The chloroform was evaporated and the extraction was resuspended in MeOH:water (7:3). This solution was partitioned, three times, with an equal volume of water-equilibrated hexane. The hexane partitions were pooled and dried under nitrogen, and resuspended in 50 μl hexane. A 1 μl volume of this solution was injected into an Agilent 5790 networked gas chromatograph-mass spectrometer maintaining an inlet temperature of 250 °C, a transfer line temperature of 290 °C, and oven programmed from 180 °C to 300 °C (with the initial temperature maintained for 1 min and the final temperature for 20 min, and a ramp rate of 10 °C/min). The column used was a capillary MS-5 column (30 m) (Restek) with a film thickness of 0.25 μm. Helium at a flow rate of 1.25 ml/min served as carrier gas. Chromatograms were analyzed using the HP ChemStation program (Agilent Technologies, Wilmington Delaware) and the steroids were identified by co-chromatography of authentic standard compounds and by comparison to previously analyzed material (Heyer et al., 2004). Ste-

roids were quantified by use of previously generated standard curves for authentic standards (Steraloids, New Port Rhode Island). The steroid profiles for both the control and sterol-modified tobacco plants used throughout this study are shown in Table 1.

2.3. Experimental design

One new fully-extended leaf was cut from each plant upon reaching the 10-leaf stage ($n = 20$). Each individual leaf was then transferred into a clear plastic box (17 × 11.5 × 6 cm) that contained three insect mounting pins (Bioquip insect pins #7 stainless), inverted, and glued to the base of the arena and arranged in the form of an isosceles triangle (each pin was approximately 5–7 cm apart). Moistened paper towel (approximately 16 × 11 cm, three layers thick) was pierced through the three pins so that it rested securely on the floor of the arena; this moistened paper towel helped maintain a constant humidity (~100%), which was necessary to insure good survival for neonate caterpillars. Next the tip of each pin was coated with a small amount of wax. Each leaf was then placed on top of the pins, and received a cohort of 7 (for *H. virescens* and *S. exigua*) or 4 (for *M. sexta*) neonates. All the boxes containing leaves and neonates were packed in a clear 56 quart storage box (Holiday Houseware Inc., Leominster, MA) and four blocks of water saturated sponges (23 × 15 × 4.5 cm) were put on the bottom of the storage box to maintain the humidity. All storage boxes were maintained in a Percival incubator, Model #I66VLC8 (Percival Scientific, Inc.) set at 27 °C with L:D 14:10 and checked daily. Water was reapplied to paper towels and sponges when necessary. After caterpillars had become established on tobacco leaves (4 days for *H. virescens*, 5 days for *S. exigua*, and 2 days for *M. sexta*), the largest larvae were collected and transferred to plants in the greenhouse (in the case of *H. virescens* and *S. exigua*, four of these caterpillars were transferred (per plant); in the case of *M. sexta*, only three caterpillars were transferred to each plant). The individual plant to which caterpillars were transferred was the same one that was the source for the leaf fed to the developing neonates. The caterpillars assigned to a particular plant were equally distributed among the top three non-apical leaves; apical leaves were avoided because they are very glandular, and young larvae often get stuck on these glands and die.

To prevent caterpillars from moving between plants, each plant was housed inside specially designed cages. These cages (45 × 45 × 100 cm) were made using 1/2 in. PVC pipe, and were wrapped in Summerweight Garden Fabric (Gardener's Supply Company (Burlington, VT)), which allows high light transmission (approximately 85%) to the plants. Plants were then examined every 3 days, and the number of caterpillars on each plant was recorded. During the first few days of the experiment it was difficult to find and accurately count caterpillars, but accurate numbers relating to survival could be obtained by recording numbers as insects grew and became bigger.

The methods used to determine the time of pupation differed between the species. For *H. virescens* and *S. exigua* a clear semicircular plastic wrap (diameter: 50 cm) was folded into the shape of a cone around the base of each plant, with the edges of the cone secured using double-sided tape (see Supplementary materials); this approach was necessary to prevent caterpillars from entering the soil to pupate. Each cone was secured at the base of its plant by wrapping a garden twist-tie around a long bamboo stick (approximately 50 cm long); this also helped stabilize the plant. Additionally, inside each cone, at the base, cotton was inserted to seal any gaps that may have allowed the caterpillar to reach the soil. Individuals were collected 2 days after pupation, their sex determined, and their mass recorded. Pupae were transferred individually to 1 oz. Fabri-Kal translucent portion cups with lids. All the pupae were kept in the Percival incubator set at 27 °C with L:D 14:10, and observed daily until they eclosed.

For *M. sexta*, multiple tobacco plants (2–3) were needed for each group of three caterpillars (a single plant was entirely eaten well in advance of pupation). Larvae were collected 3–5 days before the pre-pupal stage (depending upon body size, the head capsule to body size ratio, and feeding behavior) and transferred into 2 oz. Fabri-Kal translucent portion cups that were capped with lids containing five small air holes. All the larvae were kept in the Percival incubator set at 27 °C with L:D 14:10, and were fed leaves from their original host plants until they reached the pre-pupal stage. They were then transferred to 9 oz. SOLO clear cups that contained approximately a half-cup of potting soil (the same used for the plants), where they were allowed to pupate. Three days after pupation, their sex was determined, and their mass recorded.

For *H. virescens* and *S. exigua* (but not *M. sexta*) we explored the effects of different plant sterol profiles on reproduction. Upon eclosion, one female and two males were paired in a 32 oz. plastic deli cup (11.5 cm tall) that was covered with paper towel (20 × 20 cm) that was secured using a rubber band. Inside each container was a 1 oz. Fabri-Kal portion cup that provided a 10% sucrose solution via a cotton wick. Additionally, two paper towel strips (20 × 1.5 cm), upon which females could lay their eggs, hung from the top of the cup (across from each other). The number of mating pairs was dependent on the number of successful eclosions, but in no case were more than 15 mating pairs established. Occasionally mating pairs could not separate following copulation; when this occurred this replicate was removed from the analysis. All the mating cups were kept under L:D 14:10 photoperiod with radiant heat of 28–31 °C during the light phase (supplied by 25 W full spectrum incandescent bulbs) and 26–28 °C during the dark phase. Mating containers were monitored daily and the day on which eggs first appeared was recorded. Females were allowed to lay eggs for 6 successive days from the appearance of the first batch of eggs (typically more than 95% eggs were produced during this 6-day period (unpublished data)). The paper towel cover and strips were changed every 2 days, and transferred to a 28 oz Hefty® white foam bowl (Pactiv Corporation) that contained a ring of tanglefoot® pest barrier (Planet Nature Company) applied to the rim of the bowl.

Table 1
Percentage (mean ± SEM) of different steroids in control and modified tobacco plants. Four individual plants were analyzed for each tobacco line.

Tobacco line	Sterols	3-Ketosteroids	Stanols
Control			
Stigmasterol	37.9 (2.3)	n.d.	n.d.
Campesterol	28.0 (1.4)	n.d.	n.d.
Sitosterol	14.1 (1.4)	n.d.	n.d.
Cholesterol	10.8 (1.6)	n.d.	n.d.
Isofucosterol	9.3 (1.5)	n.d.	n.d.
Total	100.0	–	–
Modified			
Stigmasterol	11.5 (2.2)		
Sitosterol	4.9 (1.9)		
Cholesterol	3.8 (1.5)		
Isofucosterol	3.2 (0.4)		
Campesterol	1.5 (0.5)		
Stigmasta-4,22-dien-3-one		19.7 (1.3)	
Sitostan-3-one		13.5 (1.6)	
Fucostenone		8.6 (0.6)	
Campestan-3-one		4.2 (1.7)	
Sitosta-4-en-3-one		4.1 (2.4)	
Campesta-4-en-3-one		2.1 (1.0)	
Cholestan-3-one		1.0 (0.4)	
Campestanol			11.0 (2.4)
Sitostanol			9.1 (3.4)
Cholestanol			1.8 (0.8)
Total	24.9	53.2	21.9

n.d. = Not detected.

These egg bowls were put under the same condition as the mating pairs and monitored daily. If neonates hatched, the egg towels were removed and transferred to a new bowl ringed with tanglefoot®. This step was repeated daily until no new neonates were recorded. The number of neonates hatching in each bowl was recorded. Three days after no new neonates were observed, the egg towels were examined and the number of unhatched eggs recorded. This data, together with the neonate data, allowed us to measure total egg production and egg viability.

To determine whether there was a parental dietary sterols effect, second generation *H. virescens* and *S. exigua* neonates were reared on the same tobacco line as their parents. The eggs from the mating pairs described above were the source for neonates for the second generation. Only eggs laid on day 2, 3 and 4 were used to reduce possible variation. From each successful mating pair 14 neonates were randomly selected, and split into two cohorts. These cohorts were then transferred to an individual leaf in a small arena, as described above for the first generation. The protocols used for rearing larvae, collecting pupae, and obtaining reproductive data were similar to those described above. To reduce potential inbreeding effects, adults from different families were used for the second generation mating pairs.

2.4. Statistical analysis

Larval and adult survival, measured as the number of individuals/plant pupating and eclosing, respectively, were analyzed using a one-tailed Wilcoxon test (Normal Approximation). These data, despite having a non-normal distribution, had a symmetric distribution and tails that were only slightly heavy; as such we present the summarized data as means with standard errors. Pupal mass was analyzed using a nested ANOVA approach, with individual insects nested within a plant (each plant started with four caterpillars); plants were the experimental unit and the insects on each plant were random subsamples. For larval and pupal developmental time, non-parametric survival analysis was used. The mean and standard error for different treatments generated by the non-parametric survival analysis was presented in the figures. Egg production, scored as number of eggs/female, was analyzed using a one-tailed *t*-test, while egg viability was analyzed using one-tailed Satterthwaite *t*-test. All analyses were was performed in SAS v. 9.2 (Cary, NC, USA).

3. Results

3.1. Larval, pupal and adult reproduction performance (first generation)

Seven separate measures of performance on the control and modified tobacco plants were recorded: survival to pupation, time from hatch to pupation, pupal mass, time from pupation to eclosion, survival through to eclosion, egg production and egg viability. For *M. sexta*, no eggs were collected from the mating pairs reared on the control or the modified plants.

The three caterpillar species tested responded differently to the control and modified tobacco lines (Fig. 2). For *H. virescens*, larval and pupal development time were significantly longer on the modified tobacco, and somewhat surprisingly pupae on the modified tobacco plants were significantly heavier than those reared on control tobacco. For *S. exigua*, eclosion success was the only performance variable that differed, being higher on the control tobacco plants. For *M. sexta*, the only performance measure that differed on the two tobacco lines was larval survival, and it was significantly higher on the control tobacco plants.

Egg production and egg viability were used to evaluate the reproduction ability of the insects on the two tobacco lines. No significant difference, for either variable, was observed for *H. virescens* and *S. exigua* were reared on the two tobacco lines (Fig. 3).

3.2. Larval, pupal and adult reproduction performance (second generation)

The seven measures used in the 1st generation were also recorded in the 2nd generation, but only information for *H. virescens* and *S. exigua* are presented here because no eggs from *M. sexta* were collected.

At a superficial level many of the patterns observed in the first generation held in the second generation, although there was one major difference (Fig. 4). Larval survival, for both *H. virescens* and *S. exigua* was significantly reduced on the modified plants. For *H. virescens*, pupal mass and eclosion success was significantly better on the control plants, but steroid plant profile did not significantly affect larval or pupal development time. Interestingly, *S. exigua* pupal mass was significantly higher on the modified tobacco plants, but larval and pupal development time were longer on the modified tobacco plants. Eclosion success was better on the control plants, although the difference was not statistically significant.

In contrast to the 1st generation, reproduction in both *H. virsecens* and *S. exigua* was affected by plant type (Fig. 5). Insects reared on the control plants produced significantly more eggs. Furthermore, egg viability on the control plants was higher for both species, although the difference was only marginally significant for *S. exigua*.

4. Discussion

Most studies exploring sterol/steroid use in insects have employed an artificial diet approach because it allows tight control of dietary content (reviewed in Behmer and Nes (2003)). To our knowledge only three studies (two using grasshoppers (Costet et al., 1987, 1989), the other using aphids (Behmer et al., 2011)) have explored how modifying sterol profiles in plants affects insect performance. It is also the case that most studies exploring sterol use in insects are restricted to a single generation, which may miss important information on transgenerational effects (Behmer and Grebenok, 1998). In the current study we used transgenic plants, which maintained altered sterol/steroid profiles, to document the sterol/steroid affects on two generalists, and one specialist caterpillar. Our results demonstrate that exposure to novel dietary steroids can negatively affect larval, pupal, and reproductive traits in both generalist and specialist caterpillars. However, these negative effects express themselves with greater frequency in the second generation, especially with respect to egg production and viability. The strong generational effect suggests a critical role for parental sterol allocation to eggs.

There was a general trend of reduced pupal survival, longer larval and pupal development, and reduced eclosion success on the modified tobacco in the first generation, but significant differences were observed in only a handful of instances, and each caterpillar had its own unique response on the modified tobacco. That the performance in the first generation was not reduced to a greater extent on the modified plants was somewhat surprising, but we suspect two contributing factors. First, not all of the steroids in our modified plants were atypical – almost 25% of the total sterol profile is phytosterol (i.e. cholesterol, sitosterol, campesterol and stigmasterol), which is generally easily converted to cholesterol by our three caterpillars (Behmer and Nes, 2003). Additionally, almost 22% of the total sterol profile is stanol (i.e. cholestanol, campestanol and sitostanol), and these later two stanols are readily

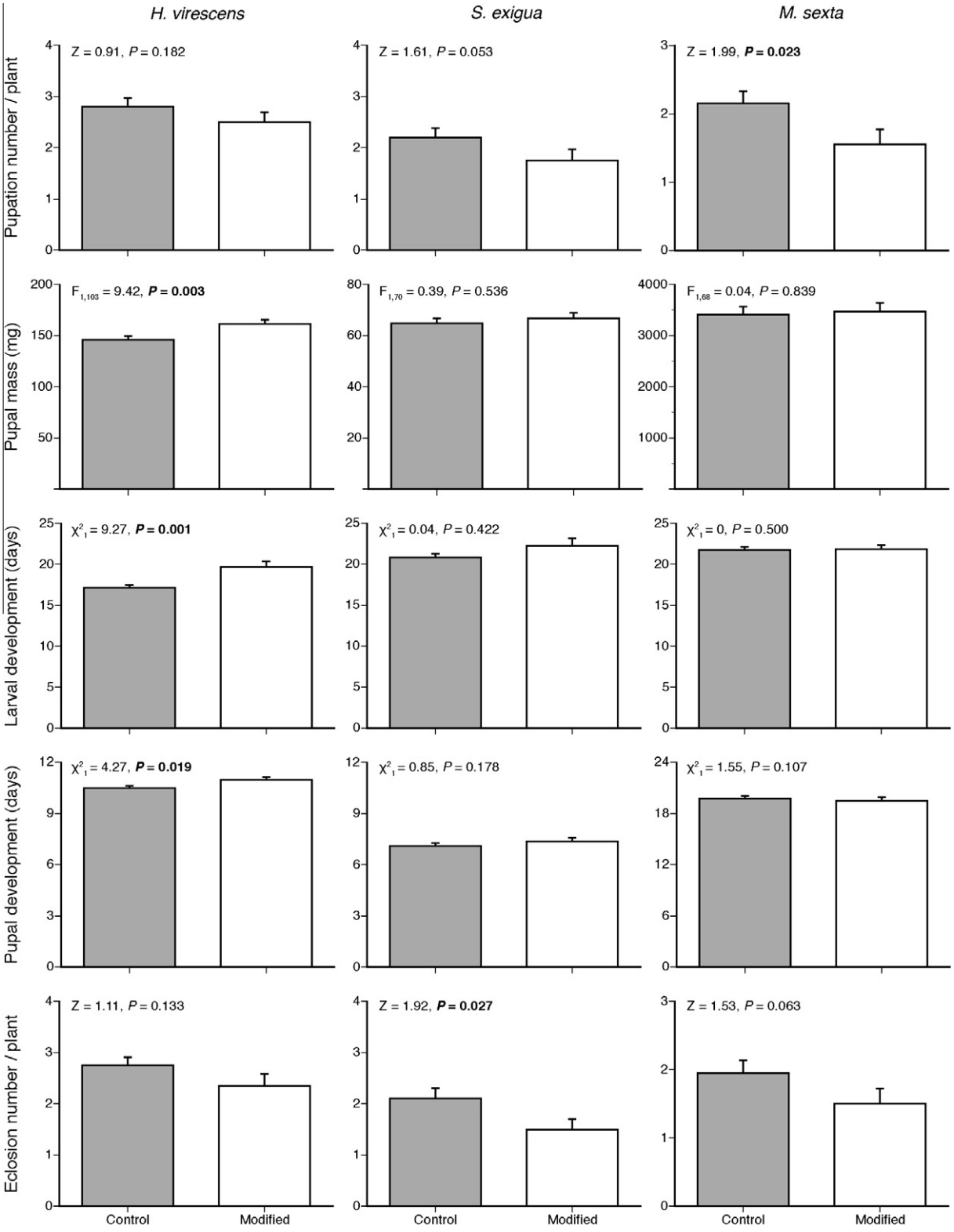

Fig. 2. Larval and pupal performance of *H. virescens*, *S. exigua*, and *M. sexta* on control and modified tobacco in the first generation. For each species we recorded pupation success (number pupating ± SEM; for *H. virescens* and *S. exigua* there were four individual neonates/plant at the start of the experiment, for *M. sexta* there were three individual neonates/plant), pupal mass (mg ± SEM), larval developmental time (days ± SEM), pupal developmental time (days ± SEM) and eclosion success (number eclosing ± SEM). Test statistics and their associated *P*-values, for each variable, are given on each individual panel.

converted to cholestanol by removal of their alkyl groups at C24 (as happens during the conversion of the campesterol and sitosterol into cholesterol (Ritter, 1984)). When cholestanol is the only dietary sterol available to insects it rarely supports strong growth, but when it is combined with a small amount of cholesterol (used for a metabolic purpose, as the required precursor for the molting hormone, 20-OH ecdysone) insect growth is often quite similar to diets that contain only cholesterol (Behmer and Nes, 2003). Thus,

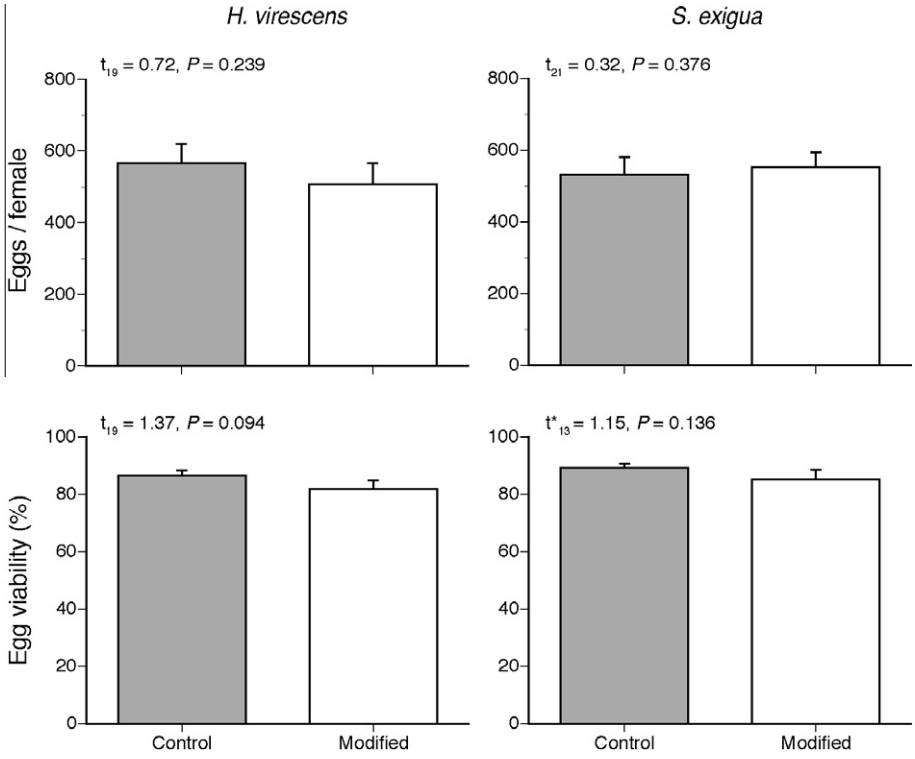

Fig. 3. Reproductive performance of *H. virescens* and *S. exigua*, on control and modified tobacco in the first generation. For both species we recorded egg production (number of eggs (±SEM) produced over the first six days that females produced eggs) and egg viability (number of eggs hatched/total number of eggs (±SEM)). Test statistics and their associated *P*-values, for each variable, are given on each individual panel. An asterisk indicates Satterthwaite's approximate *t* test was used for the egg viability analyses because the two treatments had unequal variance.

almost half of the steroid profile in the modified plants is usable for our caterpillars. The remaining portion in our modified plants was 3-ketosteroids, which do not support strong growth in insects, even when small amounts of cholesterol are available (Jing, 2011). When "good" sterols are limiting, insects may utilize a sterol sparring mechanism. In this situation, non-cholesterol sterols can be incorporated into cell membranes, with cholesterol being saved/sparred for a metabolic role (Clayton, 1964). However, the extent to which sterol sparring mechanisms are broadly practiced in insects is poorly understood. Cockroaches (Clayton and Edwards, 1961; Lasser et al., 1966) and houseflies (Dutky et al., 1967) show the strongest sterol sparring mechanism; they can grow on diets containing high ratios of non-cholesterol sterols, as long the diet contains a small amount of cholesterol (as a precursor for molting hormone). In plant-feeding insects, sterol sparring mechanisms are less efficient. The generalist caterpillar *Heliothis zea* can grow and survive (~70%) on diets that contain a 50:50 mixture of cholesterol and 24-dihydrolanosterol (which alone does not support growth), but growth and development cease as the dietary ratio of 24-dihydrolanosterol is increased (Nes et al., 1997). Generalist grasshoppers require an even higher proportion of good sterol in their diets; *Schistocerca americana* shows reduced survival as the proportion of good sterol in diet drops below 75% (Behmer and Elias, 1999, 2000).

The second factor that may have aided good growth and survival on the modified plants was parental sterol allocation to eggs. In contrast to the first generation, we observed significant differences in a number of larval and pupal traits on the two tobacco lines in the second generation. A similar generational effect was also observed when different dietary sterols were fed to diamondback moth larvae reared for two successive generations on artificial diets (Behmer and Grebenok, 1998). In both our study, and the diamondback moth study, the key difference between the two generations was the parental diet. Specifically, 1st-generation caterpillars in both studies came from parents reared on diets containing only "good" sterols. As demonstrated by Costet et al. (1987), parental dietary sterol profile directly affects egg sterol profile; adult female grasshoppers fed wheat containing phytosterols that could not be converted to cholesterol produced eggs with significantly reduced cholesterol titres (50%) compared to eggs from grasshoppers reared on wheat with typical sterol profiles. Although we did not analyze the sterol content of our caterpillar eggs, based on Costet's study we suspect that the cholesterol titres of eggs from caterpillars reared on the modified plants would have been lower compared to the cholesterol titres in eggs from normal tobacco plants. We suppose that such a reduction, when combined with the less than optimal dietary sterol profile of the modified tobacco, may have compromised any possible sterol sparring mechanism, and that this best explains why larval and pupal performance was significantly reduced in the second generation.

Interestingly, we also observed a generational effect with respect to egg production and viability, providing additional evidence of an interaction between dietary sterols and parental sterol allocation to eggs. Sterols, particularly cholesterol, can affect insect reproduction in two important ways. First, egg production (oogenesis) can be negatively affected when there is a dietary sterol deficiency (Halaweish et al., 1999), or when an insect eats a diet containing a high ratio of "bad" sterols (Behmer and Grebenok, 1998; Costet et al., 1987). Reduced egg production is most likely mediated through low ecdysteroids titres, due to low cholesterol titres in reproductive females (Dong et al., 2009; Nijhout, 1994; Shaaya et al., 1993; Shirk et al., 1990). Second, cholesterol is abundant in eggs, where it is used for structural purposes in developing embryos (Kinsella and Smyth Jr., 1966), and as the precursor to ecdysteroids that regulates embryogenesis (Truman and Riddiford, 2002). Individual caterpillar eggs are tiny compared to last stadium

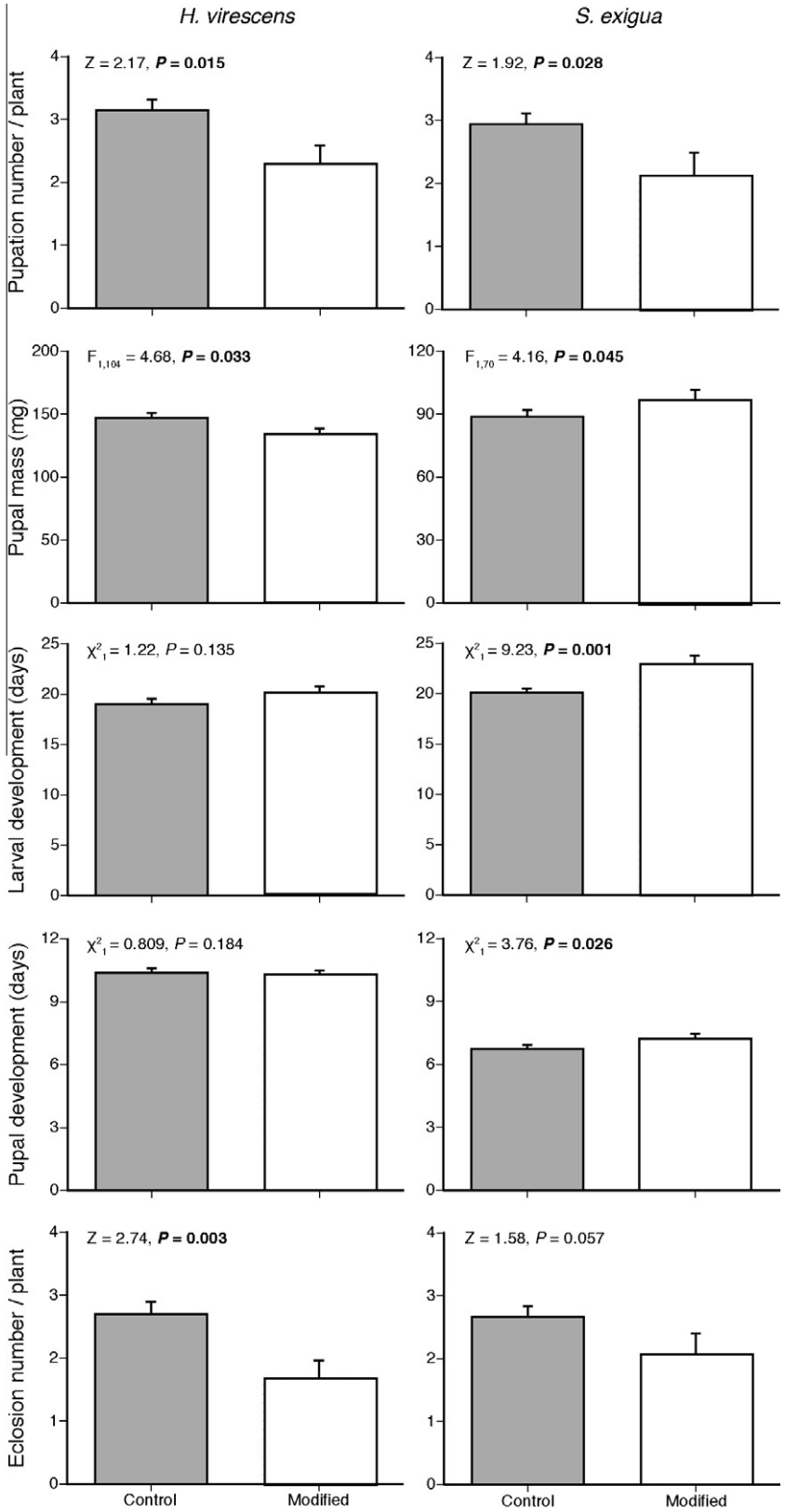

Fig. 4. Larval and pupal performance of *H. virescens* and *S. exigua* on control and modified tobacco in the second generation. For both species we recorded pupation success (number pupating ± SEM; there were four individuals/plant at the start of the experiment), pupal mass (mg ± SEM), larval developmental time (days ± SEM), pupal developmental time (days ± SEM) and eclosion success (number eclosing ± SEM). Test statistics and their associated *P*-values, for each variable, are given on each individual panel.

larvae, or adults, so it is amazing that parental sterol contribution to an egg could have such a dramatic effect on reproductive traits. Because metabolic requirements for cholesterol are much smaller than structural requirements, our best deduction is that the sterol

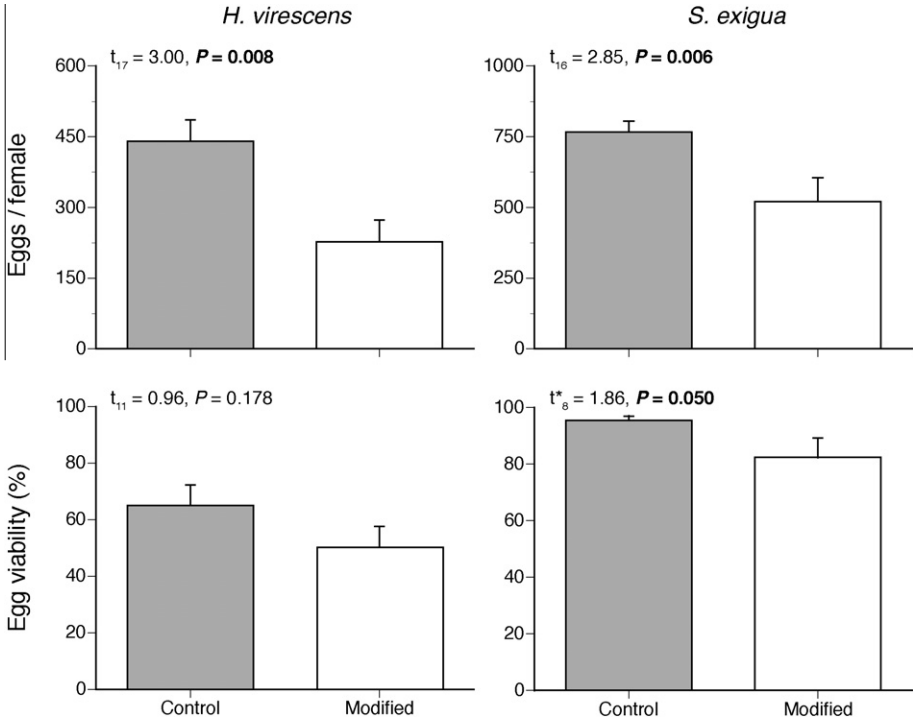

Fig. 5. Reproductive performance of *H. virescens* and *S. exigua*, on control and modified tobacco in the second generation. For both species we recorded egg production (number of eggs (±SEM) produced over the first six days that females produced eggs) and egg viability (number of eggs hatched/total number of eggs ± SEM). Test statistics and their associated *P*-values, for each variable, are given on each individual panel. An asterisk indicates Satterthwaite's approximate *t* test was used for the egg viability analyses because the two treatments had unequal variance.

contribution for metabolic purposes (i.e. ecdysteroid production) is responsible. We imagine that the sterol source from our first clutch of eggs (used to start the experiment), combined with the usable sterols in the modified tobacco, was sufficient for 1st-generation moths to produce a full batch of eggs, and that these eggs contained enough cholesterol to produce ecdysteroid titers that supported high hatching success. Nonetheless, we imagine that the cholesterol and ecdysteroid titers in these eggs would have been reduced compared to levels in eggs used as the source of neonates at the start of the experiment. Although any reduction in cholesterol and ecdysteroid titers must have been small (given the observed egg viability rate), our 2nd-generataion egg production data suggests the reduction was large enough to trigger a negative effect. Clearly an important next step in this research is to measure sterol profiles (especially cholesterol content) and ecdysteroid titers in the eggs of moths reared over successive generations on our modified tobacco plants, and correlate it with egg hatching success.

A somewhat unexpected larval performance result was that pupal mass, in two instances, was greater on the modified tobacco plants (*H. virescens* in the first generation, and *S. exigua* in the second generation). In both of these cases larval development was also extended relative to the control plants. Cholesterol is the required precursor to insect molting hormone, and because caterpillars reared on the modified plants had reduced access to cholesterol, compared to caterpillars on the control plants, development may have been delayed as a result of reduced molting hormone titers (Nijhout, 2003). Other important nutrients, particularly protein and digestible carbohydrates, would not have been limiting for caterpillars on the modified tobacco plants. However, if caterpillars practiced compensatory feeding (Behmer, 2009; De Leo et al., 1998) in response to a shortage of appropriate dietary sterols, they may have ingested greater amounts of non-limiting nutrients (e.g., protein and carbohydrates). Utilization of these excess nutrients may possibly explain why insects reared on sterol-poor plants sometimes showed larger body size.

Our data highlight how dietary sterols can affect phytophagous insects at multiple levels (e.g., growth, development, reproduction), but their true impact is most dramatically seen when we take a step back and consider their effects at the population level. Using our survival, sex ratio, egg production and egg viability data from the two different tobacco lines, we used a simple population model to estimate how large populations of our two generalist caterpillars would be at the start of their third generation, when grown on our two tobacco lines (*sensu* Behmer and Grebenok, 1998). For each caterpillar species, and on each tobacco line, we used a starting population of 100 individuals. We also assumed, for simplicity, that there were no biotic or abiotic mortality factors (see Supplementary material for full details concerning the model, and how the values shown in Fig. 6 were generated). The key result to emerge from this exercise is that for both species, population sizes at the end of the second generation were 3–5× smaller on plants containing a modified sterol profile. We also estimated development time for our generalist caterpillars on the two tobacco lines. Here we assumed it takes 2 days to produce eggs following eclosion, and 3 days for the first clutch of eggs to hatch. As shown in Fig. 6, both species are projected to take about one week longer to reach the start of the third generation when reared on the modified tobacco. Where multiple generations of caterpillars occur, this longer developmental time might ultimately result in one less generation per year. Additionally, lengthened development means greater exposure to predators, parasitoids, pathogens and abiotic mortality factors. When these additional factors are considered, the potential impact of using modified sterols as a control method against insect herbivore pests becomes quite spectacular.

The reliance of plant-feeding insects on a dietary source of steroid, and metabolic constraints that they have with respect to the type of steroids that can be converted into cholesterol, or utilized

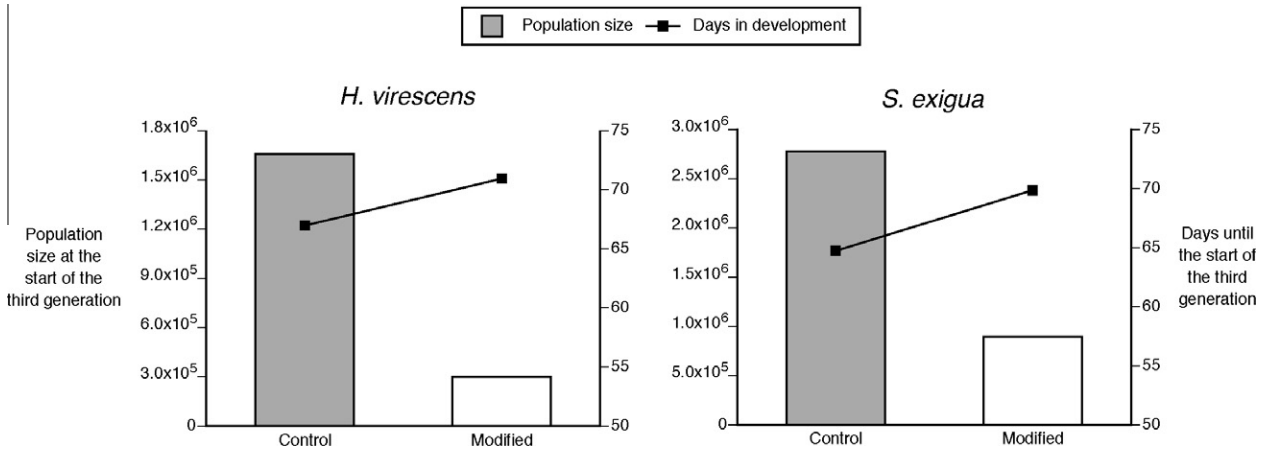

Fig. 6. Estimated population size and developmental time for *H. virescens* and *S. exigua* reared on the two tobacco lines after two generations. These projections use a starting population of 100 individuals and assume no mortality other than that related to the tobacco line (control versus modified). Full details of this model can be found in Supplementary material 1.

in place of cholesterol, has great potential to be exploited for insect pest management. Natural variation in sterol profiles in rice varieties has recently been suggested as a mechanism of resistance to the African rice gall midge (Omoloye and Vidal, 2007), but sterol profiles can also be manipulated using transgenic approaches (Corbin et al., 2001; Heyer et al., 2004). The production of new tools for managing pest insects of crop plants is especially important given the capability of insects to develop resistance to contemporary forms of pest control, including both traditional chemical approaches and transgenic techniques, such as the use of *Bacillus thuringeiensis* (Bt) (Shelton et al., 2002). Given the results from the current study, especially those projected over multiple generations, we envision a modified steroid approach complementing existing insect management practices. Another advantage of using modified plant steroids as a tool to manage insect pests is that it is a target-specific approach – there are no direct negative consequences on beneficial invertebrate predators or parasitoid, nor are there negative effects on livestock or humans, since most steroids are not toxic to vertebrates (Katan et al., 2003; McGowan and Proulx, 2009).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2011.11.013.

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