EFFECTS OF STEROL STRUCTURE ON INSECT HERBIVORE PHYSIOLOGY, BIOCHEMISTRY AND MOLECULAR BIOLOGY

A Dissertation

by

XIANGFENG JING

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Entomology

Effects of Sterol Structure on Insect Herbivore

Physiology, Biochemistry and Molecular Biology

Copyright 2011 Xiangfeng Jing

EFFECTS OF STEROL STRUCTURE ON INSECT HERBIVORE

PHYSIOLOGY, BIOCHEMISTRY AND MOLECULAR BIOLOGY

A Dissertation

by

XIANGFENG JING

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Spencer T. Behmer
Committee Members,	Frederick T. Davies
	Micky D. Eubanks
	Keyan Zhu-Salzman
Head of Department,	David Ragsdale

December 2011

Major Subject: Entomology

ABSTRACT

Effects of Sterol Structure on Insect Herbivore Physiology, Biochemistry and Molecular Biology. (December 2011)

Xiangfeng Jing, B.A., Shandong Agricultural University;

M.S., Huazhong Agricultural University

Chair of Advisory Committee: Dr. Spencer T. Behmer

Sterols serve two important biological functions in animals - they act as cellular membrane components, and as the precursor to steroid hormones. Insects require a dietary source of sterol because they cannot synthesize sterols *de novo*. Cholesterol is the most common sterol in plant-feeding insects, but because plants contain very little cholesterol, plant-feeding insects must convert plant sterols into cholesterol. In this dissertation I investigate the effect of common and novel plant sterols and steroids found in a transgenic tobacco line on several caterpillar species. I also explore the metabolism of these sterols and steroids, and use a microarray approach to identify genes involved in sterol use and metabolism in plant-feeding insects. I also study cholesterol homeostasis using a grasshopper species.

Modified tobacco plants containing a novel sterol profile negatively affected performance of three different caterpillar species, especially in the second generation. Insects reared on modified plants contained less total sterols and cholesterol than those on control plants having a normal sterol profile. Similar results were found using

artificial diets containing atypical steroids, e.g., cholestanol and cholestan-3-one, identified in the tobacco plants that were fed to my experimental caterpillars. More importantly, the sterol/steroid ratio, but not their absolute amount in the diets, determined the negative effects.

Caterpillar species could convert stigmasterol, a common plant sterol, into cholesterol. They could also convert cholestan-3-one into cholestanol and epicholestanol, although this ability varied among different species. A microarray study that focused on gene expression in midgut tissue indicated that stigmasterol, cholestanol and cholestan-3-one could induce different gene expression level, and that cholestan-3-one caused the largest pool of genes to be regulated. The genes possibly involved in the metabolism of stigmasterol and cholestan-3-one were reported. These findings are important in directing further research on the potential application of plant sterol modification to control pests in agricultural systems.

Insect herbivores could behaviorally regulate the intake of several nutrients, but they could not regulate their sterol intake. They did, however, practice cholesterol homeostasis, by post-ingestively regulating tissue sterol levels, even when feeding on diets with high cholesterol content. Collectively, the results from this dissertation provide unique insight into cholesterol regulation, which is difficult to achieve in mammals that are capable of synthesizing their own sterols.

ACKNOWLEDGEMENTS

I heartily thank my advisor, Spencer T. Behmer, for his assistance, guidance and patience during my research. Without his support in various aspects, I would not have been able to finish this dissertation. His philosophy of doing research and insight in science will influence me in my future career.

I sincerely thank Robert J. Grebenok at Canisius College, NY, for his assistance in my research and for the use of his lab. I sincerely thank Keyan Zhu-Salzman, committee member, who allowed me to use her lab resources. I also sincerely thank Heiko Vogel at the Max-Planck Institute of Chemical Ecology in Jena, Germany, for his assistance with the molecular research conducted in this disseration. Each gave me perceptive guidance in my research and important suggestions from experimental design to manuscript writing.

I also sincerely thank Micky D. Eubanks and Frederick T. Davies, committee members, for the improvement of my knowledge in insect ecology, especially insect-plant interaction, and plant physiology which are important for my knowledge as a researcher. Travis Gates provided excellent technical assistance for my research, and the advice and discussion from the members of the Behmer lab, including Steve Cook, Paul Lenhart and Marion Le Gall, were valuable in developing my research. Joshua Jones gave me important advice in my English writing. Finally, I would like to thank my family members. Their love and encouragement made it possible to finish this dissertation.

TABLE OF CONTENTS

Page

ABSTRACT iii

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

LIST OF TABLES xi

CHAPTER

I INTRODUCTION 1

II PLANT STEROLS AND HOST PLANT SUITABILITY FOR GENERALIST AND SPECIALIST CATERPILLARS 7

2.1 Introduction 7
2.2 Materials and Methods 10
2.3 Results 17
2.4 Discussion 24

III BALANCE MATTERS: HOW THE RATIO OF DIETARY STEROIDS AFFECTS CATERPILLAR DEVELOPMENT, GROWTH AND REPRODUCTION 32

3.1 Introduction 32
3.2 Materials and Methods 34
3.3 Results 42
3.4 Discussion 60

CHAPTER Page

IV STEROL AND STEROID METABOLISM PLUS ABSORPTION IN GENERALIST AND SPECIALIST CATERPILLARS 68

4.1 Introduction 68
4.2 Materials and Methods 70
4.3 Results 78
4.4 Discussion 88

V DIETARY STEROLS/STEROIDS AND THE GENERALIST CATERPILLAR *HELICOVERPA ZEA*: PHYSIOLOGY, BIOCHEMISTRY AND DIFFERENTIAL GENE EXPRESSION IN THE MIDGUT 98

5.1 Introduction 98
5.2 Materials and Methods 100
5.3 Results 107
5.4 Discussion 112

VI CHOLESTEROL HOMEOSTASIS IN A GENERALIST GRASSHOPPER, *SCHISTOCERCA AMERICANA* 120

6.1 Introduction 120
6.2 Materials and Methods 122
6.3 Results 127
6.4 Discussion 135

VII CONCLUSION 140

REFERENCES 143

APPENDIX 154

VITA 156

LIST OF FIGURES

FIGURE Page

1.1 Cholesterol and other sterols/steroids of interest 2

2.1 Cholesterol and 5 typical sterols/steroids in our modified plants 8

2.2 Larval and pupal performance of *H. virescens*, *S. exigua*, and *M. sexta* on control and modified tobacco in the first generation.. 20

2.3 Reproductive performance of *H. virescens* and *S. exigua* on control and modified tobacco in the first generation............................ 21

2.4 Larval and pupal performance of *H. virescens* and *S. exigua* on control and modified tobacco in the second generation....................... 23

2.5 Reproductive performance of *H. virescens* and *S. exigua* on control and modified tobacco in the second generation 24

2.6 Estimated population size and developmental time for *H. virescens* and *S. exigua* reared on the two tobacco lines after two generations......... 31

3.1 A total of four sterols/steroids are used in this study 38

3.2 Larval development time (a) and pupation success (b) of *H. virescens* on diets containing different dietary sterols/steroids.. 45

3.3 Pupal mass (a), pupal developmental time (b), and eclosion success (c) of *H. virescens* on diets containing different dietary sterols/steroids.. 47

3.4 Larval development time (a) and pupation success (b) of *H. zea* on diets containing different dietary sterols/steroids................................. 50

3.5 Pupal mass (a), pupal developmental time (b), and eclosion success (c) of *H. zea* on diets containing different dietary sterols/steroids.. 52

FIGURE Page

3.6 Egg production (a) and egg viability (b) of *H. virescens* on diets containing different dietary sterols/steroids................................ 56

3.7 Egg production (a) and egg viability (b) of *H. zea* on diets containing different dietary sterols/steroids................................ 58

4.1 Eight sterols/steroids in this study.. 72

4.2 Mean (± SEM) cholesterol and total body steroid profiles of *H. virescens* adults that had been reared as larvae on diets containing one, two or three steroids, at various concentrations.. 84

4.3 Mean (± SEM) cholesterol and total body steroid profiles of *M.sexta* adults that had been reared as larvae on diets containing one, two or three steroids, at various concentrations.. 87

5.1 Five sterols/steroids in this study 101

5.2 Developmental time (a) and survival (b) of *H. zea* larvae, from hatch to the start of the 3rd-stadium 108

5.3 Eclosion success of *H. zea* pupae reared on diets containing cholesterol (C), stigmasterol (S), cholestanol (A) and cholestan-3-one (K) at a concentration of 1 mg/g (dry mass).. 109

5.4 Venn diagrams showing the number of overlapping and unique 3rd-instar larval midgut genes (a) induced, or (b) suppressed by different dietary sterols/steroids relative to the cholesterol diet 112

6.1 The percent of insect survival at each of 4 stages on 6 sterol concentration diets, i.e., 0, 0.25 mg/g, 0.5 mg/g, 1mg/g, 2 mg/g and 4 mg/g.. 127

6.2 Fresh body mass of male and female insects on different sterol concentration diets.. 129

6.3 Sterol profiles of newly molted 4th-stadium grasshoppers reared on different sterol concentration diets.. 130

FIGURE Page

6.4 Sterol profiles of adults reared on different sterol concentration diets 132

6.5 Median (± MAD) non-sterol nutrients intake per day vs. sterol intake per day for male and female insects from 3rd-stadium grasshoppers to adults on different sterol concentration diets 133

6.6 Total amount of sterol ingested and total amount of sterol excreted in frass on different sterol concentration diets 135

LIST OF TABLES

TABLE Page

2.1 Percentage (mean ± SEM) of different steroids in control and modified tobacco plants 12

2.2 Statistical analysis of 1st-generation data 18

2.3 Statistical analysis of 2nd-generation data 22

3.1 Dietary sterol/steroid combinations, and their concentrations (mg/g dry mass), used in this study 37

3.2 Statistical analysis for *H. virescens* and *H. zea* 42

4.1 Sterol/steroid profiles from *H. virescens* reared on tobacco plants with normal (control) and modified sterol/steroid profiles 78

4.2 Sterol profiles from *M. sexta* reared on tobacco plants with normal (control) and modified sterol/steroid profiles 80

4.3 Body sterol profiles as a percent (mean ± SEM) for *H. virescens* reared on artificial diets containing one, two or three sterols/steroids 81

4.4 Body sterol profiles as a percent (mean ± SEM) for *M. sexta* reared on artificial diets containing one, two or three sterols/steroids 82

5.1 Sterol/steroid content in *H. zea* adults reared on cholesterol, stigmasterol, cholestanol, and cholestan-3-one 111

5.2 Genes that are up-regulated 2-fold or more by the different sterol/steroid diet treatments relative to cholesterol diet 115

CHAPTER I

INTRODUCTION

For both humans and insects, cholesterol is central in many physiological processes, most notably as structural elements in biomembranes and as essential precursors to steroid hormone (Grieneisen, 1994). In contrast to humans, though, insects lack the ability to biosynthesize cholesterol so they require a dietary source of cholesterol (Hobson, 1935).

Plants provide all kinds of nutrients including sterols for plant-feeding insects or insect herbivores. More than 100 different sterols have been identified in plants, and individual plant species always contain multiple types of sterols (Nes, 1977). Plant sterols, i.e., phytosterols, exhibit various structural difference from cholesterol, but primarily they differ in 1) the position and extent of nuclear and side chain unsaturation and 2) the extent of C24-alkylation in the side chain. For example, sitosterol and stigmasterol differ from cholesterol by the presence of an ethyl group at the C24-position and, plus a double bond at the C22-position for stigmasterol (Fig. 1.1). In contrast, cholesterol is rarely found in plants above trace levels so insect herbivores must be able to convert phytosterols into cholesterol to satisfy their physiological requirements.

This dissertation follows the style of Journal of Insect Physiology.

For most insect herbivores, the most abundant phytosterols in their hosts, e.g., sitosterol, campesterol and stigmasterol, are readily converted to cholesterol by dealkylation, and the metabolites in this process are confirmed (Ikekawa et al., 1993; Svoboda and Weirich, 1995). Recently, a reductase (DHCR24) mediating the conversion of desmosterol, the last metabolite in the conversion process from these phytosterols to cholesterol, into cholesterol was identified (Ciufo et al., 2011). However, other enzymes involved in this conversion process are still unknown (Gilbert, 2004).

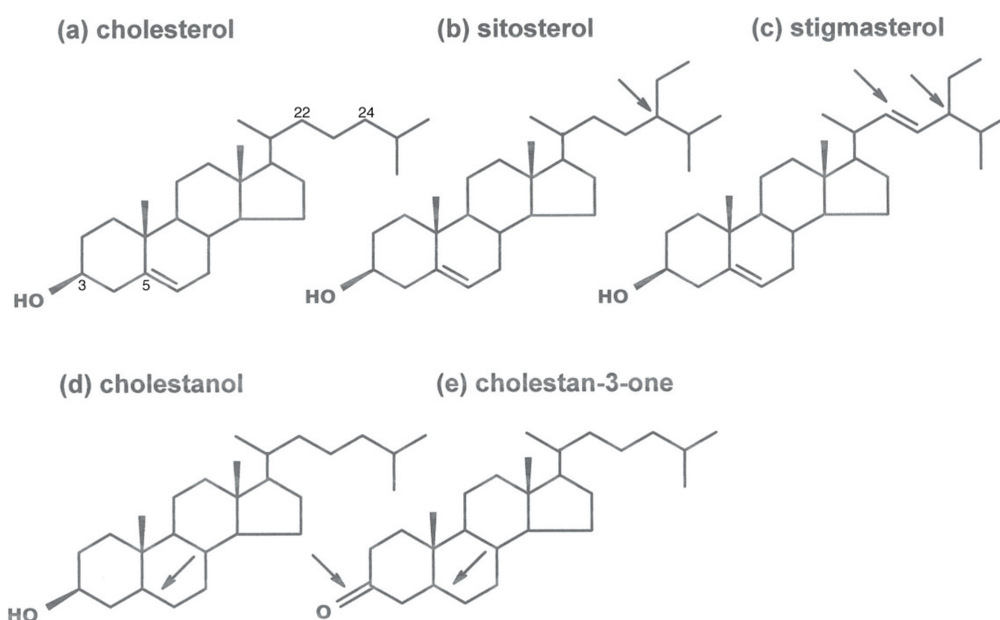


Fig. 1.1. Cholesterol and other sterols/steroids of interest. Cholesterol (a) is the normal insect sterol, but plants rarely contain it. Instead, plants contain cholesterol analogs, e.g., sitosterol (b) and stigmasterol (c), and the arrows indicate structural differences from cholesterol and many insects can remove side-chain alkyl groups and reduce side-chain double bonds at the C22-position. Cholestanol (d) is a stanol; stanols lack double bonds in the sterol nucleus. Cholestan-3-one (e) is a ketone-steroid. Ketone-steroids have a C3 ketone instead of a C3 hydroxyl.

Insect herbivores are, however, often limited in terms of which sterols can be converted into cholesterol and insect performance is poor on these unsuitable sterols (Behmer and Nes, 2003). No insect has reported to be able to insert a double bond at the C5-position, to convert cholestanol (Fig. 1.1) into cholesterol, although they can substitute cholestanol for some cholesterol function, e.g., cellular membrane component, and use the limited cholesterol as other irreplaceable function, e.g., as a precursor to steroid hormones. This is the concept of “essential” and “sparing” sterols, as originally introduced (Clayton, 1964). For example, the fruit fly, *Drosophila melanogaster*, can complete development on a diet containing mostly cholestanol and a trace amount of cholesterol, but not on a diet only containing cholestanol (Kircher and Gray, 1978). Similar findings in other insects, e.g., cockroaches and dermestid beetles caused the earlier researchers to downplay the significance of the dietary sterol composition because it was assumed that strong sparing abilities extended to most, if not all insects (Clark and Bloch, 1959a; Clark and Bloch, 1959b; Dutky et al., 1967; Kuthiala and Ritter, 1988). Recent work suggests, however, that “sparing” ability in insects is not that robust for all sterols. Dutkey found that cholestan-3-one was much worse than cholestanol as the “sparing” component in houseflies (Dutky et al., 1967). Nes *et al.* (Nes et al., 1997), using the caterpillar *Helicoverpa zea*, and Behmer & Elias (Behmer and Elias, 1999b, 2000), using the grasshopper *Schistocerca americana*, found that when the balance of suitable sterols to unsuitable sterols in the diet dropped below to 50% and 70%, respectively, growth rates dropped and mortality increased significantly, even if

the absolute amount of suitable sterols present as the sole dietary sterol supported normal growth and development.

Except for the limited ability in replacing cholesterol, unsuitable sterols may have extensively negative effects on insect herbivores. In plants, sterols are thought to stabilize membranes and modulate the activity of resident membrane enzymes (Cooke et al., 1994; Popp et al., 1995), but the functional significance of variation in phytosterol structure remains unknown. One possibility is that some phytosterols may provide a form of defense against insect herbivores, a suggestion that is supported by the finding that: 1) grasshoppers have behavioral responses that are sensitive to food sterol profiles, and can develop aversion responses, via learning, to foods containing unsuitable sterols (Behmer and Elias, 1999a; Behmer et al., 1999a; Champagne and Bernays, 1991), and 2) plants that contain unsuitable sterols often escape significant damage even from high insect herbivore population (Behmer and Nes, 2003). The exact nature of how unsuitable sterols disrupt physiological processes in insects is not clear, but the incorporation into important and active tissues like the midgut, fat body, muscles, and the nervous system may have serious consequences (Behmer and Nes, 2003). Additionally, the consumption of unsuitable sterols also impairs reproductive output, since large quantities of cholesterol are needed in eggs to ensure proper development (Costet et al., 1987). Moreover, unsuitable sterols affect insect herbivores indirectly, by increasing their susceptibility to plant toxins (Bloem et al., 1989) and viruses (Macdonald and Ritter, 1988). Finally, if unsuitable sterols extend insect development, the window of opportunity might be extended for many beneficial invertebrate predators

and parasitoids, which are often limited by the size of the prey or hosts they can attack (Behmer and Grebenok, 1998; Ritter and Nes, 1981b). It seems, therefore, that introducing unsuitable sterols into crop plants has great potential in providing a novel form of control, both directly and indirectly, against insect pests of crops.

The mechanisms governing uptake of dietary sterols are also poorly understood, current working models and data suggest that sterols, packaged as mixed micelles with phospholipids, are absorbed passively into the membranes of midgut tissue (Jouni et al., 2002; Turunen and Crailsheim, 1996). There are two supportive facts for this inference. Firstly, insect sterol profile is largely affected by the dietary sterol profile (Costet et al., 1989; Dutky et al., 1967; Kuthiala and Ritter, 1988; Nes et al., 1997; Ritter, 1984). Secondly, insects cannot get enough sterols for their development and growth when the dietary sterol concentration is low (Behmer and Elias, 1999b, 2000; Clayton et al., 1964; Ritter and Nes, 1981a). Normally, insects on low concentration sterol diet have an extended development, but some of these insects can survive to later instars/stadia, or even reach adulthood, although body characters such as weight and size are often compromised. Most previous studies have focused on the minimal dietary sterol requirement for insect development but the effect of dietary sterol concentration, and the ratio of suitable to unsuitable sterols on insect sterol content has rarely been studied.

In this dissertation, I take the advantage of a transgenic tobacco line expressing a 3-hydroxysteroid-oxidase gene that maintains elevated levels of 3-ketosteroids and stanols to study the effects of unsuitable sterols on insect performance (Corbin et al., 2001; Heyer et al., 2004). I then examine the effects of phytosterols, both common and

novel ones, on insect herbivores at physiological, biochemical and molecular level using sterol-free artificial diets. I also use a molecular approach, via microarray techniques, to study how different sterols/steroids affect gene regulation patterns in a generalist caterpillar. Finally, I use a generalist grasshopper to explore the extent to which insect herbivores practice sterol homeostasis. The collective results are then summarized in the last chapter, where I also give directions for the further research.

CHAPTER II

PLANT STEROLS AND HOST PLANT SUITABILITY FOR GENERALIST AND SPECIALIST CATERPILLARS

2.1 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. 2.1a) 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. 2.1b) and stigmasterol (Fig. 2.1c). 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, 1999b; Behmer et al., 1999b), 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).

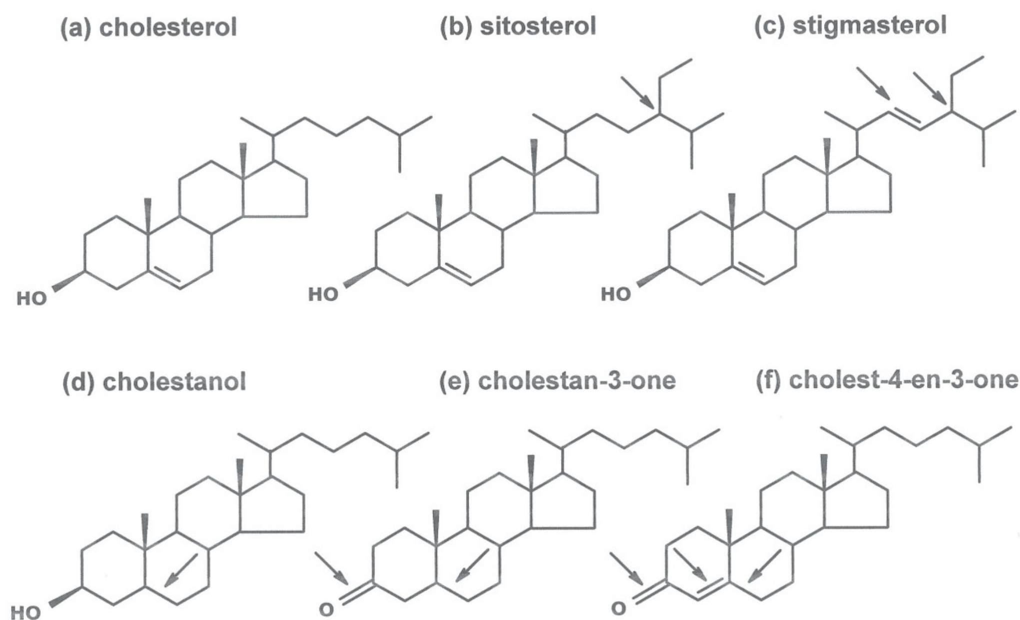


Fig. 2.1. Cholesterol and 5 typical sterols/steroids in our modified plants. Cholesterol (a), the most common sterol in insects, is seldom found in plants. Two phytosterols, sitosterol (b) and stigmasterol (c), are common sterols in plants. Both sitosterol and stigmasterol have a C24 ethyl group; stigmasterol 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 cholesten-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., cholesten-4-en-3-one).

Artificial diets have been an critical tool for studying sterol use in insects (Dadd, 1957, 1960a; 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 & Nes, 2003). In contrast, relatively few studies have attempted to manipulate

dietary sterols in real foods, especially in plants. Costet et al., using the systemic fungicide fenpropimorph, modifed the sterol profile in wheat (replacing typical wheat sterols with 9β,19-cyclopropylsterols (up to 95% of total sterols))(Costet et al., 1989; Costet et al., 1987). 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. 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 (Behmer et al., 2011). Stanols (e.g., cholestanol (Fig. 2.1d)), lack double bonds in the sterol nucleus. The defining feature for a ketone-steroid is a ketone at the C3-position (e.g., cholestane-3-one (Fig. 2.1e)); in some instances ketone-steroids contain a double bond in the sterol nucleus (e.g., cholest-4-en-3-one). Behmer et al. showed these atypical sterols occurred in both the vegetative tissue, and phloem, and that green peach aphids (*Myzus persicae*) reared on plants expressing high levels of atypical steroids suffered reduced survival, growth and fecundity (Behmer et al., 2011).

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 used the data generated from our generalist caterpillars to estimate the effect of modified phytosteroid profiles at the insect population level (Behmer and Grebenok, 1998).

2.2 Materials and Methods

2.2.1 Insects

Three caterpillars that have been recorded as feeding on tobacco were used for this study. Two of these, *Heliothis virescens* (Noctuidae) and *Spodoptera exigua* (Noctuidae), are generalists that feed on a wide range of plants. The third species, *Manduca 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.

2.2.2 Tobacco plants

Two tobacco (*Nicotiana tabacum*) lines were used. The first expressed the chloroplast-targeted 3-hydroxysteroid oxidase gene, pMON33814. 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 by Heyer et al.(Heyer et al., 2004). Each leaf was individually freeze-dried, pulverized and extracted in chloroform for 24 hrs. 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 tempertature 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 um. 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). Steroids 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 modified tobacco plants used throughout this study are shown in Table 2.1.

Table 2.1 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

2.2.3 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 everyday. Water was refilled to paper towel and sponges when necessary. After insects were adapted to tobacco leaves, 4 (4 days for *H. virescens* and 5 days for *S. exigua*) or 3 (2 days for *M. sexta*) of the largest larvae were collected and transferred to plants in the greenhouse. 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 inch 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 based of each plant, with the edges of the cone secured using double-sided tape (see supplemental 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 two 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 3 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 5 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 (same used for the plants), where they were allowed to pupate. Three days after pupation, their sex 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, 1 female and 2 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 the radiant heat of 28-31 °C during the light phase (supplied by 25 W full spectrum incandescent bulbs) and the heat

of 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 two 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. 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. 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.

2.2.4 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 4 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 the analysis was performed in SAS v. 9.2 (Cary, NC, USA).

2.3 Results

2.3.1 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 on both control and modified plants.

Table 2.2 Statistical analysis of 1st-generation data. No reproduction data were obtained for *Manduca sexta*.

Variable	*H. virescens*	*S. exigua*	*M. sexta*
Survival to pupation (number per plant)	Z = 0.91 (P = 0.182)	Z = 1.61 (P = 0.053)	Z = 1.99 (P = 0.023)
Pupal mass (mg)	$F_{1, 103}$ = 9.42 (P = 0.003)	$F_{1, 70}$ = 0.39 (P = 0.536)	$F_{1, 68}$ = 0.04 (P = 0.839)
Larval development (days)	χ^2_1 = 9.27 (P = 0.001)	χ^2_1 = 0.04 (P = 0.422)	χ^2_1 = 0.00 (P = 0.500)
Pupal development (days)	χ^2_1 = 4.27 (P = 0.019)	χ^2_1 = 0.85 (P = 0.178)	χ^2_1 = 1.55 (P = 0.107)
Survival to eclosion (number per plant)	Z =1.11 (P = 0.133)	Z =1.92 (P = 0.027)	Z =1.53 (P = 0.063)
Egg production (number per female)	t_{19} = 0.72 (P = 0.239)	t_{21} = 0.32 (P = 0.376)	- -
Egg viability * (% of eggs hatching)	t_{16} = 1.40 (P = 0.090)	t_{13} = 1.15 (P = 0.272)	- -

* A Satterthwaite test was used for the egg viability analyses because the two treatments had unequal variance.

The three caterpillar species tested responded differently to the control and modified tobacco plants, and *H. virescens* was the species that seemed most affected. 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 (Fig. 2.2; Table 2.2). For *S. exigua*, eclosion success was the only performance variable that differed (Table 2.2), being higher on the control tobacco plants (Fig. 2.2). For *M. sexta*, the only performance measure that differed on the two tobacco lines was larval survival (Table 2.2), and it was significantly higher on the control tobacco plants (Fig. 2.2).

Egg production per female and egg viability were used to evaluate the reproduction ability of the insects on the two tobacco plants. No significant difference was found for either species, *H. virescens* and *S. exigua*, whose eggs were successfully collected in the experiments (Fig. 2.3, Table 2.2).

2.3.2 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.

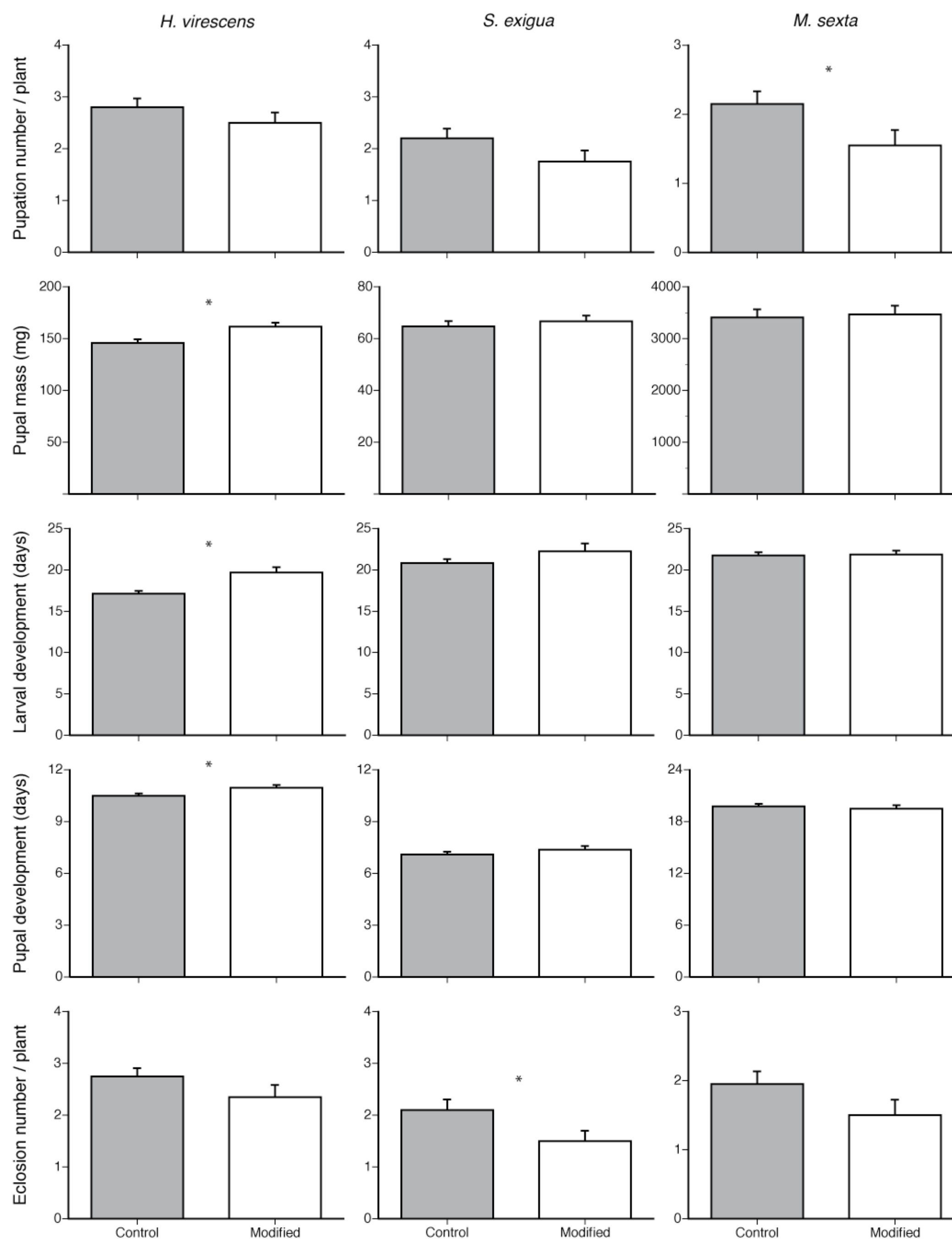


Fig. 2.2. Larval and pupal performance of *H. virescens*, *S. exigua*, and *M. sexta* on control and modified tobacco in the first generation. For each species we recorded pupation success (number pupating ± SEM; for *H. virsecens* and *S. exigua* there were four individual neonates/plant at the start 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). An asterisk above the bars (in a panel) indicates a statistically significant difference between treatments ($\alpha = 0.05$). See Table 2.2 for the statistical test used, the test statistic value, and the exact P-value.

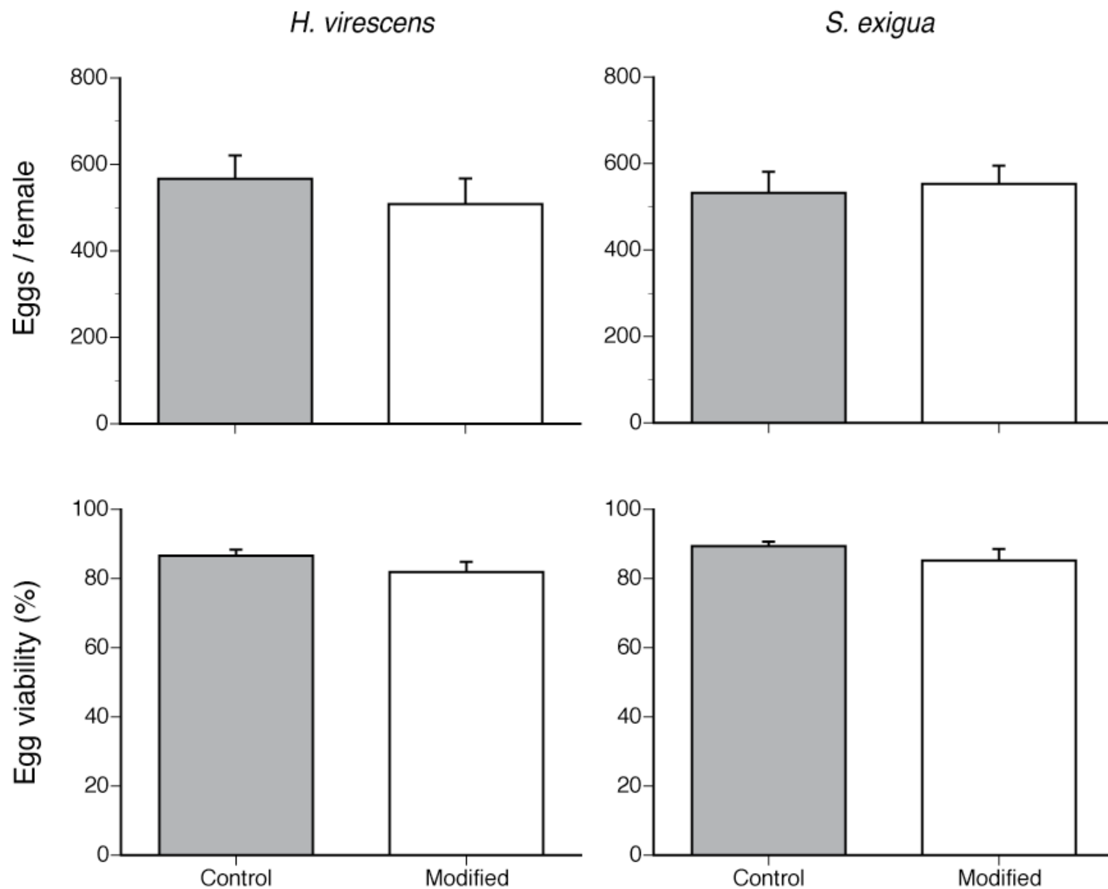


Fig. 2.3. Reproductive performance of *H. virescens* and *S. exigua*, on control and modified tobacco in the first generation. For both species we recorded egg production (number of eggs (± SEM) produced over the first six adult) and egg viability (number of eggs hatched/total number of eggs (± SEM)). An asterisk above the bars (in a panel) indicates a statistically significant difference between treatments (α = 0.05). See Table 2.2 for the statistical test used, the test statistic value, and the exact *P*-value.

At a superficial level many of the patterns observed in the first generation held in the second generation, although there was one major difference. Larval survival, for both *H. virescens* and *S. exigua* was significantly reduced on the modified plants (Fig. 2.4; Table 2.3). For *H. virescens*, pupal mass and eclosion success was significantly better on the control plants, but sterol plant profile did not significantly affect larval or pupal development time (Fig. 2.4: Table 2.3). 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 (Fig. 2.4; Table 2.3). Eclosion success was better on the control plants, although the difference was not statistically significant (Fig. 2.4; Table 2.3).

Table 2.3 Statistical analysis of 2nd-generation data.

Variable	*H. virescens*	*S. exigua*
Survival to pupation (number per plant)	Z = 2.17 (*P* = 0.015)	Z = 1.92 (*P* = 0.028)
Pupal mass (mg)	$F_{1, 104} = 4.68$ (*P* = 0.032)	$F_{1, 70} = 4.16$ (*P* = 0.045)
Larval development (days)	$\chi^2_1 = 1.22$ (*P* = 0.135)	$\chi^2_1 = 9.23$ (*P* = 0.001)
Pupal development (days)	$\chi^2_1 = 0.81$ (*P* = 0.184)	$\chi^2_1 = 3.76$ (*P* = 0.026)
Survival to eclosion (number per plant)	Z = 2.74 (*P* = 0.003)	Z = 1.58 (*P* = 0.057)
Egg production (number per female)	$t_{17} = 3.00$ (*P* = 0.008)	$t_{16} = 2.85$ (*P* = 0.006)
Egg viability * (% of eggs hatching)	$t_{11} = 0.96$ (*P* = 0.178)	$t_{13} = 0.96$ (*P* = 0.050)

* A Satterthwaite test was used for the egg viability analyses because the two treatments had unequal variance.

In contrast to the 1st generation, reproduction in both *H. virsecens* and *S. exigua* was affected by plant type. Insects reared on the control plants produced significantly more eggs (Fig. 2.5; Table 2.3). Furthermore, egg viability on the control plants was also higher for both species, although the difference was only significant for *S. exigua* (Fig. 2.5, Table 2.3).

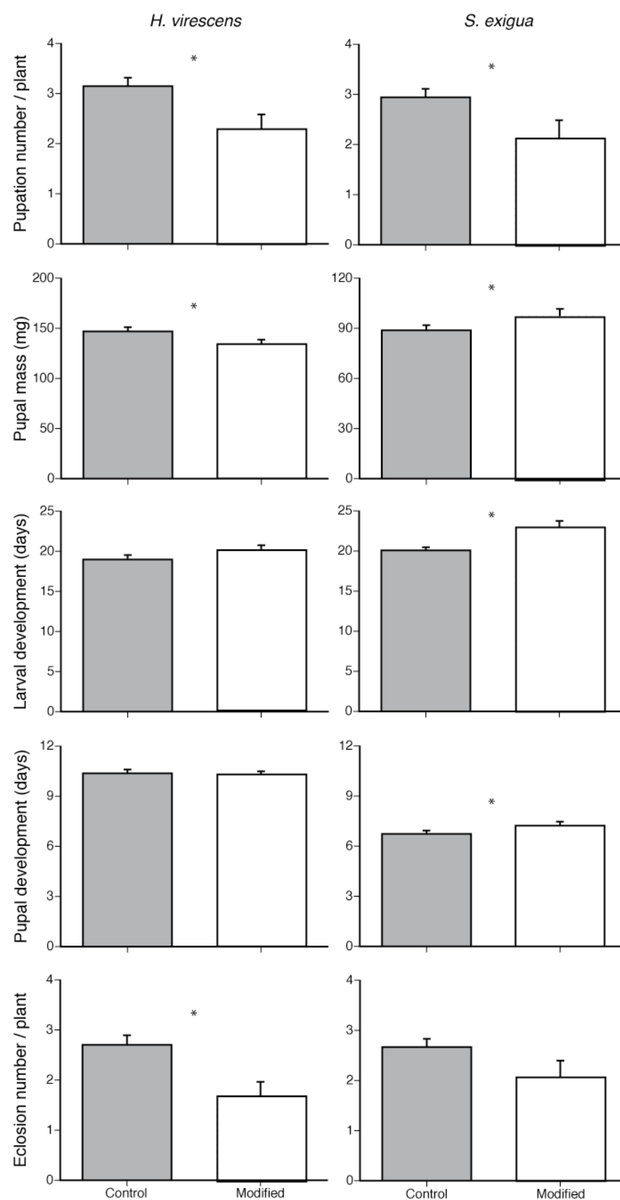


Fig. 2.4. Larval and pupal performance of *H. virescens* and *S. exigua* on control and modified tobacco in the second generation. For both species we recorded pupation success (number pupating ± SEM; there were four individuals/plant at the start of the experiment), pupal mass (mg ± SEM), larval developmental time (days ± SEM), pupal developmental time (days ± SEM) and eclosion success (number eclosing ± SEM). An asterisk above the bars (in a panel) indicates a statistically significant difference between treatments (α = 0.05). See Table 2.3 for the statistical test used, the test statistic value, and the exact *P*-value.

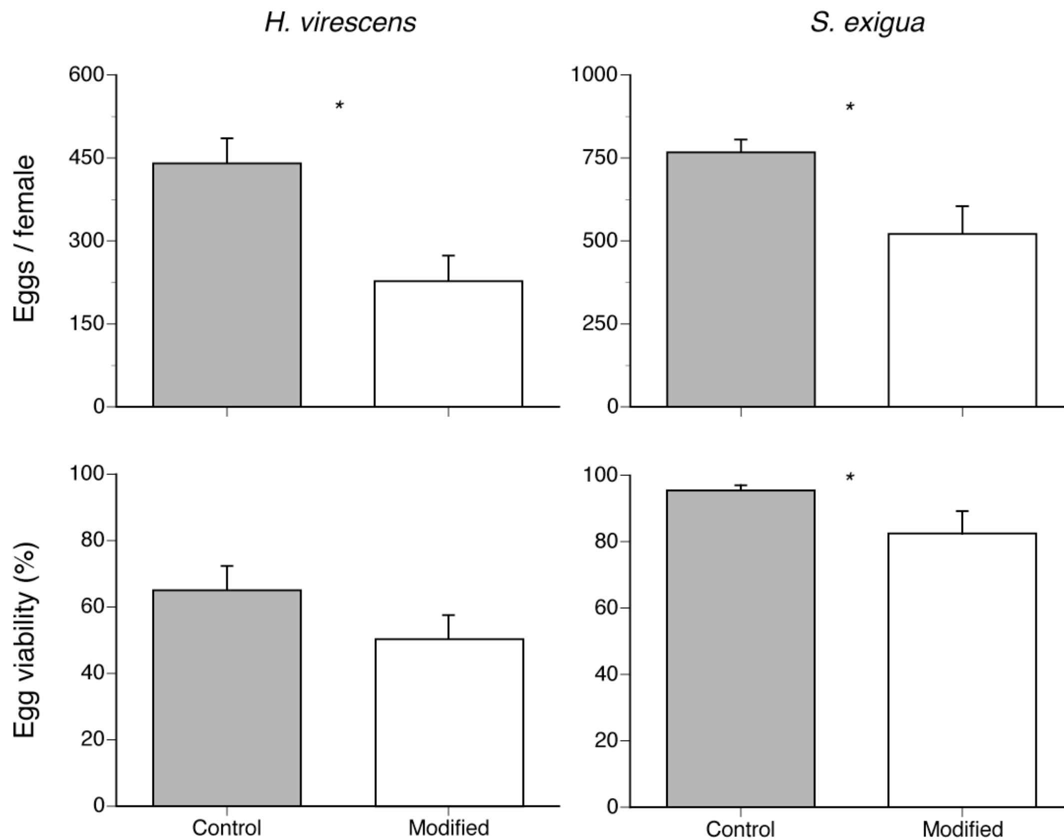


Fig. 2.5. Reproductive performance of *H. virescens* and *S. exigua*, on control and modified tobacco in the second generation. For both species we recorded egg production (number of eggs (± SEM) produced over the first six adult) and egg viability (number of eggs hatched/total number of eggs ± SEM). An asterisk above the bars (in a panel) indicates a statistically significant difference between treatments ($\alpha = 0.05$). See Table 2.3 for the statistical test used, the test statistic value, and the exact *P*-value.

2.4 Discussion

Most studies exploring sterol use in insects have employed an artificial diet approach because it allows tight control of dietary sterol content (reviewed in Behmer and Nes, 2003). To our knowledge only three studies (two using grasshoppers (Costet et al., 1989; Costet et al., 1987), the other using aphids (Behmer et al., 2011)) have explored how modifying sterol profile 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 of dietary sterols (Behmer and Grebenok, 1998). In the current study we used transgenic plants, which maintained altered sterol profiles, to document the sterol affects on two generalists, and one specialist caterpillar. Our results demonstrate that exposure to novel dietary sterols can negatively affect larval, pupal, and reproductive traits in both generalist and specialist caterpillars, but that their negative effects are more pronounced in the second generation. 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 species 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 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, almost half of the sterol profile in the modified plants is suitable for our caterpillars. The remaining portion of the sterol profile in our modified plants was 3-ketosteroids, which do not support strong growth in insects, even when small amounts of cholesterol are available (Chapter III). When "good" sterols are limiting, insects may utilize a sterol sparing mechanism. In this situation, non-cholesterol sterols can be incorporated into cell membranes, with cholesterol being saved/spared for a metabolic role (Clayton, 1964). However, the extent to which sterol sparing 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 sparing 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 sparing 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, 1999b, 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. (Costet et al., 1987), parental dietary sterol profile directly affects egg sterol profile; adult female grasshoppers fed wheat that 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 sparing 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 (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 larvae, or adult, 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 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 titres that supported high hatching success. Nonetheless, we imagine that the cholesterol and ecdysteroid titres 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 titres must have been small (given the observed egg viability rate), our 2nd-generataion egg

production data suggests the reduction was a 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 titres 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 titres (Nijhout, 2003). Other important nutrients, particularly protein and digestible carbohydrates, would not have been limiting for caterpillars on the modified tobacco plants. Thus, one explanation for larger body size is that because caterpillars lived longer, they consumed greater amounts of protein and digestible carbohydrates that were ultimately converted into body tissues (e.g., integument, muscle and visceral organs, and lipid stores).

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, 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 in a 1:1 sex ratio (this ratio was typical for both *H. virescens* and *S. exigua* in both generations (unpublished data)). We also assumed, for simplicity, that there were no biotic or abiotic mortality factors. The key result to emerge from this exercise is that for both species, population sizes at the start of the third generation were 3-5x 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. 2.6, 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 in place of cholesterol, has great potential to be exploited for insect 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.

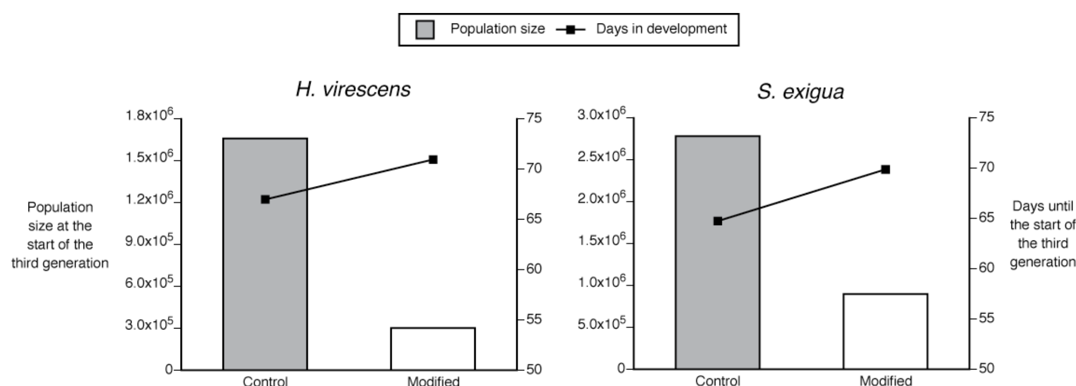


Fig 2.6. Estimated population size and developmental time for *H. virescens* and *S. exigua* reared on the two tobacco lines after two generations. These projections use a starting population of 100 individuals (50 males and 50 females) and assume no mortality other than that related to the tobacco line (control versus modified).

CHAPTER III

BALANCE MATTERS: HOW THE RATIO OF DIETARY STEROIDS AFFECTS CATERPILLAR DEVELOPMENT, GROWTH AND REPRODUCTION

3.1 Introduction

Unlike mammals, insects do not have the enzyme squalene synthase to synthesize cholesterol from farnesyl pyrophosphate so that they need acquire it from their food (Klowden, 2007). Sterols, especially cholesterol, are important for two main reasons: 1) they are a cellular membrane component that regulates cell membrane fluidity and permeability (Lönnfors et al., 2011), and the 3-hydroxyl group at the C3 position is important in fulfilling this function (Simons and Ikonen, 2000), and 2) sterols are critical precursors to steroids hormones, in particular insect molting hormones (i.e., ecdysteroids) (Behmer and Nes, 2003). Among various hormonal ecdysteroids, 20-hydroxyecdysone (20E) and ecdysone (E), both with a 3-hydroxyl group, are the most common molting hormones in insects (Gilbert et al., 2002).

The most common plant sterols (e.g., sitosterol, stigmasterol and campesterol) differ from cholesterol by having 24(R)-alkyl. Most insect herbivores studied can convert these phytosterols into cholesterol by removing the 24-alkyl group (Gilbert et al., 2002). In a recent plant study (Chapter II), we found the caterpillars *Heliothis virescens*, *Spodoptera exigua*, and *Maduca sexta*, grew worse on genetically transformed plants having novel sterols (i.e., 3-keto-steroids and 3-hydroxyl-stanols), compared to caterpillars reared on normal tobacco plants; this study also revealed that caterpillar

growth, development and reproduction were more negatively affected in the 2nd-generation (Chapter II). It is important for researchers to have an overall evaluation on insect performance on plants, a complex dietary system, but modifying one character may cause the change of others in plants which are unidentified, and subsequently, unaddressed (Wolfenbarger and Phifer, 2000). Therefore, it seems necessary and important to confirm the negative effects of modifying plant sterols by using artificial diets, not to mention the convenience of sterols manipulation in diets.

The importance of artificial diets in insects research has been discussed extensively by Cohen (Cohen, 2004), and the important finding that sterols are an essential dietary nutrient was discovered using artificial diet developed for blowflies (Hobson, 1935). After Hobson's seminal finding, sterol requirement in insects were studied extensively based on artificial diets. Later, researchers used artificial diets to explore how, when insect cholesterol levels are low, other sterols can be used to spare tissue cholesterol requirements (e.g., in cell membranes), so that the metabolic demand of cholesterol for insect hormonal ecdysteroids can be met (Clayton and Bloch, 1963). For example, cockroaches (Clayton and Edwards, 1961; Lasser et al., 1966) and houseflies (Dutky et al., 1967) fed with an artificial diet containing sterols that cannot be converted to cholesterol, plus a very small amount of cholesterol, had equal performance compared insects on diets containing only sterols that can be converted to cholesterol. Caterpillars and grasshoppers, however, are less tolerant to the presence of unconvertible dietary sterols. Caterpillars show negative effects when unconvertible sterols to comprise more than 50% of the total dietary sterol profile (Nes et al., 1997).

Grasshoppers are even less tolerant; their performance drops when unconvertible sterols begin to exceed 25% of the total dietary sterol profile (Behmer and Elias, 1999b, 2000).

In this study we used artificial diets that contained sterols and steroids found in our modified tobacco plants to explore how different sterol/steroid structures, and different ratios and amounts of these sterols and steroids affected caterpillar growth and development, and reproduction. Two caterpillar species, *Heliothis virescens* and *Helicoverpa zea*, were reared from hatching to eclosion, and a range of performance values was recorded on a range of different experimental diets. Experiments were conducted for two generations, which allowed the effects of parental dietary sterols to be evaluated. We discuss how the balance of different dietary sterols/steroids affects caterpillars, and the potential application of sterol/steroid modification in crops.

3.2 Materials and Methods

3.2.1 Insects

Two generalist noctuid caterpillars, *Helicoverpa zea* and *Heliothis virescens*, were used in this experiment. Eggs of both species were purchased from commercial available cultures maintained at Benzon Research Inc. (Carlisle, PA). The eggs were incubated at 27 °C and neonates hatching within 6 hrs were used as a source for the start of the experiments. Individual neonates were randomly distributed onto the different experimental diets (described below) using a small paintbrush.

3.2.2 Experimental diets

Both species were reared on the same basic diet, which was originally developed for *H. zea* (Ritter and Nes, 1981a). However, because this diet did not support insect development beyond one generation, and insect performance expressed as larval development, pupal mass and reproduction was greatly reduced compared to caterpillars fed a more standard artificial diet (i.e., the corn-soy-milk) (Roeder et al., 2010), some modifications to the original Ritter and Nes diet were made. These included the addition of torula yeast, non-fat dry milk, a vitamin mix, and forbic acid (Appendix).

However, both the torula yeast and non-fat dry milk contained high amount of sterols, so these two components were combined, and their sterols were extracted, using the following extraction procedure. First, the combined torula yeast/non-fat dry milk mixture was extracted 5 times with ethanol, followed by 5 extractions with hexane. The extract demonstrated little contamination with esterified sterols, therefore free sterols were the focus of the cleaning procedure (Moreau et al., 2002). The ethanol and hexane fractions were recombined and evaporated to dryness. The dried extract was resuspended in hexane using sonication, and then applied to a 50 g silica column. The extract was eluted from the silica column using 3 column volumes each of hexane, hexane/toluene (50/50 v/v), toluene, ether, ether/methanol (50/50 v/v) and methanol in succession (Ripa and Adler, 1987). Free sterols eluted in the ether fraction. The hexane, hexane toluene and toluene fractions, which were now sterol free, were added back to the extracted diet components, and homogenized using a Kitchen Aid Professional 600 mixer (Kitchen Aid Inc., St. Joseph, MI).

Next, the ether, ether/methanol, and methanol fractions containing the free sterol were combined and resuspended in ether and subjected to Thin Layer Chromatography (TLC; silica gel G, 2000 microns, Analtech, Newark, DE). These TLC plate sat in a small volume of toluene ethyl acetate (90/10 v/v). Authentic standards of sterols, purchased from Sigma Chemical Inc., (St. Louis, MO), were co-chromatographed on all TLC plates (Ripa and Adler, 1987), and the developed plates were incubated with Iodine vapor to elucidate the position of the sterols. Following evaporation of the Iodine, the areas of the TLC plates other than those that co-migrated with sterol standards were eluted from the TLC silica and added back to the extracted diet components as described above. The compounds co-chromatographing with the sterol containing regions were eluted and subjected to Solid Phase extraction, (DSC-Si, Silica Tube, 3 ml, 500 mg; purchased from Supelco Inc., Bellefonte, PA); this was done using identical conditions applied to the silica columns, described above.

The ether, ether/methanol, and methanol fractions containing the sterols were discarded, while the hexane, hexane/toluene, and toluene fractions were added back to the original diet components. These recombined components were homogenized using a kitchen aid mixer, until the consistency of the components reached pre-extraction form. The recombined components were then analyzed for sterol content using standard extraction techniques, followed by GC-MS analysis (as described in Chapter II). The reconstituted mixture of torula yeast and dry milk was found to contain only a very trivial amount of sterol (estimated to be 5 nanograms of sterol per gram dry weight, as determined using GC-MS with Selective Ion Monitoring analysis). All other peaks

looked comparable to profiles from preextraction samples analysis. The only other diet component containing sterol is casein (1.3 µg cholesterol/g casein). However, because the sterol concentration is so low, no steps were taken to remove cholesterol from the casein.

Table 3.1 Dietary sterol/steroid combinations, and their concentrations (mg/g dry mass), used in this study. Four different sterols/steroids were used to construct 12 different sterol/steroid diets, including diets containing single, double or triple sterol/steroid combinations. The four sterols/steroids used were: (1) cholesterol, (2) stigmasterol, (3) cholestanol, and (4) cholestan-3-one. These four sterols/steroids are shown in Fig. 3.1.

	Sterols/steroids (µg/g)			
Diet Treatments	Cholesterol	Stigmasterol	Cholestanol	Cholestan-3-one
Single sterol/steroid	1.0	-	-	-
	-	1.0	-	-
	-	-	1.0	-
	-	-	-	1.0
Sterol plus steroid	-	0.25	1.75	-
	-	1.0	-	1.0
	-	0.5	-	1.5
	-	0.25	-	1.75
	-	1.0	-	3.0
Sterol plus 2 steroids	-	1.0	0.33	0.67
	-	0.5	0.5	1.0
	-	0.25	0.58	1.17

In total, 12 unique diets were generated from the basal diet described above, using various combinations of 4 different sterols/steroids. The sterols/steroids used in this study are shown in Fig. 3.1. They are: (1) 5α-cholester-3β-ol (cholesterol, ≥ 95%), (2) 5,22-cholestadien-24β-ethyl-3β-ol (stigmasterol, ≥ 98%), (3) 5α-cholestan-3β-ol

(cholestanol, 95%), and (4) 3-keto-5α-cholestane (cholestan-3-one, ≥ 98%); cholesterol and cholestanol were purchased from Sigma Chemical (St. Louis, MO, USA), while stigmasterol and cholestan-3-one were purchased from Steraloids Inc. (Newport, RI, USA). Previous studies have suggested that cholestanol and cholestan-3-one do not support good insect growth and development well when they are fed to insects (see Chapter II). Thus, we expect these two steroids to be "bad" for our caterpillars.

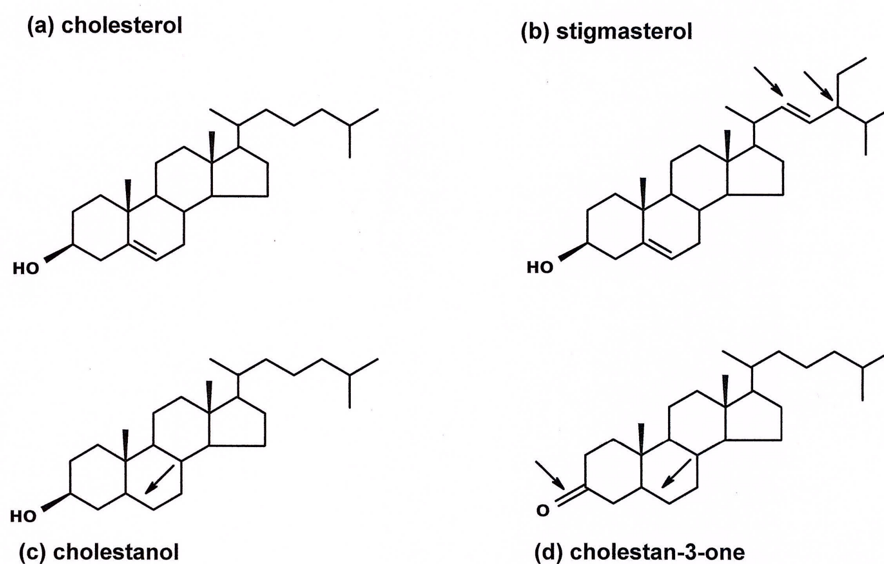


Fig. 3.1. A total of four sterols/steroids are used in this study. Cholesterol (a) is the most common sterol found in insects. Stigmasterol (b), a common phytosterol, differs from cholesterol by having a C24 ethyl group, plus a C22 double-bond. Cholestanol (c) is identical to cholesterol, except that it lacks a Δ5 double bond. Cholestan-3-one (d) is a keto-steroid. In contrast to cholesterol, cholestan-3-one has a C3 ketone instead of a C3 hydroxyl, and like cholestanol, it has no Δ5 double bond in the B-ring.

The first four diets generated were single-steriod diets, with cholesterol, stigmasterol, cholestanol, and cholesta-3-one added at a concentration of 1 mg/g dry mass (Table 3.1). The next five diets contained two steroids (one "good", one "bad"), at

various concentrations. The first double-steroid diet contained stigmasterol and cholestanol at 0.25 mg/g and 1.75 mg/g, respectively (a 1:7 ratio). The other four double steroid diets contained stigmasterol (S) and cholestan-3-one (K), paired at various concentrations: (1) 1.0 mg/g of S, paired with 1.0 mg/g K (a 1:1 ratio), (2) 0.5 mg/g S, paired with 1.5 mg/g K (a 1:3 ratio), (3) 0.25 mg/g S, paired with 1.75 mg/g K (a 1:7 ratio), and (4) 1.0 mg/g S, paired with 3.0 mg K (a 1:3 ratio). The last three diets contained 3 steroids: stigmasterol (S), cholestanol (A), and cholestan-3-one (K). The ratio of A to K in the diet was meant to reflect the ratio in which they occur, relative to each other, in transgenic tobacco lines that have modified steroid profiles (Chapter II). The three triple-steroid diets had the following steroid combinations: (1) 1.0 mg/g S, 0.33 mg/g A, and 0.67 mg/g K (a 1:1 ratio of “good” to “bad” steroids), (2) 0.5 mg/g S, 0.5 mg/g A, and 1.0 mg/g K (a 1:3 ratio of “good” to “bad” steroids), and (3) 0.25 mg/g S, 0.58 mg/g A, and 1.17 mg/g K (a 1:7 ratio of “good” to “bad” steroids).

Each diet was made using the ingredients listed in the Appendix (with the exception of sterols/steroids). First, the casein and cellulose were combined and mixed. Second, the sterols/steroids for each treatment were dissolved in chloroform. This solution was added to the casein-cellulose mixture, and stirred so that the chloroform solution was evenly distributed. The mixture was left for 24 hrs under a fume hood, so that the chloroform could completely evaporate. The third and fourth steps were to add the other main ingredients, and antibiotic mixture, respectively, with both steps followed by thorough mixing. At step five, a number of ethanol-soluble components were added to 100% ethanol, while at step six a number of water-soluble components were added to

water. These two solutions, plus 1/3 of the water from step 7, were combined with the previously mixed diet components. After a thorough mixing, an agar solution using the remaining 2/3 of the water in step 7 was created, and mixed with the combined diet ingredients. Around 20 ml of this liquid diet was then poured into 1 oz plastic condiment cups (Fabri-Kal), and allowed to set. After the diets had cooled, caps were placed on each container of food. Pilot studies showed that no insects survived on this reconstituted diet without sterols added, and that insect performance was completely recovered by adding proper amounts of suitable sterols.

3.2.3 Experiment design

Upon hatching neonates were transferred individually to small rearing chambers (1 oz plastic condiment cups (Fabri-Kal)) containing diet (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. Each container was examined every three days to record development and mortality. Upon reaching the last instar, caterpillars were observed daily to record pupation. 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) and the same method was used to collect eggs and count the viable ones (Chapter II). Occasionally mating pairs did not separate following copulation; eggs from these individuals were not included in the statistical analysis,

although their viable eggs were used as a source of neonates for the next generation (see below).

To determine whether paternal dietary sterols had any effect on successive generations, a second generation was reared on the same paternal diet 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 on the same diet (n = 60 individuals per sterol treatment).

3.2.4 Statistical analysis

Non-parametric survival analysis was used for larval and pupal development. The larval developmental time for those that died before pupation was incorporated into the analysis as censored data, while only the pupal developmental time for those that eclosed was used because it is difficult to identify the death of a pupa without any damage. A Log-rank tests was used instead of Wilcoxon test in our analysis because the former tends to be more sensitive to distributional difference late in time, which is exactly the behavior of our data (Martinez and Naranjo, 2010). Larval survival and adult survival (i.e., pupation and eclosion) were binomial distributions and analyzed using Likelihood Ratio Chi-Square statistics. No difference was found between males and females in pupal mass, so the two sets of data were combined for analysis. Pupal mass, egg production and egg viability was analyzed by ANOVA. For all analysis, type I error was 0.05, and False Discovery Rate, which controls the expected proportion of falsely

rejected hypotheses, was used for the adjustment in multiple comparison (Benjamini and Hochberg, 1995). All analysis was performed in SAS v. 9.2 (Cary, NC, USA).

Table 3.2 Statistical analysis for *H. virescens* and *H. zea*. Seven variables included in this table were used for measuring insect performance.

	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)	$F_{10, 101} = 5.4$ ($P < 0.001$)	$F_{8, 80} = 3.0$ ($P = 0.006$)	$F_{11, 85} = 2.3$ ($P = 0.015$)	$F_{10, 66} = 4.9$ ($P < 0.001$)

3.3 Results

For both *H. virescens* and *H. zea*, a total of seven different performance variables were recorded, including: 1) time from hatch to pupation, 2) survival to pupation, 3) pupal mass, 4) time from pupation to eclosion, 5) survival from hatch to eclosion, 6) egg

production, and 7) egg viability. These variables were recorded over two successive generations.

3.3.1 Larval and pupal performance

3.3.1.1 Single-steroid treatment

There were four single sterol/steroid treatments (cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)), each with their respective sterol/steroid in the diet at 1.0 mg/g (dry mass). Generally, insect performance on C1.0 and S1.0 (which can be readily metabolized to cholesterol) was quite similar, although some differences were observed for both species (Table 3.2). For example, for *H. virescens*, 1st generation larval development (Fig. 3.2a), and 2nd generation pupal development (Fig. 3.3b), were slightly longer on S1.0, while pupae on S1.0 were slightly heavier in the 2nd generation (Fig 3.3a). For *H. zea*, pupal development was longer on C1.0 for both generations (Fig. 3.5b).

Larval and pupal performance on the A1.0 treatment (which is structurally similar to cholesterol, but lacks a double bond at position C5), relative to the cholesterol treatment, differed between the two caterpillar species. For *H. virescens*, larval development and pupation success were reduced (Fig. 3.2), as were pupal mass, pupal development time, and eclosion success (Fig. 3.3). In the second generation, however, dramatic reductions in larval performance were observed (Fig 3.2), most notably the

Fig. 3.2. Larval development time (a) and pupation success (b) of *H. virescens* on diets containing different dietary sterols/steroids. Data, presented as means (± SEM), are shown for two generations. Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 12 diets shown in this figure. The first four treatments were single steroid diets, with each steroid at a concentration of 1 µg/g (dry mass). The next five treatments represent diets with two steroids (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (µg/g) of each of these steriods is shown in parentheses directly below the bars. The last three treatments represent diets with three steroids (stigmasterol, cholestanol and cholestan-3-one), with the concentration of each steroid, in each diet, shown below each bar. Different lower case and upper case letters above bars indicate statistically significant differences between the treatments in the 1st and 2nd generation, respectively. For each treatment, and asterisk represents a statistically significant difference between the two generations; ns = no statistically significant difference.

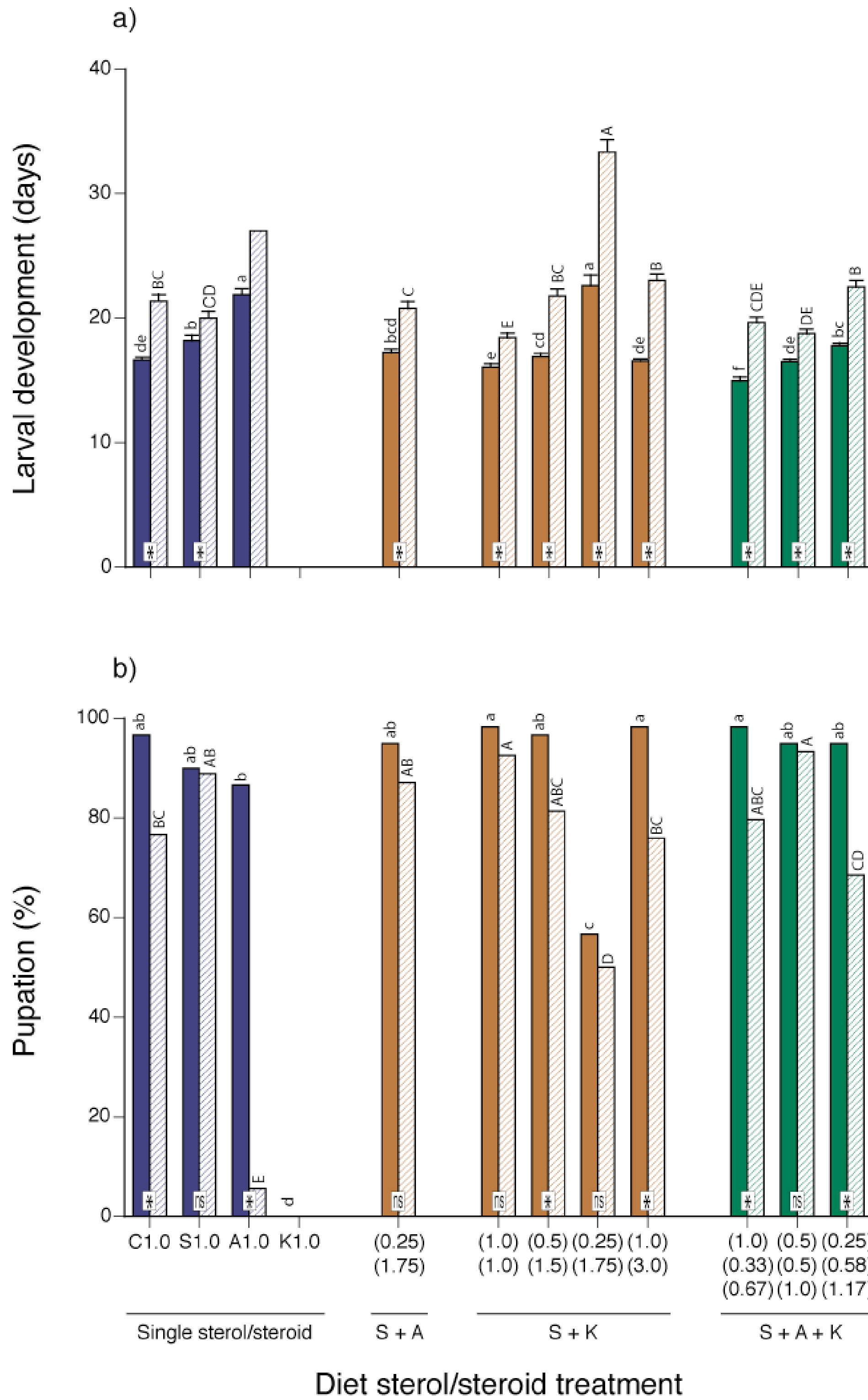
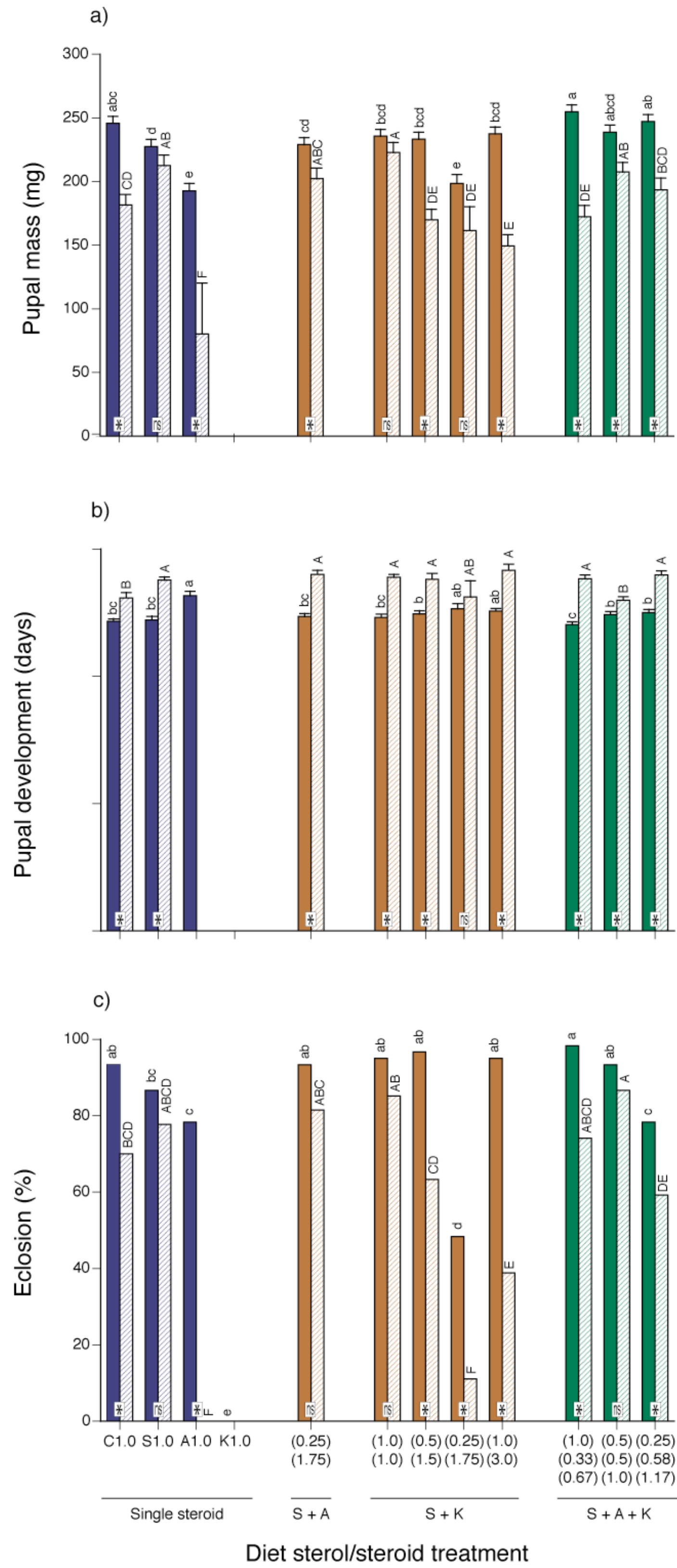

a)
Larval development (days)
40
30
20
10
0
b)
Pupation (%)
100
80
60
40
20
0
C1.0
S1.0
A1.0
K1.0
(0.25)
(1.75)
(1.0)
(1.0)
(0.5)
(1.5)
(0.25)
(1.75)
(1.0)
(3.0)
(1.0)
(0.33)
(0.67)
(0.5)
(0.5)
(1.0)
(0.25)
(0.58)
(1.17)
Single sterol/steroid
S + A
S + K
S + A + K
Diet sterol/steroid treatment

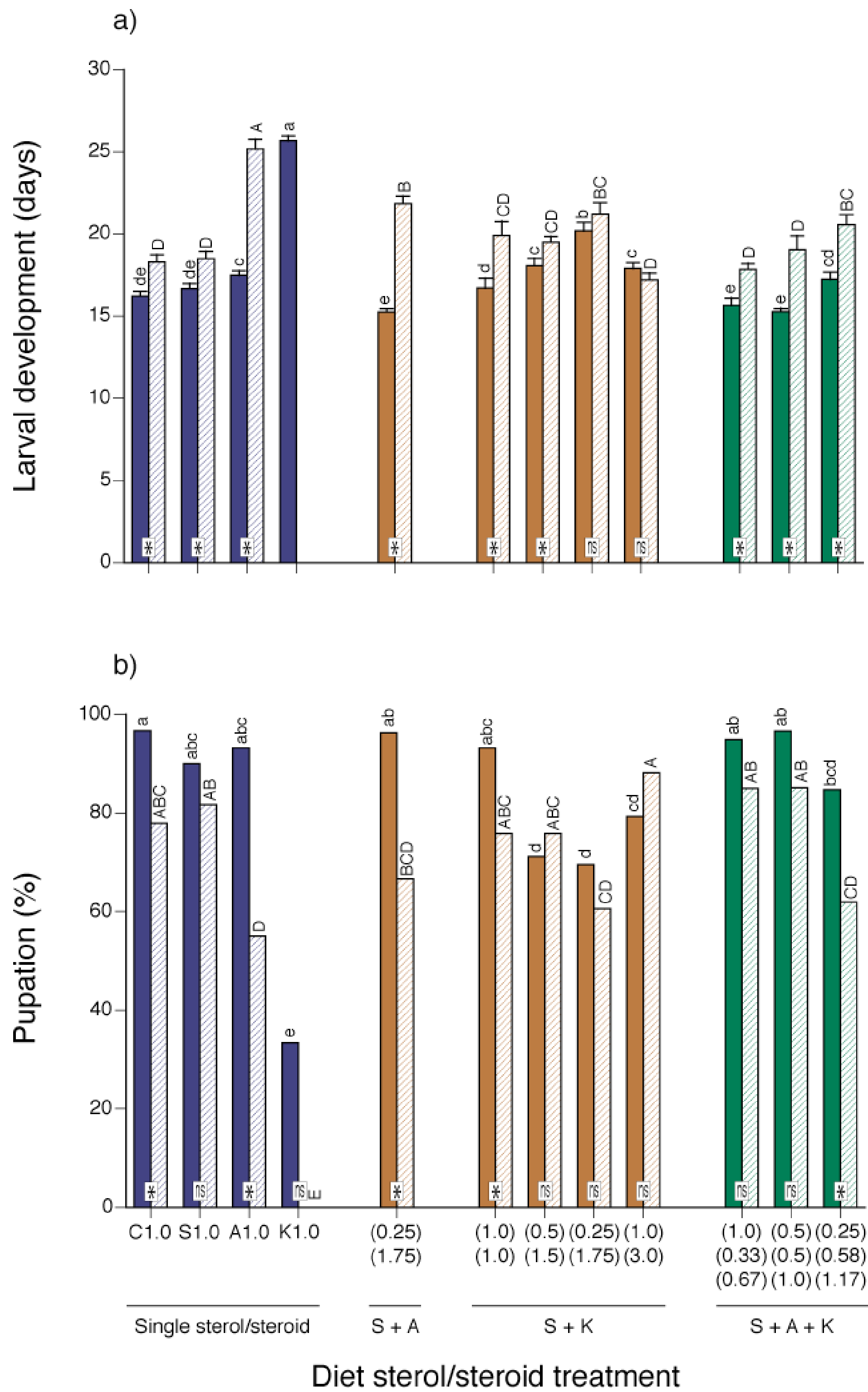
Fig. 3.3. Pupal mass (a), pupal developmental time (b), and eclosion success (c) of *H. virescens* on diets containing different dietary sterols/steroids. Data, presented as means (± SEM), are shown for two generations. Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 12 diets shown in this figure. The first four treatments were single steroid diets, with each steroid at a concentration of 1 µg/g (dry mass). The next five treatments represent diets with two steroids (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (µg/g) of each of these steriods is shown in parentheses directly below the bars. The last three treatments represent diets with three steroids (stigmasterol, cholestanol and cholestan-3-one), with the concentration of each steroid, in each diet, shown below each bar. Different lower case and upper case letters above bars indicate statistically significant differences between the treatments in the 1st and 2nd generation, respectively. For each treatment, and asterisk represents a statistically significant difference between the two generations; ns = no statistically significant difference.


a)
Pupal mass (mg)
b)
Pupal development (days)
c)
Eclosion (%)
C1.0 S1.0 A1.0 K1.0
(0.25) (1.75)
(1.0) (1.0)
(0.5) (1.5)
(0.25) (1.75)
(1.0) (3.0)
(1.0) (0.33) (0.67)
(0.5) (0.5) (1.0)
(0.25) (0.58) (1.17)
Single steroid
S + A
S + K
S + A + K
Diet sterol/steroid treatment

failure of any pupae to successfully eclose (Fig 3.3). For *H. zea*, there was only a single significant 1st-generation effect - larval development was slightly extended (Fig 3.4a). The effects of cholestanol were much more pronounced in the 2nd-generation. Larval development was much longer, and pupation success was greatly reduced (Fig. 3.4). Likewise, pupal mass was much lower, and eclosion success was greatly reduced (Fig. 3.5); interestingly there was effect on pupal developmental time (Fig. 3.5b).

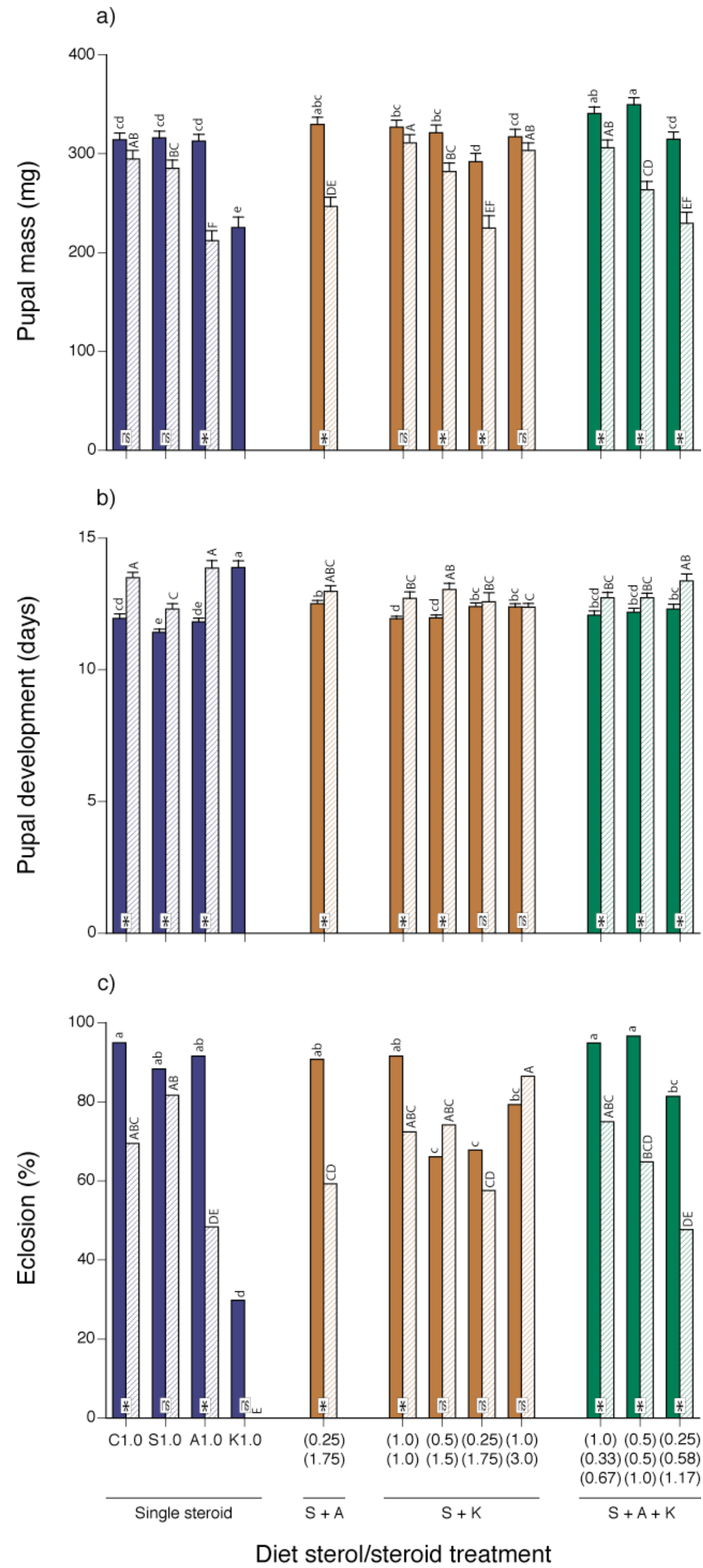
Cholestan-3-one as the sole dietary steroid was detrimental for both caterpillar species, although its negative effects impacted *H. virescens* more strongly – no 1st-generation *H. virescens* larvae completed development to the pupal stage (Fig. 3.2). In contrast, about 40% of *H. zea* larvae pupated (Fig. 3.4b), although their developmental time was significantly longer relative to cholesterol-reared caterpillars (Fig. 3.4a). The *H. zea* pupae on this diet were significantly smaller compared to cholesterol-reared caterpillars, plus they had extended developmental time and significantly reduced eclosion rates (Fig. 3.5). The pupae that eclosed and were mated produced eggs and viable offspring (see below), but none of these 2nd-generation caterpillars pupated.

Fig. 3.4. Larval development time (a) and pupation success (b) of *H. zea* on diets containing different dietary sterols/steroids. Data, presented as means (± SEM), are shown for two generations. Four different sterols/steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 12 treatments shown in this figure. The first four treatments were single sterol/steroid diets, with each sterol/steroid at a concentration of 1 µg /g (dry mass). The next five treatments represent diets that combine a sterol and steroid (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (µg/g) of each sterol/steriod is shown in parentheses directly below the bars. The last three treatments represent diets with one sterol (stigmasterol) and two steroids (cholestanol and cholestan-3-one), with the concentration of each sterol/steroid shown below each bar. Different lower case and upper case letters above bars indicate statistically significant differences between the treatments in the 1st and 2nd generation, respectively. For each treatment, an asterisk represents a statistically significant difference between the two generations; ns = no statistically significant difference.



a)
Larval development (days)
b)
Pupation (%)
C1.0
S1.0
A1.0
K1.0
(0.25) (1.75)
(1.0) (1.0)
(0.5) (1.5)
(0.25) (1.75)
(1.0) (3.0)
(1.0) (0.33) (0.67)
(0.5) (0.5) (1.0)
(0.25) (0.58) (1.17)
Single sterol/steroid
S + A
S + K
S + A + K
Diet sterol/steroid treatment

Fig. 3.5. Pupal mass (a), pupal developmental time (b), and eclosion success (c) of *H. zea* on diets containing different dietary sterols/steroids. Data, presented as means (± SEM), are shown for two generations. Four different sterols/steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 12 treatments shown in this figure. The first four treatments were single sterol/steroid diets, with each sterol/steroid at a concentration of 1 µg/g (dry mass). The next five treatments represent diets that combine a sterol and steroid (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (µg/g) of each sterol/steriod is shown in parentheses directly below the bars. The last three treatments represent diets with one sterol (stigmasterol) and two steroids (cholestanol and cholestan-3-one), with the concentration of each sterol/steroid shown below each bar. Different lower case and upper case letters above bars indicate statistically significant differences between the treatments in the 1st and 2nd generation, respectively. For each treatment, an asterisk represents a statistically significant difference between the two generations; ns = no statistically significant difference.



a)
Pupal mass (mg)
b)
Pupal development (days)
c)
Eclosion (%)
C1.0 S1.0 A1.0 K1.0
(0.25) (1.75)
(1.0) (1.0)
(0.5) (1.5)
(0.25) (1.75)
(1.0) (3.0)
(1.0) (0.33) (0.67)
(0.5) (0.5) (1.0)
(0.25) (0.58) (1.17)
Single steroid
S + A
S + K
S + A + K
Diet sterol/steroid treatment

3.3.1.2 Two-steroids mixture treatment

Stigmasterol was mixed with cholestanol (1:7 ratio; 0.25 mg/g stigmasterol (S) paired with 1.75 mg/g cholestanol (A)), or with cholestan-3-one (4 different pairings; see Table 3.1); neither cholestanol nor cholestan-3-one can be converted to cholesterol. On the S + A treatment, *H. zea* was more negatively affected than was *H. virescens*. For example, the performance of *H. virescens* on the S + A diet was similar to that on the stigmasterol only diet, for both generations. *H. zea* also had similar performance on these two diets in the 1st generation, but in the 2nd generation the larvae took longer to reach the pupal stage, the pupae was smaller, and eclosion success was lower compared to the stigmasterol only diet.

In contrast, both species performance decreased as the ratio of cholestan-3-one in the diet increased, and this effect was most apparent in the 2nd-generation (Fig. 3.2-3.5), but the responses by the two species differed depending on the ratio (three S + K ratios: 1:1, 1:3, and 1:7; there were also two 1:3 treatments, that differed in the absolute amounts of sterols/steroids present). In the 1st generation, the performance of both species on 1:7 treatment was significantly worse compared to the stigmasterol only diets. Here larval development was longer, pupation was lower, the pupae were lighter, and eclosion success decreased. This reduced performance was consistent for both species in the 2nd generation. *H. zea* also had these negative effects on the 1:3 diets (0.5 mg/g S + 1.5 mg/g K, and 1 mg/g S + 3 mg/g K) in the 1st generation, but *H. virescens* did not. For example, larval and pupal development, for *H. zea* were longer compared to the stigmasterol only treatment; the same was true for pupation and eclosion. In the 2nd

generation, significantly negative effects also appeared for *H. virescens* on the 1:3 diets, including lower pupal mass, longer larval development, and lower eclosion success. Interestingly, in contrast to the 1st generation, there were no negative effects of the 1:3 diets on *H. zea* in the 2nd generation. Finally, when the S:K ratio was 1:1, performance was similar to the stigmasterol only diet across two generations, and for both species.

3.3.1.3 Three-steroids mixture treatment

Insect performance on this series of diets for both species was negatively related to the ratio of cholestanol and cholestan-3-one in the diet although their performance was not reduced as significantly as that on the S + K mixture diets. In the 1st generation, *H. virescens* was not affected by mixing cholestanol and cholestan-3-one with stigmasterol, while *H. zea* was only slightly affected by showing the longer pupal development on all three diets than that on the stigmasterol only diet (Fig. 3.2-3.5). In the 2nd generation, both experimental insects were affected extensively. For example, on the S0.25 + A0.58 + K1.17 treatment, less *H. virescens* larvae survived to the pupal stage, and the larval development was longer compared to the stigmasterol only diet. Additionally, it seemed that this series of sterol/steroids mixtures could reduce *H. virescens* pupal mass, although the only significant difference was found on the S1.0 + A0.67 + K0.33 treatment. The performance of *H. zea* was reduced only when they were given the S0.25 + A0.58 + K1.17 treatment. Here the negative effects included lower pupation and eclosion success, smaller pupae, and longer larval development.

Fig. 3.6. 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. Four different sterols/steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 12 treatments shown in this figure. The first four treatments were single sterol/steroid diets, with each sterol/steroid at a concentration of 1 µg/g (dry mass). The next five treatments represent diets that combine a sterol and steroid (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (µg/g) of each sterol/steriod is shown in parentheses directly below the bars. The last three treatments represent diets with one sterol (stigmasterol) and two steroids (cholestanol and cholestan-3-one), with the concentration of each sterol/steroid shown below each bar. Different lower case and upper case letters above bars indicate statistically significant differences between the treatments in the 1st and 2nd generation, respectively. For each treatment, an asterisk represents a statistically significant difference between the two generations; ns = no statistically significant difference.

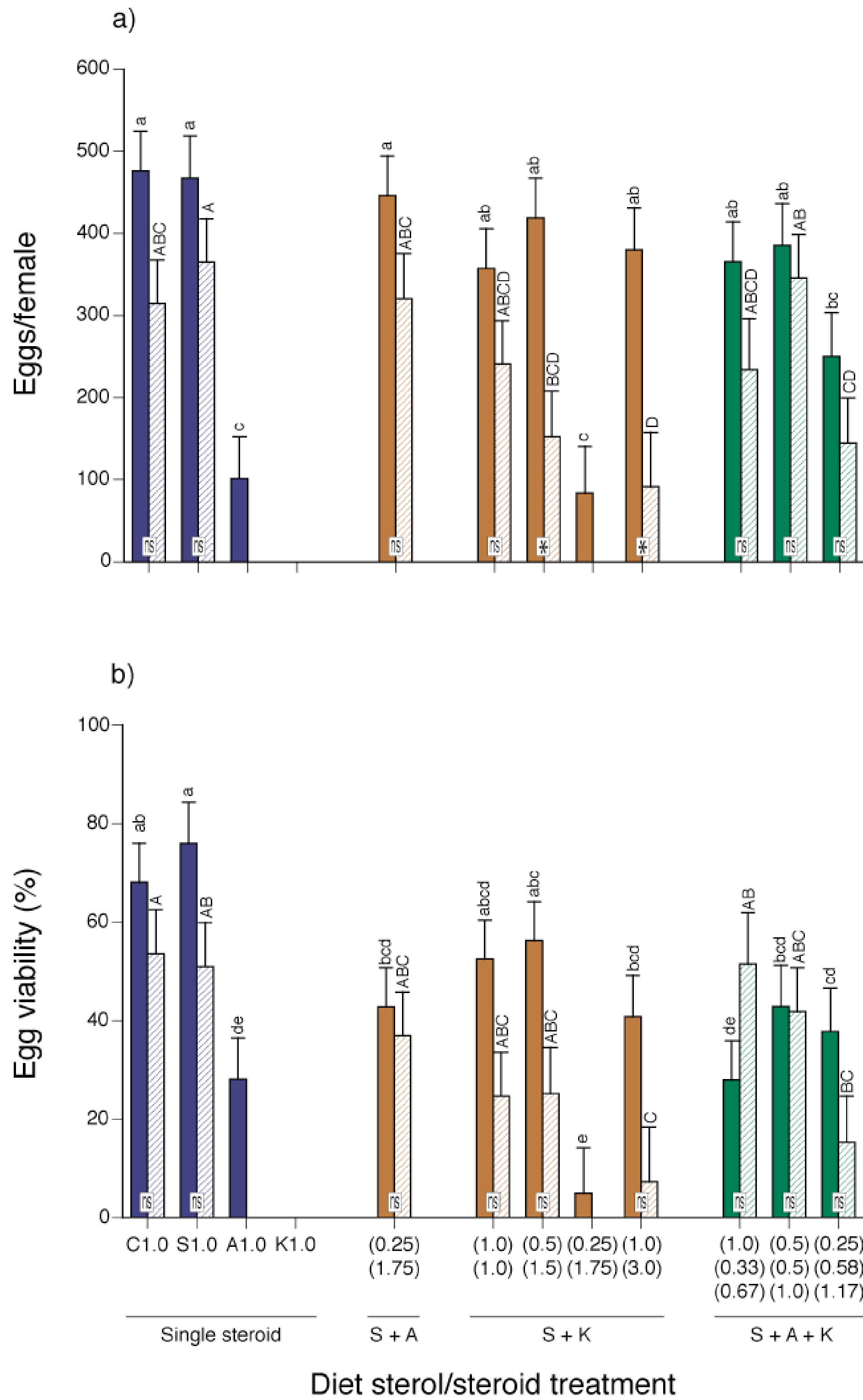
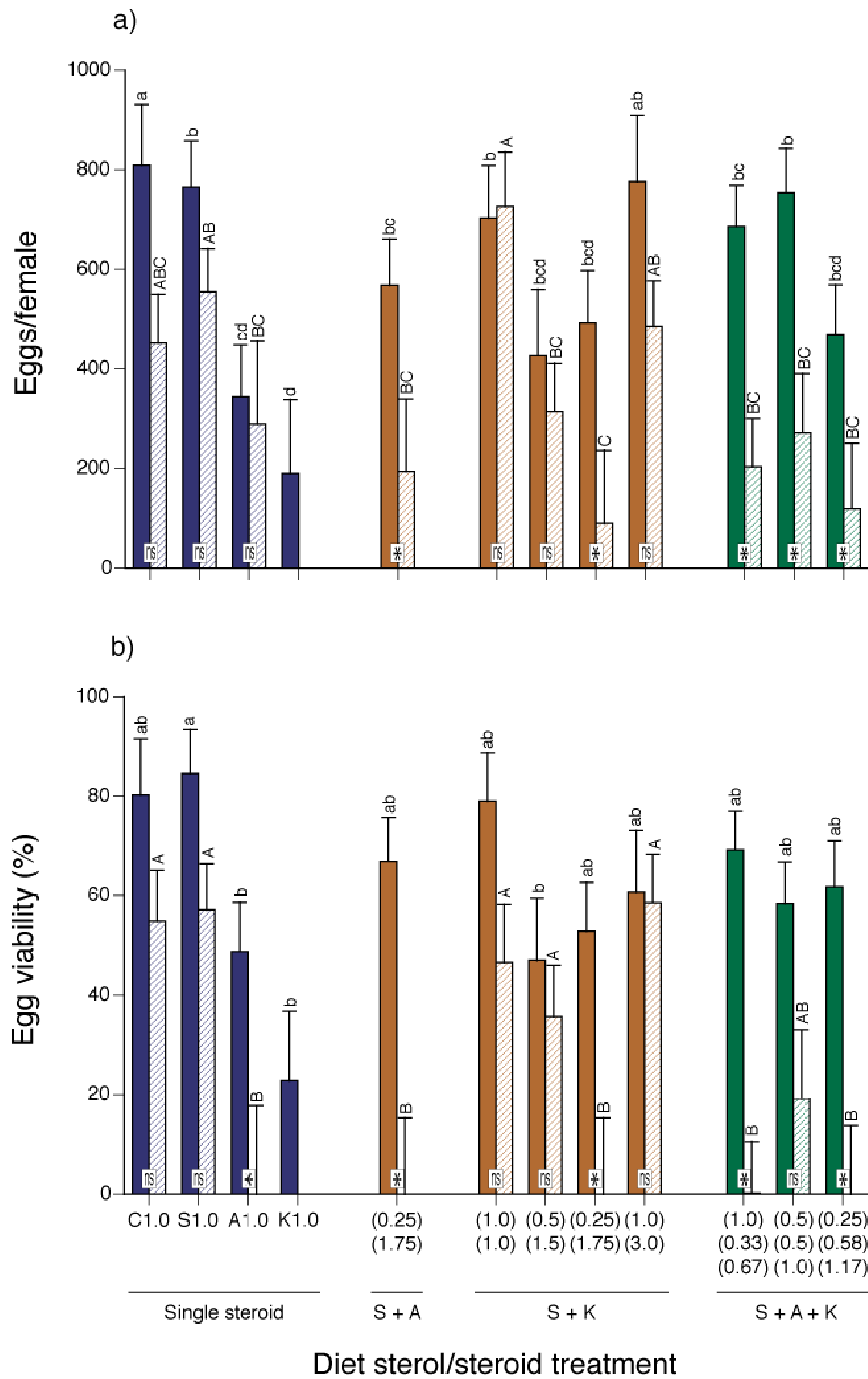

a)
Eggs/female
600
500
400
300
200
100
0
b)
Egg viability (%)
100
80
60
40
20
0
C1.0 S1.0 A1.0 K1.0
(0.25)
(1.75)
(1.0)
(1.0)
(0.5)
(1.5)
(0.25)
(1.75)
(1.0)
(3.0)
(1.0)
(0.33)
(0.67)
(0.5)
(0.5)
(1.0)
(0.25)
(0.58)
(1.17)
Single steroid
S + A
S + K
S + A + K
Diet sterol/steroid treatment

Fig. 3.7. 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. Four different sterols/steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 12 treatments shown in this figure. The first four treatments were single sterol/steroid diets, with each sterol/steroid at a concentration of 1 µg/g (dry mass). The next five treatments represent diets that combine a sterol and steroid (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (µg/g) of each sterol/steriod is shown in parentheses directly below the bars. The last three treatments represent diets with one sterol (stigmasterol) and two steroids (cholestanol and cholestan-3-one), with the concentration of each sterol/steroid shown below each bar. Different lower case and upper case letters above bars indicate statistically significant differences between the treatments in the 1st and 2nd generation, respectively. For each treatment, an asterisk represents a statistically significant difference between the two generations; ns = no statistically significant difference.



a)
Eggs/female
1000
800
600
400
200
0
b)
Egg viability (%)
100
80
60
40
20
0
C1.0 S1.0 A1.0 K1.0
(0.25) (1.75)
(1.0) (1.0)
(0.5) (1.5)
(0.25) (1.75)
(1.0) (3.0)
(1.0) (0.33) (0.67)
(0.5) (0.5) (1.0)
(0.25) (0.58) (1.17)
Single steroid
S + A
S + K
S + A + K
Diet sterol/steroid treatment

3.3.2 Reproduction performance

Adults produced similar amount of eggs on the cholesterol and stigmasterol only treatments, and egg viability was similar on these two diets for both generations (Fig. 3.6 and 3.7). No *H. virescens* survived to adult stage on cholestan-3-one treatment in the 1st-generation, and on the cholestanol treatment in the 2nd-generation so there was no reproduction data. Similarly, no data was presented for *H. zea* on the cholestan-3-one treatment in the 2nd generation.

For *H. virescens*, egg production and egg viability was significantly reduced on cholestanol treatment than that on cholestan-3-one treatment in the 1st generation (Fig. 3.6 and 3.7). For *H. zea*, egg production, but not egg viability on the cholestanol and cholestan-3-one treatments was significantly lower than that on cholesterol treatment in the 1st generation, while egg viability but not egg production on cholestanol treatment was significantly reduced in the 2nd generation.

Egg production and egg viability decreased as the ratio of cholestanol and cholestan-3-one was increased in the diets. For *H. virescens*, 1st-generation egg production was only significantly lower on highest novel steroids ratio diets (including S0.25 + K1.75, and S0.25 + A0.58 + K1.17). Egg production on the S0.25 + K1.75 treatments was also lower than that on other steroids mixtures. In contrast, egg viability was affected more extensively in the 1st generation. For example, egg viability on the S0.25 + A1.75, S1.0 + K3.0, S1.0 + A0.33 + K0.67, S0.5 + A0.5 + K1.0, and S0.25 + A0.58 + K1.17 was lower than that on the stigmasterol only treatment. Furthermore, egg viability on S0.25 + K1.75 was also lower than that on the other mixtures, except for the

S1.0 + A0.33 + K0.67 treatment. In the 2nd generation, egg production on the S0.5 + K1.5 and S1.0 + K3.0 treatment was lower than that on that on the stigmasterol only treatment. For *H. zea*, 1st-generation egg production on various sterol/steroids mixtures was similar to that on the stigmasterol only treatment, but it was significantly reduced on S0.25 + K1.75 in the 2nd generation. In contrast, *H. zea* egg viability was more affected. In the 1st generation, on the S0.5 + K1.5 treatment, it was significantly lower than that on stigmasterol only treatment. However, in the 2nd generation, a much greater range of sterol/steroid mixtures significantly impacted egg viability. These included the S0.25 + A1.75, S0.25 + K1.75, S1.0 + A0.33 + K0.67, and S0.25 + A0.58 + K1.17 treatments, which all had lower viability compared to the stigmasterol only treatment.

3.4 Discussion

3.4.1 Larval and pupal performance

H. virescens, an important crop pest across the United States, has never been studied in terms of its sterol use, and it is interesting to compare it to its close relative *H. zea*, which is also a notorious pest. Moreover, transgenerational information of dietary sterols is important in evaluating sterols effects on insect herbivores, but only one published paper using *Plutella xylostella* ever reported this effect (Behmer and Grebenok, 1998). In this experiment, we used these two caterpillars as our model and tested their response to a series of different ratio of sterols/steroids for two generations.

Both species performed well on cholesterol and stigmasterol for two generations, indicating that they are proficient at converting stigmasterol into cholesterol. Typically

caterpillar species can use various phytosterols, including stigmasterol, in their host plants (Behmer and Nes, 2003; Ritter and Nes, 1981b; Svoboda et al., 1969a; Svoboda et al., 1988; Svoboda and Weirich, 1995). This is in contrast to grasshoppers, which can only use sitosterol, another common phytosterol (Behmer and Elias, 2000; Behmer et al., 1999b). The strong diet mixing ability that is only feasible in highly mobile insect herbivores, may enables grasshoppers to be extreme specialists with respect to sterol use (Behmer and Elias, 2000).

Cholestanol, a sterol different from cholesterol only by lacking a C5 double bond (Fig. 3.1), has been shown to be good at replacing cholesterol for structural purpose (i.e., cellular membrane) in beetles, cockroaches and other insects, as long as a minimum supply of cholesterol is available for steroid hormone (Clark and Bloch, 1959b; Clayton, 1964; Dutky et al., 1967). In the current experiment, both species performed well in the 1st-generation even if only cholestanol was added into the diet. The sterol contamination in diet components, for instance from casein, and cholesterol in eggs, provisioned by reproducing females, might act as the supply of cholesterol in this generation.

However, caterpillars on the cholestanol only treatment could not function well in the 2nd generation, and eggs were the only difference between the two generations insects. Eggs for the start of the 1st-generation were produced by the parents reared on stock diet that contains suitable sterols (i.e., sitosterol, another phytosterol commonly used by insect herbivores, and stigmasterol), while eggs for the start of the 2nd-generation came from females fed cholestanol. Eggs are believed to contain enough cholesterol to support insect development when a proper sparing sterol (e.g.,

cholestanol) is used (Dupont, 1982), and parental dietary sterols can directly affect sterol content in eggs (Costet et al., 1987). Therefore, in contrast to those in the 1st generation, 2nd-generation insects could not get a proper amount of cholesterol or suitable sterols from their parents and, consequently, cholestanol was limited in fulfilling its sparing function. Similar results have also been found in fruitflies, *Drosophila melanogaster* (Kircher and Gray, 1978). For example, fruitflies produced by parents fed cholesterol had a much better performance on cholestanol than those produced by parents fed cholestanol.

The two species seemed to have different ability in using cholestanol as a "sparing" sterol. In our experiment, when they were fed the S0.25 + A1.75 diet, *H. virescens* performance was similar to that on the stigmasterol only treatment, for both generations, which indicated that sterol content in the eggs was not that important when a certain amount of "good" sterol (i.e., stigmasterol) was incorporated into the diet. Interestingly, for *H. zea*, performance was significantly reduced in the 2nd-generation, compared to performance on the stigmasterol only diet. This indicated that cholestanol is not good for *H. zea* in sparing cholesterol, but this effect was only detected when *H. zea* could not get beneficial sterols from the eggs. And this difference was more obvious in reproduction performance.

Cholestan-3-one is obviously an unsuitable steroid in this experiment, but it is also the case that insects seldom encounter this steroid in nature. Cholestan-3-one differs from cholestanol by replacing a C3 hydroxyl by a C3 ketone (Fig. 3.1). In this experiment, the caterpillars on cholestan-3-one diet had significantly poorer performance

than those on the cholestanol diets, and this was the case in both generations. This suggests that insects could not use cholestan-3-one as efficiently as cholestanol. From a structural perspective, this suggests a C3 hydroxyl is a very important structural feature for insects.

Interestingly, some insects, including houseflies (Dutky et al., 1967), are able to convert C3 ketone into C3 hydroxyl. However; it appears the efficiency at which they do this is very low (Dutky et al., 1967). We also found that both species could convert cholestan-3-one into epicholestanol (3α-hydorxyl isomer) and cholestanol (3β-hydroxyl isomer) but they differed in this ability (Chapter IV). For example, there was more cholestanol than epicholestanol in *H. zea,* but vice verse in *H. virescens* when they fed cholestan-3-one. From a structural perspective, a cis-structure (3α-hydroxyl isomer, epicholestanol) does not function as well as a flat structure (3β-hydroxyl isomer, cholestanol) in cellular membranes (Demel and De Kruyff, 1976). Therefore, the result that *H. zea* was more tolerant to cholestan-3-one than *H. virescens* could be caused by different ability between *H. zea* and *H. virescens* in converting cholestan-3-one.

Insect performance improved as the ratio of stigmasterol to cholestan-3-one increased in the diet. It is worth noting, however, that even when stigmasterol was at its lowest concentration (S0.25 + K1.75), it is still present at amounts near the minimum sterol requirement (0.025% vs. 0.015%) (Ritter and Nes, 1981a). Given that there is sufficient total stigmasterol in the diet, this suggests that dietary sterols are passively absorbed. This supposition is supported by the finding that insect performance on the S0.5 + K1.50 and S1.0 + K3.0 diets (both of which have the same ratio, but the total

amount of sterols in the later one is twice as that in the former one) was similar. A handful of other studies have shown a similar result (Behmer and Elias, 1999b, 2000; Nes et al., 1997; Ritter, 1984).

Insects only need a tiny amount of ecdysteroids (0.4 – 4 μg / g fresh weight) in larval stage (Lafont et al., 2005). The poorer performance on higher cholestan-3-one ratio diets was possibly due to the sterol deficiency in structure purpose. Lower pupal mass on these diets also suggested a deficiency in related to structural purposes. Behmer and Elias also found that grasshopper, *Schistocerca americana*, had a lower body mass when unsuitable sterol was mixed with suitable sterol in their diet (Behmer and Elias, 1999b). From the results, we also found that 75% (i.e., S0.5 + K1.50 and S1.0 + K3.0) seems to be the threshold where the unsuitable steroid, cholestan-3-one, began to affect insect performance negatively. This threshold can vary upon different unsuitable sterols/steroids and among different insect species. For example, Nes et al (1997) found *H. zea* growth was arrested in the 3rd-instar when it was fed diets containing more than 50% unsuitable sterol, 24-dihydrolanosterol. Likewise, the grasshopper *Schistocerca americana* shows reduced survival when its diets containing more than 75% unsuitable sterol (Behmer and Elias, 1999b, 2000).

In this experiment, we also tested the effects of three sterol/steroids mixture (Table 3.1), which is a common combination in our modified tobacco plants (see Chapter II). Not surprisingly, cholestanol, as a "sparing" sterol, could alleviate the negative effects of cholestan-3-one. For instance, in contrast to performance on the S0.25 + K1.75 treatment, performance on S0.25 + A0.58 + K1.17 was not worse than

that on stigmasterol diet, and the reduced performance only appeared in the 2nd generation, including longer larval development, lower pupation for both species, lower eclosion and lower pupal mass for *H. zea*.

3.4.2 Reproduction

Adult females have follicle cells in developing ovaries that synthesize ecdysteroids, and then package them in their eggs (Swevers and Iatrou, 2003); this is in contrast to larvae, which produce ecdysteroids in the prothoracic glands. Ecdysteroids are important for embryogenesis. For instance, they are actively involved in vitellogenesis, the uptake of yolk protein (Swevers et al., 2005), and deficiencies in ecdysteroids can result in embryonic lethality (Gilbert, 2004). In our experiment, reproduction is more sensitive to cholestanol and cholestan-3-one than larval and pupal performance because egg production and viability were reduced significantly for both species fed the cholestanol only treatment, even in the first generation. This means that more cholesterol is needed in reproduction than larval and pupal development. In fact, the concentration of ecdysteroids in eggs/embryos of Orthoptera and Lepidoptera is 10 to 100 times that in larvae or pupae (Lafont et al., 2005). Furthermore, this sensitivity is also species-specific and not related to the ability of insects in converting cholestan-3-one into cholestanol, because reproduction, especially egg viability, is reduced more for *H. zea* than for *H. virescens* when cholestanol was included in the diet (Fig. 3.6 and 3.7). Similar to that in the larval and pupal performance, the strong reproduction performance in the first generation also indicated the importance of cholesterol in eggs.

3.4.3 Abnormal phenomenon in this experiment

There was abnormal phenomenon in our experiments. For example, even on suitable sterol, insect performance was still reduced in the second generation than that in the first generation (Fig. 3.2-3.5). High concentrations of suitable sterols generally do not produce deleterious effects (Ritter and Nes, 1981a), so this was probably caused by: 1) a potentially suboptimal diet, and 2) insect inbreeding. In our experiment, a semi-synthetic diet was used so many unknown important nutrients may not be induced in the experimental diets, compared with the stock diet. However, this semi-synthetic diet may have many advantages in insect sterol research over the stock diet since it has less sterol contamination. Moreover, subtle negative effects between treatments are more likely detected by using suboptimal diet (Blanco et al., 2009). Inbreeding can lower fitness-related traits including development, survival and reproduction (Charlesworth and Charlesworth, 1987; Harano, 2011). In this experiment, there is unavoidable inbreed although we tried to reduce this problem through cross-family breeding. In spite of this abnormal phenomenon, our results provide some very novel insights concerning insect sterol utilization, especially the effects of variation in the ratios of dietary sterols/steroids.

3.4.4 Significance and application

In this experiment, we can conclude that: 1) sterol structure is important in determining sparing mechanism capabilities, 2) there is variation of different insect

species in using different sterols/steroids, and 3) insect performance will decrease as the ratio of suitable sterols increases. Therefore, it is possible to develop different sterol profile targeting specific pests in crops. Phytosterols (e.g., sitosterol, stigmasterol and campesterol) are important in physiological process including cellular membrane components and signal transduction in plants (Moreau et al., 2002; Piironen et al., 2000). This experiment confirms that it is not necessary to remove all "good" phytosterols from plants, and that modifying the sterols/steroids ratio can have significant negative effects on insect herbivores.

CHAPTER IV

STEROL AND STEROID METABOLISM PLUS ABSORPTION IN GENERALIST AND SPECIALIST CATERPILLARS

4.1 Introduction

Sterols are important for both insects and mammals in various aspects, and two well-known functions for insects are: 1) cellular membrane components which provide rigidity and adjust permeability, and 2) as essential precursors to steroid hormone, e.g., ecdysone, which actively participates in insect physiological processes, including molting and metamorphosis. However, only very low amounts of sterols are needed for the production of steroid hormones (Behmer and Nes, 2003; Lafont et al., 2005). Both insects and mammals have cholesterol as their dominant body sterol, but unlike mammals, insects do not possess the enzymatic system needed to generate cholesterol from farnesyl pyrophosphate (Klowden, 2007). Consequently, insects must acquire sterols from their food.

Insect herbivores get all kinds of nutrients including sterols from plants. However, plants only contain a low amount of cholesterol (Piironen et al., 2000). Instead, they contain a range of various phytosterols (Behmer et al., 2011; Nes et al., 1977; Salt et al., 1991). Caterpillar species can convert a variety of phytosterols, e.g., stigmasterol, sitosterol and campesterol, into cholesterol by dealkylation (Svoboda, 1999; Svoboda and Weirich, 1995). However, atypical steroids may cause problem for insects. In a plant study (Chapter II), several caterpillar species including *H. virescens*

and *M. sexta* were given two tobacco lines – one a control line containing normal plant sterols, and the other a modified line containing both normal and novel steroids,(e.g., stanols (21.9%) and 3-ketosteroids (53.2%)). Different performance patterns were found between the two lines, and the inability of insects in using novel steroids, especially the conversion to cholesterol, was proposed. To date, no studies have recorded whether plant-feeding insects could convert these two groups of steroids into cholesterol, although some species can metabolize them. For example, cockroaches, an omnivorous insect, can convert cholestanol into Δ^7-5α-cholesten-3β-ol slowly (Clayton and Edwards, 1963), and houseflies can convert cholestanone into cholestanol and epicholestanol (Dutky et al., 1967). Both of these studies only used the target sterol/steroid as their sole dietary sterol, and it is interesting to see what sterol profile in insect herbivores looks like when they are given different ratios of these steroids, a mimic in the plants.

In mammals, plant sterols, especially phytostanols, can interfere with cholesterol, i.e., replacing or precipitating cholesterol in mixed micelles absorbed by intestine (Behmer et al., 2011; Ikeda et al., 1988; Moreau et al., 2002), but mammals rarely contains any plant sterols because the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family can preferentially secretes noncholesterol sterols back into the gut lumen, along with bile (Allayee et al., 2000; Berge et al., 2000). As a result, dietary plant sterols can help reduce cholesterol levels in human. In contrast, atypical steroids, i.e., noncholesterol steroids, are often found in insects even though their dominant sterol is cholesterol when they feed on normal dietary sterols (reviewed by Behmer and Nes, 2003). This difference may be an evolutionary result of the different ability in

synthesizing cholesterol *de novo* between insects and mammals. Although ABC proteins are also found in insects, their only known function is the transportation of pigment precursors (Ewart et al., 1998; Komoto et al., 2009).

For plant-feeding insects, a high ratio of unsuitable sterols can negatively affect performance, through lowering survival, increasing development, reducing body mass, and reducing reproduction (Behmer and Elias, 1999b, 2000; Costet et al., 1987; Dutky et al., 1967; Nes et al., 1997); although the degree to which performance is reduced varies depending upon sterol structure. This gives a promising opportunity of controlling phytophagous insect pests by increasing the amount of nonconvertible sterols in plants. In this study, we use two genetically modified tobacco lines, one expressing normal sterol profile and the other expressing highly novel sterols/steroids, to explore how sterols profile in insects changes upon different dietary sterols. We further use artificial diets to simulate and simplify the phytosterol profile in the modified tobacco plants to have a deep investigation in sterol absorption and metabolism in insects. We use *Heliothis virescens*, a generalist, and *Manduca sexta*, a specialist, in this study, which allows us to compare and contrast how insects with different feeding-biology respond to different dietary sterol/steroid combinations and ratios.

4.2 Materials and Methods

4.2.1 Experimental insects

One generalist caterpillar, *Heliothis virescens* (Noctuidae) and one specialist caterpillar, *Manduca sexta* (Sphingidae), were used in this experiment. Both caterpillars

accept tobacco as their host in nature. The *H. virescens* eggs were purchased from Benzon Research Inc. (Carlisle, PA) and the *M. sexta* eggs were purchased from Carolina Biological Supply Company (Burlington, NC). The eggs were incubated at 27 °C and neonates hatching within 6 hrs were used as a source for the start of the experiments.

4.2.2 Experimental plants

The two tobacco lines were used in this study are the same described in Chapter II. These two tobacco lines had different steroid profiles (see Table 2.1 in Chapter II), but no other differences are known (Corbin et al., 2001). The plants and two caterpillar species were grown and reared, respectively, using the methods previously described in Chapter II. Adults, immediately upon eclosion, were collected and frozen at -20 °C, and then freeze-dried until needed for sterol analysis.

4.2.3 Artificial diets

The artificial diets used in this study are similar to those described in Chapter III. In total, 11 unique diets were generated from various combinations of 4 different sterols/steroids. The sterols/steroids used in this study are shown in Fig. 4.1. They are: (1) 5α-cholester-3β-ol (cholesterol, ≥ 95%), (2) 5,22-cholestadien-24β-ethyl-3β-ol (stigmasterol, ≥ 98%), (3) 5α-cholestan-3β-ol (cholestanol, 95%), and (4) 3-keto-5α-cholestane (cholestan-3-one, ≥ 98%); cholesterol and cholestanol were purchased from Sigma Chemical (St. Louis, MO, USA), while stigmasterol and cholestan-3-one were

purchased from Steraloids Inc. (Newport, RI, USA). The caterpillars used in this study do not grow well when cholestanol and cholestan-3-one are the only steroids in the diet (see Chapter III). Thus, these two steroids are considered "bad" for our caterpillars.

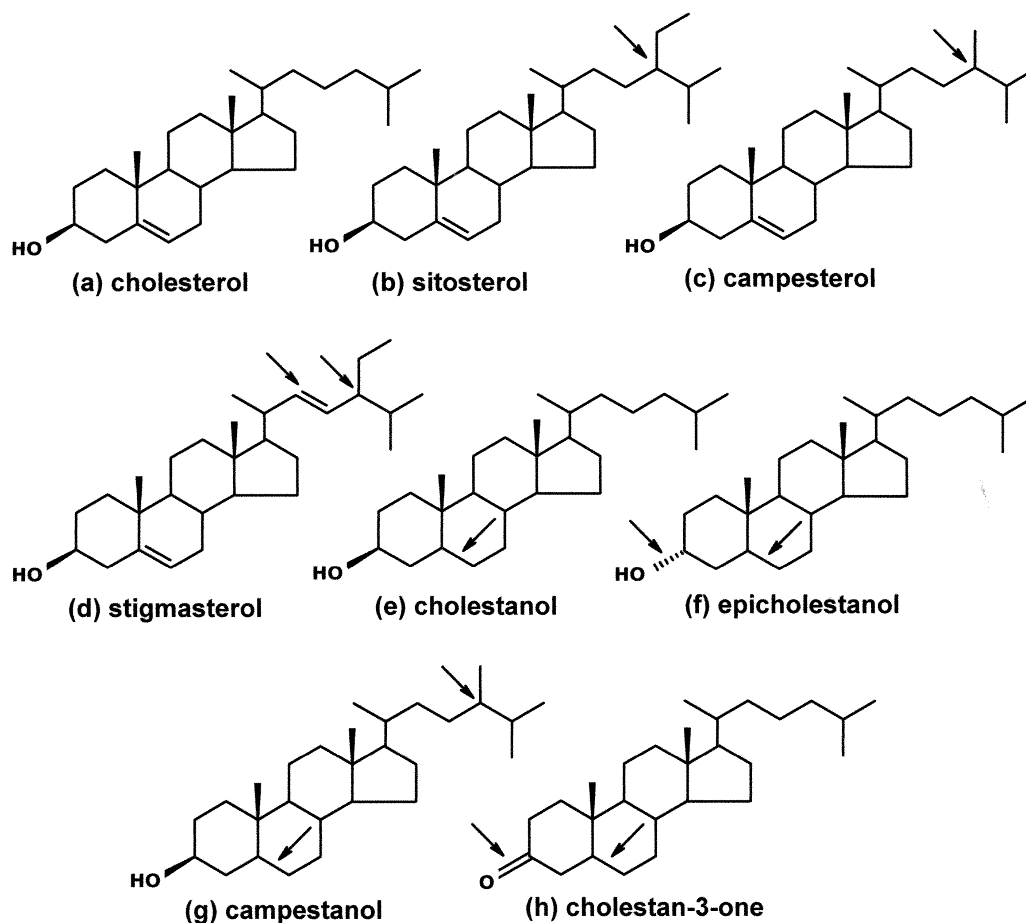


Fig. 4.1. Eight sterols/steroids in this study. Cholesterol (a) is the dominant sterol in most insects including those that feed on plants. Sitosterol (b), campesterol (c), and stigmasterol (d) are all common phytosterols that many caterpillars readily convert to cholesterol. Sitosterol and campesterol differ from cholesterol by having a C24 ethyl and methyl group, respectively. Stigmasterol differs from cholesterol by having a C24 ethyl group, plus a C22 double bond. Cholestanol (e), epicholestanol (f), and campestanol (g) are steroids lacking a $\Delta 5$ double bond. Epicholestanol is an isomer of cholestanol (it has a 3α-hydroxyl group instead of 3β-hydroxyl). Campestanol differs from cholestanol and epicholestanol by having a C24 methyl group. Cholestan-3-one (h) is a keto-steroid. In contrast to cholesterol, this steroid has a C3 ketone instead of a C3 hydroxyl, and there is no $\Delta 5$ double bond in the sterol nucleus.

The 11 different diets used in this study represent a range of single and mixed-sterol diets. Three of these diets were single-steriod diets, with cholesterol, stigmasterol and cholestanol added at a concentration of 1 mg/g dry mass. Five diets contained two steroids (one "good", one "bad"), at various concentrations. The first double-steroid diet contained stigmasterol and cholestanol at 0.25 mg/g and 1.75 mg/g, respectively (a 1:7 ratio). The other four double steroid diets contained stigmasterol (S) and cholestan-3-one (K), paired at various concentrations: (1) 1.0 mg/g of S, paired with 1.0 mg/g K (a 1:1 ratio), (2) 0.5 mg/g S, paired with 1.5 mg/g K (a 1:3 ratio), (3) 0.25 mg/g S, paired with 1.75 mg/g K (a 1:7 ratio), and (4) 1.0 mg/g S, paired with 3.0 mg K (a 1:3 ratio). The last three diets contained 3 steroids: stigmasterol (S), cholestanol (A), and cholestan-3-one (K). The ratio of A to K in the diet was meant to reflect the ratio in which they occur, relative to each other, in transgenic tobacco lines that that have modified steroid profiles (Chapter II). The three triple-steroid diets had the following steroid combinations: (1) 1.0 mg/g S, 0.33 mg/g A, and 0.67 mg/g K (a 1:1 ratio of "good" to "bad" steroids), (2) 0.5 mg/g S, 0.5 mg/g A, and 1.0 mg/g K (a 1:3 ratio of "good" to "bad" steroids), and (3) 0.25 mg/g S, 0.58 mg/g A, and 1.17 mg/g K (a 1:7 ratio of "good" to "bad" steroids).

The two caterpillar species were reared on these diets using protocols previously described in Chapter III. Adults, immediately upon eclosion, were collected and frozen at -20 °C, and then freeze-dried until needed for sterol analysis.

4.2.4 Sterol identification and quantification

4.2.4.1 H. virescens *on tobacco plants*

The freeze-dried insect bodies (3-5 individuals) were homogenized and weighed and then extracted in ethanol. The debris-ethanol mixture was shaken and sonicated for 3 consecutive 5-minute bouts. To facilitate proper quantification of the identified steroids in each sample, 50 μg of the internal standard, cholestane, was added to each sample. Following a 12 hrs incubation period, 5 ml of chloroform and 5 ml of H_2O were added to each sample, mixed and allowed to separate for 12 hrs. Following separation, the chloroform (lower layer) was removed and evaporated under nitrogen to a volume of 200 μl.

The release of base hydrolysable sterol esters was accomplished by the addition of 8 ml of 70% methanol/ H_2O (5% KOH w/v) to each sample. The samples were incubated at 55 °C in a water bath for 2.5 hrs, with constant shaking. Following base saponification, 3 ml of H_2O was added to each sample, and each sample was washed twice with 4 ml volumes of hexane. The samples were then reconstituted in hexane and water, and shaken vigorously; the samples were then left until the layers separated. The hexane layer (containing the released sterols) was removed, combined and backwashed to neutrality against an equal volume of 50% methanol/H_2O, with neutrality determined by PHydrion Mikro Ion paper (Micro Essential Laboratories, Brooklyn, New York). The neutral hexane fraction was evaporated to a 50 μl volume. Sterols/steroids were identified by gas chromatography – mass spectroscopy (GC-MS) and quantified by gas

chromatography. All of the described procedures utilized solvents that were preequilibrated to the paired solvent.

Free sterols and sterols freed following base saponification were quantified by gas liquid chromatography (GLC) using authentic stigmasterol and cholesterol as standards. Identification of sterols by GLC was based on their relative retention times to cholesterol on the DB-17 column (Agilent Technologies) with dimensions of 30 meters, 0.25 mm diameter and 0.25 micron film thickness in an Agilent 6890 Networked GC-fid. The system maintained a carrier gas flow rate of 1.3 ml per minute, inlet temperature of 280 °C, detector temperature of 290 °C and an oven ramp beginning at 80 °C ascending at 25 °C per minute to a temperature of 240 °C and ascending to a final temperature of 290 °C at a rate of 5 °C per minute and holding and the final temperature for 20 minutes. The elution pattern of the sterols on the GC was in agreement with those reported previously by Heyer et al., 2004 (Heyer et al., 2004) and confirmed by Agilent 5973 GC-MS running the identical column, gas and temperature protocols as those described for the GC-fid.

4.2.4.2 H. virescens on *diets*

Each individual dry body was ground and weighed in a 1.5 ml VWR eppendorf centrifuge tube. A 0.5 ml volume of chloroform, a 0.5 ml volume of methanol, 10 µg of cholestane and 2 glass beads (size 3, Kimble Kontes LLC) were added to each sample. The samples were shaken vigorously using a Pneumatic paint shaker (Central Pneumatic, stock No. 422) powered by a 3-Gallon air compressor (Craftsman) for 30

mins. A 0.5 ml volume of H_2O was added to each tube and each sample was vortexed, and the solvent layers were allowed to separate for 12 hrs. Following separation, the chloroform (lower layer) was removed and evaporated under nitrogen to a volume of 200 µl. Then the base hydrolysable sterol esters were released as stated above.

All free sterol and free sterols evolved following base saponification were converted to TMSi derivatives prior to identification and quantification by GC-MS. Conversion of all free sterols to their TMSi derivatives was accomplished by the addition of 100 µl BSTFA + TMCS, 99:1 (Sylon BFT) kit (Supelco, Bellefonte, PA) to the hexane fraction. The solution was well-mixed and incubated for 2 hrs. Following the 2 hrs incubation, equal volumes of both hexane and 70% methanol/water were added to each sample to stop the reaction. Each sample was then well-mixed before being allowed to separate. Following separation, the hexane layer was removed and evaporated to a 50 µl volume. Sterols/steroids were identified and quantified by gas chromatography – mass spectroscopy (GC-MS) (see system described above). All derivatized sterols maintained characteristics that were typical of TMSi derivatized standards and were also in agreement with those published by Nasir and Noda, 2003(Nasir and Noda, 2003). All of the described procedures utilized solvents that were preequilibrated to the paired solvent.

4.2.4.3 M. sexta *on tobacco plants and diets*

Each individual dry body was ground and weighed in a 50 ml VWR centrifuge tube. An 8 ml volume of chloroform, 8 ml volume of methanol, 10 µg of cholestane and

15 glass beads (size 3, Kimble Kontes LLC) were added to each sample. The samples were shaken vigorously by using a Pneumatic paint shaker powered by a 3-Gallon air compressor for 30 mins. A 700 µl volume of the extraction was transferred into a 1.5 ml VWR eppendorf centrifuge tube and a 350 µl volume of H_2O was added into each tube. Each sample was vortexed and allowed to separate for 12 hrs. Following separation, the chloroform (lower layer) was removed and evaporated under nitrogen to a volume of 200 µl. Then the total steroids amount including free and esterified ones was measured following the same method used before.

4.2.5 Statistical analysis

The steroid profiles for *H. virescens* reared on our two tobacco lines came from pooled samples, which precluded any statistical analysis. For all other experiments, sterol analyses were performed on individuals (with samples size of between 4-6 for *H. virescens* and *M. sexta* on diet and 12 for *M. sexta* on plant). For the *M. sexta* plant experiment, a t-test was used for comparisons of total steroid body content and total cholesterol body content on the two tobacco lines (normal and modified steroid profiles). For the diet experiments, total body steroid content and total cholesterol body content was analyzed by ANOVA. For all analysis, type I error was 0.05 and False Discovery Rate which controls the expected proportion of falsely rejected hypotheses was used for the adjustment in multiple comparison (Benjamini and Hochberg, 1995). All analyses were performed in SAS v. 9.2 (Cary, NC, USA).

4.3 Results

4.3.1 Body sterol/steroid composition of caterpillars reared on plants

For *H. virescens*, individual dry mass was not recorded. Instead, 3-5 individuals were pooled, and their combined total total dry mass was recorded. As a result, no statistical comparison of body size on the two tobacco lines was possible. For *M. sexta*, individual dry mass was recorded, and here no difference was observed in adult dry mass between the two tobacco lines ($t_{22} = 1.33$, $P = 0.196$). Therefore, the relative amount of steroids (µg/g dry mass), instead of absolute amount of steroids, was used in the analyses.

Table 4.1 Sterol/steroid profiles from *H. virescens* reared on tobacco plants with normal (control) and modified sterol/steroid profiles. The values presented for each tobacco line represent a single sample of 3-5 pooled individuals.

		Tobacco line	
Sterol type	Variable	Control	Modified
Total Steroids	Relative amount (µg/g)	1658	951
	Tissue profile (%)	100	100
Cholesterol	Relative amount (µg /g)	1445	715
	Tissue profile (%)	87.2	75.2
Stigmasterol	Relative amount (µg /g)	213	24
	Tissue profile (%)	12.8	2.6
Cholestanol	Relative amount (µg /g)	not detected	186
	Tissue profile (%)	-	19.5
Cholestan-3-one	Relative amount (µg /g)	not detected	26
	Tissue profile (%)	-	2.7

Different steroids were recovered from insects on the two tobacco lines. For *H. virescens*, cholesterol and stigmasterol were the only sterols recovered when this caterpillar was reared on the control tobacco plants (Table 4.1). In contrast, two additional steroids, cholestanol and cholestan-3-one, were found in *H. virescens* fed tobacco plants with modified sterol profiles (Table 4.1). For *M. sexta*, campesterol, but not stigmasterol, was recovered from caterpillars reared on both the control and modified tobacco plants (Table 4.2). Cholesterol was the dominant sterol recovered from both *H. virescens* and *M. sexta*, on both the control and modified tobacco plants. Interestingly, the cholesterol profile (expressed as a % of the total sterol/steroid profile) for these two caterpillar species differed. For *H. virescens*, the percent cholesterol was similar in caterpillars from the control and modified tobacco plants (Table 4.1). In contrast, the proportion of cholesterol in *M. sexta* from the control lines was significantly higher than that from larvae on modified plants ($t_{22} = 15.50$, $P < 0.001$, Table 4.1 and Table 4.2). Moreover, the relative cholesterol amount (μg/g dry body mass) as well as the relative total sterol/steroid amount (μg/g dry body mass) in both species on the control plants was much higher than those on the modified plants (*M. sexta*: relative cholesterol, $t_{22} = 8.78$, $P < 0.001$; relative total, $t_{22} = 3.01$, $P = 0.006$; Table 4.1 and Table 4.2). Interestingly, a large percentage of cholestanol, but only a very small amount of cholestan-3-one was found when both species were reared on the modified tobacco plants (Table 4.1 and Table 4.2).

4.3.2 Artifical diet experiment

There was no difference in adult dry mass between different treatments (*H. virescens*: $F_{10, 53} = 1.86$, $P = 0.073$; *M. sexta*: $F_{9, 51} = 1.94$, $P = 0.067$) so the relative amount of steroids (µg/g dry body mass), not the absolute amount of steroids, was used for the analysis. Additionally, no *M. sexta* adults developed on the stigmasterol only treatment, so the sterol profile of 3rd-instar larvae was presented. These data are not, however, included in the statistical analysis; they are presented for reference purposes only.

Table 4.2 Sterol profiles from *M. sexta* reared on tobacco plants with normal (control) and modified sterol/steroid profiles. The data are presented as means (± SEM). * indicates a higher value for the measure of the specific sterol type between two tobacco lines.

		Tobacco line	
Sterol type	Variable	Control (± SEM)	Modified (± SEM)
Total Steroids	Relative amount (µg/g)	4046 (191)*	3468 (150)
	Tissue profile (%)	100	100
Cholesterol	Relative amount (µg /g)	3731 (179)*	1808 (126)
	Tissue profile (%)	92.2 (0.4)*	52.1 (2.6)
Campesterol	Relative amount (µg /g)	315 (22)	99.3 (117)
	Tissue profile (%)	7.8 (0.4)	2.9 (0.5)
Cholestanol	Relative amount (µg /g)	not detected	1556 (124)
	Tissue profile (%)	-	44.9 (2.7)
Cholestan-3-one	Relative amount (µg /g)	not detected	5 (3)
	Tissue profile (%)	-	0.2 (0.1)

Table 4.3 Body sterol profiles as a percent (mean ± SEM) for *H. virescens* reared on artificial diets containing one, two or three sterols/steroids. In total 4 different sterols were used to make these diets: cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K). For each treatment, the sterol present in the diet, and its concentration (the number adjacent to a capital letter), are presented. For example, the first double sterol diet contained stigmasterol and cholestanol at concentrations of 0.25 mg/g and 1.75 mg/g, respectively. Comparisons across treatments were limited to the cholesterol. ANOVA was performed, and treatments with different letters indicate a significant difference ($P < 0.05$).

	Sterol treatments										
	Single sterol diets			Double steroid diets					Triple steroid diets		
Body sterol type (%)	C 1.0	S 1.0	A 1.0	S 0.25 A 1.75	S 1.0 K 1.0	S 0.5 K 1.5	S 0.25 K 1.75	S 1.0 K 3.0	S 1.0 A 0.33 K 0.67	S 0.5 K 0.5 K 1.0	S 0.25 K 0.58 K 1.17
Cholesterol	100[a]	96.9[a]	29.3[e]	43.8[d]	59.8[b]	40.3[d]	27.1[e]	48.9[c]	54.1[bc]	37.9[d]	24.9[a]
(± SEM)	(0)	(1.4)	(1.0)	(1.6)	(2.0)	(2.2)	(1.9)	(4.4)	(2.3)	(2.0)	(1.2)
Stigmasterol	-	3.1	-	1.5	3.6	2.2	1.3	3.3	3.6	1.7	1.1
(± SEM)		(1.3)		(0.7)	(0.7)	(0.6)	(0.7)	(0.7)	(0.7)	(0.6)	(0.4)
Cholestanol	-	-	70.7	55.7	5.0	7.2	13.3	4.1	10.7	23.3	23.6
(± SEM)			(1.0)	(1.4)	(1.3)	(1.3)	(0.8)	(1.1)	(0.7)	(0.8)	(2.1)
Epicholestanol	-	-	-	-	31.2	49.5	57.3	42.7	33.8	36.1	49.3
(± SEM)					(2.0)	(2.9)	(2.4)	(3.8)	(2.6)	(2.2)	(3.2)
Cholestan-3-one	-	-	-	-	0.5	1.0	1.0	1.1	0.8	1.1	1.1
(± SEM)					(0.2)	(0.2)	(0.2)	(0.2)	(0.2)	(0.3)	(0.1)

Table 4.4 Body sterol profiles as a percent (mean ± SEM) for *M. sexta* reared on artificial diets contain one, two or three sterols/steroids. In total 4 different sterols were used to make these diets: cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K). For each treatment, the sterol present in the diet, and its concentration (the number adjacent to a capital letter), are presented. For example, the first double sterol diet contained stigmasterol and cholestanol at concentrations of 0.25 mg/g and 1.75 mg/g, respectively. Comparisons across treatments were limited to the cholesterol. ANOVA was performed, and treatments with different letters indicate a significant difference ($P < 0.05$).

	Sterol treatments										
	Single sterol diets			Double steroid diets					Triple steroid diets		
Body sterol type (%)	C 1.0	S 1.0*	A 1.0	S 0.25 A 1.75	S 1.0 K 1.0	S 0.5 K 1.5	S 0.25 K 1.75	S 1.0 K 3.0	S 1.0 A 0.33 K 0.67	S 0.5 K 0.5 K 1.0	S 0.25 K 0.58 K 1.17
Cholesterol	100 [a]	72.5	24.9 [e]	31.2 [d]	42.9 [b]	38.0 [c]	30.1 [d]	36.7 [c]	42.9 [b]	36.8 [c]	26.1 [e]
(± SEM)	(0)	(2.7)	(1.3)	(1.0)	(1.4)	(0.5)	(1.2)	(0.8)	(1.7)	(0.9)	(1.6)
Stigmasterol	-	27.5	-	-	0.8	0.6	-	0.2	-	-	-
(± SEM)		(2.7)			(0.6)	(0.5)		(0.2)			
Cholestanol	-	-	72.4	64.9	48.4	51.6	56.1	52.6	51.1	53.4	63.1
(± SEM)			(1.2)	(1.1)	(1.3)	(1.5)	(1.5)	(1.5)	(1.2)	(1.1)	(1.5)
Epicholestanol	-	-	-	-	3.8	7.9	9.6	7.7	3.8	7.1	6.7
(± SEM)					(1.2)	(1.4)	(0.8)	(0.8)	(0.9)	(0.8)	(0.7)
Cholestan-3-one	-	-	2.8	3.9	4.1	1.8	4.1	2.8	2.2	2.7	4.2
(± SEM)			(0.6)	(0.8)	(0.8)	(0.3)	(0.9)	(0.6)	(0.3)	(0.4)	(0.8)

*For stigmasterol treatment, sterol profile in the 3rd instar larvae was presented and it was excluded from the cholesterol comparison.

4.3.2.1 Body sterol/steroid composition of caterpillars reared on artificial diets

There were many similarities in body sterol/steroid composition between the two species (Table 4.3 and 4.4). For example, cholesterol was always found in *H. virescens* and *M. sexta*, regardless of the diet sterol/steroid content. Likewise, in both species, cholestanol was recovered from insects fed cholestanol and/or cholestan-3-one, and it was at a high percentage in caterpillars fed cholestanol-containing diet. Finally, epicholestanol was only found in insects fed the diets containing cholestan-3-one.

But there were also differences in body sterols/steroids between the two species (Tables 4.3 and 4.4). One very noticeable difference was that when caterpillars were fed cholestan-3-one, a high percentage of epicholestanol and low percentage of cholestanol was found in *H. virescens*, but in *M. sexta* this trend was reversed. Cholestan-3-one profiles in the bodies also differed between the two species. In *H. virescens*, cholestan-3-one was only found when the diets contained this steroid, but it was also found in *M. sexta* when the insect fed cholestanol-containing diets. Additionally, stigmasterol was found in *H. virescens* when stigmasterol was mixed in the diets and, in contrast, it was not always recovered from *M. sexta* reared on stigmasterol containing diets.

Cholesterol was the dominant sterol for both species fed diets containing only cholesterol or stigmasterol, and insects on these two diets had significantly higher cholesterol profiles (as a % of the total sterol profile) compared to the other treatments (*H. virescens*: $F_{10, 53} = 130.31$, $P < 0.001$; *M. sexta*: $F_{9, 51} = 55.45$, $P < 0.001$; Table 4.3 and Table 4.4). Cholesterol percentage in bodies decreased as the percentage of stigmasterol decreased in the diets, for both *H. virescens* and *M. sexta*. For example, the

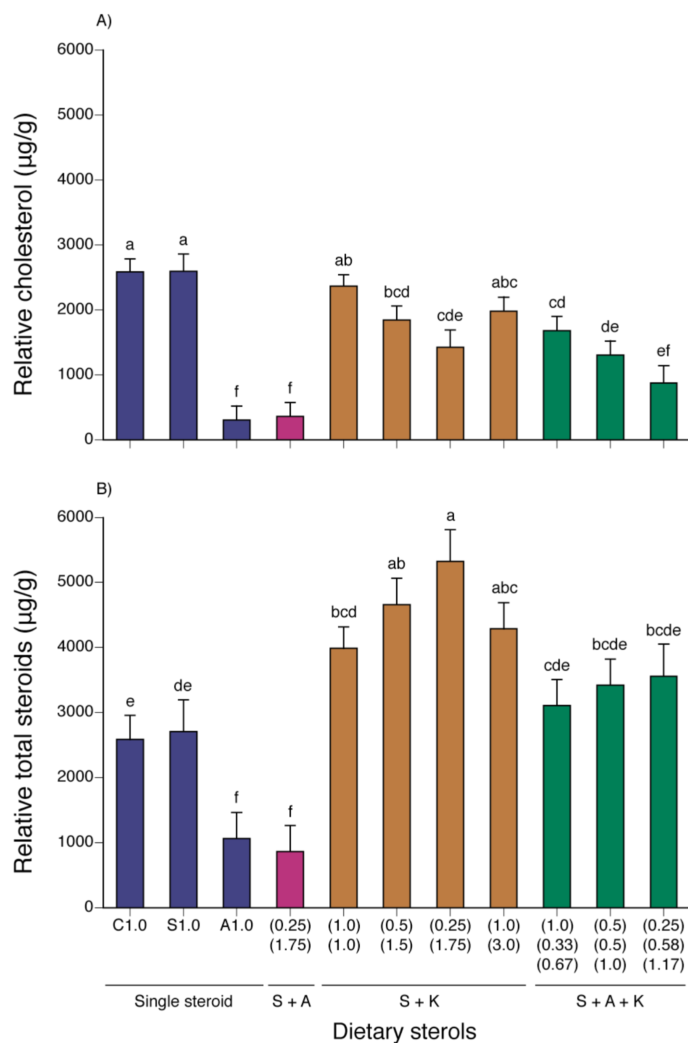


Fig. 4.2. Mean (± SEM) cholesterol and total body steroid profiles of *H. virescens* adults that had been reared as larvae on diets containing one, two or three steroids, at various concentrations. A) Relative cholesterol amounts, B) Relative total steroids. Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-oid (K)] were used to make the 11 diets shown in this figure. The first three treatments were single steroid diets, with each steroid at a concentration of 1 mg/g (dry mass). The next five treatments represent diets with two steroids (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (mg/g) of each of these steriods is shown in parentheses directly below the bars. The last three treatments represent diets with three steroids (stigmasterol, cholestanol and cholestan-3-one), with the concentration of each steroid in each diet shown below each bar. Data were analyzed using ANOVA, and different letters above the bars indicate statistically significant differences among the treatments.

cholesterol percentage on the S1.0 + A0.33 + K0.67 and S1.0 + K1.0 treatments was higher than that on S0.5 + A0.5 + K1.0, S0.5 + K1.5, and S1.0 + K3.0 treatments, and the later was higher than that on the S0.25 + A0.58 + K1.17 and S0.25 + K1.75 treatments.

4.3.2.2 Relative cholesterol amounts and total steroids in H. virescens

For *H. virescens*, there was significant difference in relative cholesterol amount between different treatments ($F_{10, 53} = 13.93$, $P < 0.001$; Fig. 4.2A). The relative cholesterol amount in the insects fed the C1.0 and S1.0 treatments was highest, and significantly higher than other treatments, except for the S1.0 + K1.0 and S1.0 + K3.0 treatments, and it was lowest on the A1.0 treatment. The relative cholesterol amount decreased as the percentage of stigmasterol in the diets decreased, although significant differences were only detected between treatments where there were large differences in the ratio of stigmasterol, for example, when comparing the S1.0 + K1.0 and S0.25 + K1.75 treatments, and when comparing the S1.0 + A0.33 + K0.67 and S0.25 + A0.58 + K1.17 treatments. The relative cholesterol amount on the S0.5 + K1.5 and S1.0 + K3.0 treatments were almost equal, although the dietary amount of stigmasterol on the S1.0 + K3.0 treatment was twice that on the S0.5 + K1.5 treatment. Furthermore, relative cholesterol levels were lower on cholestanol containing-diet compared to the cholestan-3-one containing-diet, even if similar amounts of stigmasterol were added. For example, relative cholesterol levels were higher on S0.25 + K1.75 treatment than that on the S0.25 + A1.75 treatment, and it was higher on S1.0 + K1.0 treatment compared to the S1.0 + A0.33 + K0.67 treatment.

Relative total steroids in *H. virescens* also differed between treatments ($F_{10, 53}$ = 11.17, $P < 0.001$; Fig. 4.2B). There was no difference in relative total steroids between the cholesterol and stigmasterol treatments. Interestingly, the relative total steroids amount increased as the percentage of cholestan-3-one in the diets increased. The total steroids amount was highest on the S0.25 + K1.75 treatment (the highest cholestan-3-one percentage diet), and it was higher than that on other treatments except for the S0.5 + K1.5 and S1.0 + K3.0 treatments, whose percentage of cholestan-3-one was closest to the S0.25 + K1.75 treatment. The lowest relative total steroids amount was on the A1.0 and S0.25 + A1.75 treatments, the two diets having cholestanol but no cholestan-3-one. Similar to the relative cholesterol amount, relative total steroids amount were lower on cholestanol containing-diet compared to the cholestan-3-one containing-diet, even if same amounts of stigmasterol were added, e.g., S0.25 + A1.75 vs S0.25 + K1.75.

4.*3.2.3 Relative cholesterol amounts and total steroids in* M. sexta

For *M. sexta*, relative cholesterol amount differed among the various treatments ($F_{9, 51}$ = 55.45, $P < 0.001$; Fig. 4.3A). It was highest on the cholesterol only treatment, and it was significantly higher compared to all the other treatments. The lowest relative cholesterol amount was on the A1.0, S0.25 + A1.75, and S0.25 + A0.58 + K1.17. On diets containing stigmasterol, cholesterol percent decreased as the stigmasterol percentage in the diets decreased (Table 4.4), but the relative cholesterol amount on the S1.0 + K1.0, S0.5 + K1.5, and S1.0 + K3.0 was similar and higher than that on the S0.25 + K1.75 (Fig. 4.3A). The relative cholesterol amount on the S0.25 + A1.75 was lower

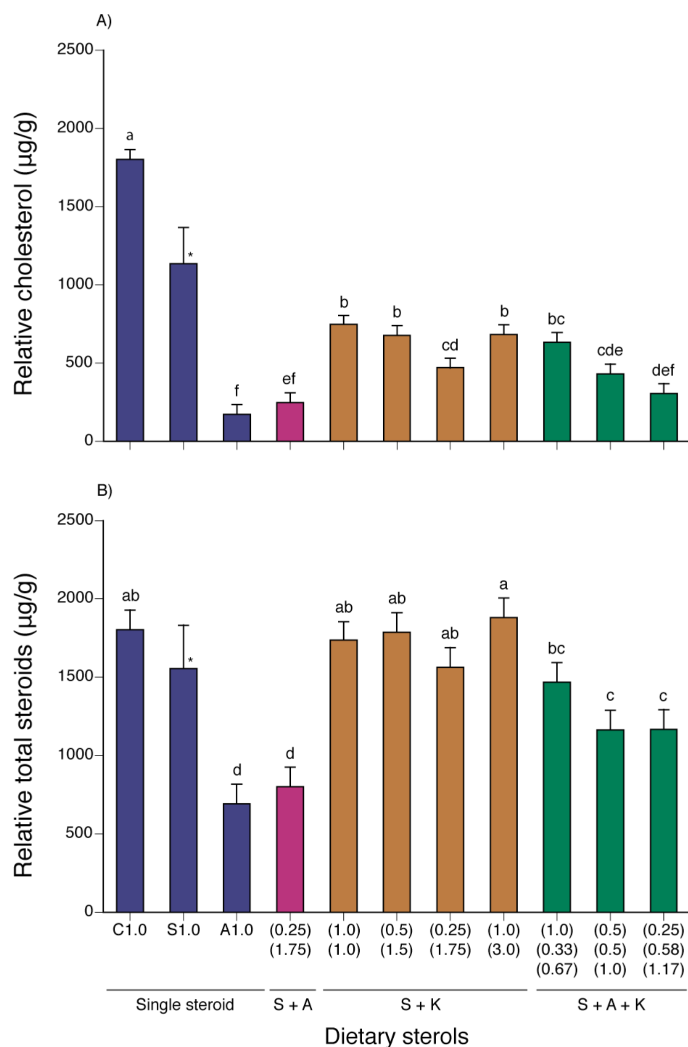


Fig. 4.3. Mean (± SEM) cholesterol and total body steroid profiles of *M.sexta* adults that had been reared as larvae on diets containing one, two or three steroids, at various concentrations. A) Relative cholesterol amounts, B) Relative total steroids. Four different steroids [cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K)] were used to make the 11 diets shown in this figure. The first three treatments were single steroid diets, with each steroid at a concentration of 1 mg/g (dry mass). The next five treatments represent diets with two steroids (stigmasterol paired with cholestanol, and stigmasterol paired with cholestan-3-one); the concentration (mg/g) of each of these steriods is shown in parentheses directly below the bars. The last three treatments represent diets with three steroids (stigmasterol, cholestanol and cholestan-3-one), with the concentration of each steroid in each diet shown below each bar. Data were analyzed using ANOVA, and different letters above the bars indicate statistically significant differences among the treatments. There is an asterisk on each of the stigmasterol at 1 mg/g bars; these data was collected from 3rd stadium caterpillars, because no adults were produced on this treatment (in two separate experiments). These data are shown for reference only, and have not been included in the formal statistical analysis.

than that on the S0.25 + K1.75 treatment, although the two diets had the same amount of total steroids and stigmasterol. The decreasing pattern was also found among three-steroid mixture, but the only difference was found between the S1.0 + A0.33 + K0.67 and S0.25 + A0.58 + K1.17 treatments. Interestingly, it was lower on the S0.5 + A0.5 + K1.0 compared with S0.5 + K1.5 treatment, although these two diets contained the same amount of stigmasterol and total steroids respectively.

Relative total steroids amount also differed between treatments for this species ($F_{9, 51} = 11.84$, $P < 0.001$; Fig. 4.2B). The highest appeared on the cholesterol treatment, and the stigmasterol/cholestan-3-one mixtures, and lowest on the cholestanol only and the S0.25 + A1.75 treatment. Total relative steroid levels were intermediate on the two triple sterol/steroid treatments where cholestanol exceeded 0.5 mg/g in the diets (Fig. 4.2B).

4.4 Discussion

In previous studies, insect sterol profiles and metabolism were studied mostly on artificial diets (Behmer and Elias, 1999b, 2000; Behmer et al., 1999b; Behmer and Grebenok, 1998; Clayton, 1964; Lasser et al., 1966; Robbins et al., 1971; Svoboda and Weirich, 1995), because of the convenient manipulation of dietary sterols. There are, however, a handful of studies that have used plants with manipulated sterol profiles (Behmer et al., 2011; Connor et al., 2006; Costet et al., 1987; Janson et al., 2009). In this study, we used a pair of tobacco lines containing different sterol profile to investigate the sterol metabolism and absorption in insects, in concert with artificial diets

containing fixed amounts and ratios of sterols/steroids. This later approach provides greater control. Two different insects, a specialist and a generalist, were used so we can compare the sterol utilization between two species having different feeding strategies.

4.4.1 Tobacco plants experiment

In this experiment, two tobacco lines containing different sterol profile were fed to two species, *H. virescens* and *M. sexta.* Different sterols and relative total sterol amount were retrieved respectively when both species fed different tobacco lines. Interestingly, different sterols were also found between two species on the same tobacco line.

4.4.1.1 Metabolism and absorption of normal phytosterols

More than 100 different sterols have been identified in plants, and individual plants always contain multiple types of sterol (Nes, 1977). Plant sterols exhibit various structural differences from cholesterol, but primarily they differ in: (1) the position and extent of nuclear and side chain unsaturation, and (2) the extent of C24-alkylation in the side chain. For example, stigmasterol differs from cholesterol by the presence of an ethyl group at the C24-position and the occurrence of a double bond at the C24-position (Fig. 4.1); campesterol only differs from cholesterol by a methyl group at the C24-position (Fig. 4.1). In the control plants, there are five different phytosterols: stigmasterol, campesterol, sitosterol, cholesterol, and isofucosterol (Chapter II), yet cholesterol was the dominant body sterol in both caterpillar species reared on the control

tobacco line (Table 4.1 and Table 4.2). Because sterols that enter the midgut are absorbed passively into the membranes of the midgut cells, generally being packaged as mixed micelles, with phospholipids, (Behmer and Elias, 1999b; Canavoso et al., 2001; Jouni et al., 2002), our results indicate that the two caterpillar species can generally convert all of these typical phytosterols into cholesterol. Svoboda did significant work on sterol metabolism using *M. sexta* and found that this species could convert a number of C28 and C29 phytosterols, including sitosterol, campesterol, stigmasterol, and fucosterol into cholesterol (Svoboda, 1968; Svoboda and Weirich, 1995). No sterol metabolism analysis on *H. virescens* has been conducted prior to this current study, but our findings indicate that this insect species has similar phytosterol metabolic capability as other caterpillar species.

Interestingly, different phytosterols were recovered from the two species on the control line (Table 4.1 and Table 4.2), so this suggests that different species that share the same host plant may have different abilities with respect in metabolizing phytosterols. For instance, *H. virescens* was not partricularly efficient at converting stigmasterol into cholesterol, while *M. sexta* was less efficient at converting campesterol into cholesterol. Campesterol (C24 methyl) is very similar to sitosterol (C24 ethyl), and sitosterol is generally the most abundant sterol in most plants (Itoh et al., 1973); but stigmasterol is the most abundant sterol in tobacco (Heyer et al., 2004). *M. sexta* is a specialist on the family of tobacco, while *H. virescens* is a generalist that feed on various plants (including tobacco), so the different phytosterol metabolic ability may reflect their diet breadth. Coincidentally, the sterol composition in plants, and the plant speciation of

insect herbivore mostly varies at plant family level (Behmer et al., 2011; Schoonhoven et al., 2005), so adaptation of insect herbivores to phytosterol fingerprints in plants was possibly one example of adaptive radiation, the central concept of the coevolution between insect herbivore and plants introduced by Ehrlich and Raven (Ehrlich and Raven, 1964).

4.4.1.2 Metabolism and absorption of novel steroids

In the modified plants, both normal phytosterols and phytosterol derivatives (e.g., stanols and 3-ketosteroids) were retrieved (Chapter II). In both caterpillar species fed these plants, the same sterols were found in those on the control line, e.g., cholesterol and stigmasterol for *H. virescens*, and cholesterol and campesterol for *M. sexta*. Moreover, cholestanol and cholestan-3-one, but not their derivatives, were also found in both species. This indicated that both caterpillar species could dealkylate various stanols and 3-ketosteroids into cholestanol and cholestan-3-one, respectively. Therefore, the enzymes mediating dealkylation would not be affected much by the different structures in the sterol nucleus, i.e., no double bond at the C5 position (i.e., stanols), and a C3 keto instead of C3 hydroxyl (i.e., 3-ketosteroids).

It is not surprising that both the cholesterol amount and percent cholesterol were reduced in caterpillars fed the modified tobacco lines, because the normal phytosterols only accounted for 24.9% of the total steroids in the modified plants. Interestingly, though, the relative total steroids amount for both species on the modified plants also decreased. This occurred despite the fact that the total steroids amount in the modified

plants is much higher (approximately 4 times greater) than that in the control plants (Heyer et al., 2004). Thus, for some reason, novel steroids can lower body sterol content in insects. This is the first report of this phenomenon in insects, but it is well known in mammals. Phytosterols and, to a greater extent, stanols, both of which can be excreted back into gut lumen by mammalian ABC transporters (Allayee et al., 2000; Berge et al., 2000), can block the dietary cholesterol absorption by replacing or precipitating cholesterol in intestine (Moreau et al., 2002). Therefore, our data suggest that stanols and/or 3-ketosteroids might have the similar function in blocking phytosterol absorption in insects.

The significant contrast in the 3-ketosteroid profile between modified tobacco plants, and the insects fed these plants, indicates significant metabolism of 3-ketosteriods by our caterpillars. Two mechanisms may explain why the 3-ketosteroid amounts were so low: 1) insects could metabolize this steroid, and 2) insects had low efficiency at absorbing these steroid, e.g., these steroids were prone to be replaced by other sterols. However, it is very hard to identify exactly which mechanism functioned in the plant experiment because of the complexity of plant sterol profile.

4.4.2 Artificial diet experiment

4.4.2.1 Single sterol diet

Both *H. virescens* and *M. sexta* can absorb cholesterol and stigmasterol, and convert stigmasterol into cholesterol. Relative total sterol amounts, relative cholesterol amounts, and the cholesterol percent were all similar between the S1.0 and C1.0

treatments. Interestingly, when *H. virescens* and *M. sexta* were fed cholestanol, i.e., A1.0, a sterol differing from cholesterol only by lacking a C5 double bond (Fig. 4.1), the above three measures were all much lower compared to caterpillars reared on cholesterol. This indicated that both species had low, or no ability to insert a double bond at C5 position, a common phenomenon in insects (Behmer and Nes, 2003). Interestingly, there was still some cholesterol in the insects. This could have come from two sources: 1) parental cholesterol transferred by the egg, and 2) cholesterol contamination in the diet. Egg sterol profile is closely related to parental dietary sterol profile (Costet et al., 1987), and it could even determine the insect performance in the 2nd-generation (Kircher and Gray, 1978). Furthermore, the relative lower total steroids amount on A1.0 probably indicated that the insects could preferentially secrete cholestanol out of their bodies, which was also found in plant experiment. But this excretion process cannot stop the absorption of cholestanol completely because there was still appreciable amounts of cholestanol in both insects. Actually, insects have no problem in incorporating cholestanol into their body, and cholestanol is a good "sparing" sterol in insects, for example, houseflies use it as a cellular membrane components when cholesterol is limited (Clayton, 1964).

4.4.2.2 Two-steroids mixture

In this experiment, one mixture of stigmasterol and cholestanol (S0.25 + A1.75), and a series of stigmasterol and cholestan-3-one mixture (S1.0 + K1.0, S0.5 + K1.5,

S0.25 + K1.75, and S1.0 + K3.0) were fed to both species and many interesting sterol profile results were observed.

Surprisingly, the relative cholesterol amount and the total steroid amount on the S0.25 + A1.75 treatment was similar to that on the A1.0 treatment, but the percentage of cholesterol did increase significantly for both species. The similar total steroid amount indicated that the insects could not absorb more sterols even if they were fed a higher sterol concentration diet (2 mg/g vs 1 mg/g), when a large ratio of cholestanol, i.e., S0.25 + A1.75, was in the diet. The similar amount of cholesterol indicated that cholestanol could block the absorption of cholesterol, which is common in mammals (Behmer et al., 2011; Moreau et al., 2002), but cholestanol could not inhibit the absorption of cholesterol completely because the percent cholesterol still increased.

There were many interesting phenomena from the insects fed diets containing cholestan-3-one. Obviously, insects could metabolize cholestan-3-one because there was cholestanol recovered from both species fed the cholestan-3-one containing diets, and these insects could not convert stigmasterol into cholestanol. Interestingly, there was also epicholestanol in these insects, and this sterol did not appear in cholestanol containing diet, so epicholestanol must also be a metabolic product of cholestan-3-one. Two enzymes, 3α-reductase and 3β-reductase, both of which are actively involved in converting 3-keto-ecdysone into 3-hydroxyl-ecdysone (Gilbert, 2004), are possibly responsible for this metabolism. The product catalyzed by 3α-reductase is epiecdysone, while the product catalyzed by 3β-reductase is ecdysone, which matches the two metabolic isomers of cholestan-3-one in this experiment. The fact that much more

cholestanol was in *M. sexta*, while much more epicholestanol was in *H. virescens* probably indicated that 3β-reductase activities is dominant in *M. sexta*, while 3α-reductase activities is dominant in *H. virescens*. Additional evidence also supports the notion that 3β-reductase was more abundant in *M. sexta*. In this experiment, cholestan-3-one was detected in *M. sexta* on the A1.0 and S0.25 + A1.75 treatments, but not in *H. virescens*, and the reaction mediated by 3β-reductase is reversible (Gilbert, 2004; Yang et al., 2010). This difference between these two species probably reflects their different demand of steroids hormone because 3α-reductase is an important enzyme in the process of ecdysone inactivation and degradation, and 3β-reductase plays key role in maintain the ecdysone titer in insects (Yang et al., 2010).

When different ratio of cholestan-3-one and stigmasterol were fed to insects, the cholesterol percentage and relative cholesterol amount in both species decreased as stigmasterol in the diets decreased. Therefore, unlike cholestanol, insects probably absorbed stigmasterol and cholestan-3-one nonselectively, which means cholestan-3-one would not block the absorption of stigmasterol. In contrast to cholestanol containing diet, relative total steroids amounts on cholestan-3-one containing diets were much higher than observed on the A1.0 and S1.0 treatments for *H. virescens*. Therefore, the excretion mechanism for stanols did not work for cholestan-3-one. On the contrary, cholestan-3-one might be more easily absorbed into the midgut cells because the relative total steroids amount in *H. virescens* was increasing, along with the increase of the percentage of cholestan-3-one in the diets, although the total steroids were constant in the diet.

Interestingly, and in contrast to patterns observed in *H. virescens*, relative total steroids amount in *M. sexta* on cholestan-3-one containing diets were equal to that on cholesterol. This may not be surprising because *M. sexta* could preferentially convert cholestan-3-one into cholestanol (unlike *H. virescens*). And both 3-keto reductases were abundant in the midgut (Gilbert, 2004; Weirich et al., 1993), where the absorption and excretion of sterols takes place (Behmer et al., 1999b; Joshi and Agarwal, 1977; Jouni et al., 2002). Therefore, cholestanol in *M. sexta*, but not epicholestanol in *H. virescens*, converted from cholestan-3-one, was possibly excreted immediately in the midgut. This species difference also suggests that the secretion system in insects is sensitive to the confirmation of chemical, i.e., the difference between two isomers.

4.4.2.3 Three steroids mixture

In this experiment, a series of stigmasterol, cholestanol and cholestan-3-one mixtures (S1.0 + A0.33 + K0.67, S0.5 + A0.5 + K1.0, and S0.25 + A0.58 + K1.17), which mimic the sterol profile in modified tobacco plants (a mixture of phytosterols, stanols and 3-ketosteroids), were fed to both species and, interestingly, relative total steroids and relative cholesterol amount were reduced on these mixures compared to the stigmasterol/cholestan-3-one mixture diets. This further confirms that cholestanol was selectively secreted in insects.

4.4.3 Conclusion

Both species we used in this experiment had no problem converting normal phytosterols into cholesterol, via dealkylation, and the structure in the sterol nucleus did not affect dealkylation related enzymatic activity. Interestingly, compared with cholestanol, cholestan-3-one was always kept at a very low level in insects. The structure of cholestan-3-one is similar to insect molting hormones (ecdysteroids) so it is highly possible that insects try to prevent this steroid from intervening their normal physiological process. The mechanisms for controlling these 3-ketosteroids probably include: 1) converting 3-ketosteroids into cholestanol, and 2) excreting cholestanol out of the body. The two enzymes possibly involved in this process were identified, although enzymatic analysis *in vitro* is still needed. The excretion of cholestanol is very interesting but more evidence is necessary for making a conclusion, for example, identification of the possible transporters involved in this process, and our study point out a promising future for the research in this area.

CHAPTER V

DIETARY STEROLS/STEROIDS AND THE GENERALIST CATERPILLAR *HELICOVERPA ZEA*: PHYSIOLOGY, BIOCHEMISTRY AND DIFFERENTIAL GENE EXPRESSION IN THE MIDGUT

5.1 Introduction

Insects require a dietary sterol source because they lack the ability to synthesize sterols, which are used as a cellular membrane component that modulate membrane permeability and fluidity, and serve as an important precursor for molting hormones (Behmer and Nes, 2003). Cholesterol is the dominant sterol in most insect species, and it is the precursor to ecdysone, the most typical insect steroid hormone controlling molt, metamorphosis, reproduction and other important physiological process (Lafont et al., 2005; Nijhout, 1994). However, plants contain little cholesterol. Intstead, they contain a variety of phytosterols (Piironen et al., 2000; Svoboda et al., 1978), so the cholesterol demand in phytophagous insects is mostly satisfied by dealkylating phytosterols (Ikekawa et al., 1993). The metabolic pathway in converting phytosterols into cholesterol by dealkylation has been elegantly described in *Manduca sexta* and other caterpillar species by using inhibitors to track sterol intermediates and several enzymes, e.g., reductase, oxidase and hydrolase, are possibly involved in this process (Robbins et al., 1971; Svoboda et al., 1972; Svoboda, 1967; Svoboda, 1968; Svoboda et al., 1988; Svoboda and Weirich, 1995; Thompson et al., 1972). These studies suggest that the

midgut is the main tissue where dealkylation of phytosterols occurs (Svoboda and Weirich, 1995; Svoboda et al., 1969b).

Sterol absorption is another important topic in sterol use and different absorption sites have been reported in insects. For omnivorous and carnivorous insects (e.g., *Eurycotis floridana*, *Gryllodes sigilatus*, *Camponotus compressus* and *Dytiscus sp.*), the crop is the main site for sterol absorption, but in phytophagos insects the midgut is the principle site of sterol absorption (Behmer et al., 1999b; Clayton et al., 1964; Joshi and Agarwal, 1977; Jouni et al., 2002; Turunen and Chippendale, 1977). Sterol absorption in insect midguts, and in vertebrate intestines, is a non-selective process (Behmer and Nes, 2003; Igel et al., 2003). The epithelial gut cells of mammals, however, can be preferentially pump out noncholesterol sterols, i.e., phytosterols, via ATP-binding cassette (ABC) transporters in mammals (Allayee et al., 2000; Berge et al., 2000). No similar mechanism has been found in insects, although ABC proteins have been reported to function as pigment transporters in *Drosophila* and in silkworms (Ewart et al., 1998; Komoto et al., 2009).

In a plant study using two transgenic tobacco lines, we found that several caterpillar species can metabolize a range of plant sterols and steroids (Chapter II and Chapter IV), but that novel steroids found in one of our transgenic tobacco lines can have negative consequences both at the individual and population level; these results were confirmed using artificial diets (Chapter III and Chapter IV). In the current study, we reared *H. zea* caterpillars on artificial diets containing three different sterols/steroids; (1) stigmasterol, (2) cholestanol, and (3) cholestan-3-one. These three sterols occur in

the modified tobacco line used in previous chapters. We also reared *H. zea* caterpillars on cholesterol diet, which acted as a control. On each of these diets we measured larval performance (growth and pupation success), and analyzed the tissue sterol profile of freshly eclosed adults from these four treatments. We also took the advantage of an available midgut specific microarray cDNA library for *Helicoverpa armigera*, a very close relative of *H. zea* that has high genetic similarity with *H. zea* (Behere et al., 2007; Pogue, 2004). We were particularly interested in understanding how the different sterol/steroid diet treatments affected gene expression patterns in the midgut, the tissue that is central to both sterol metabolism and absorption. To our knowledge, this is the first study to examine the effects of sterols on gene expression in any insects. We report and discuss some gene candidates involved in sterol/steroid metabolism, and how variation in dietary sterol/steroid structure might affect other physiological processes in the midgut.

5.2 Materials and Methods

5.2.1 Insects

Helicoverpa zea (Noctuidae), one of the most important crop pests in New Worlds, is a generalist caterpillar that can feed on a broad range of plants representing different plant families (Behere et al., 2007). Eggs of *H. zea* were purchased from Benzon Research Inc. (Carlisle, PA), and the hatchlings from these eggs were used for these experiment.

5.2.2 Sterols and insect diet

Four sterols/steroids were used in this experiment (Fig. 5.1): 5α-cholester-3β-ol (cholesterol, ≥ 95%), 5,22-cholestadien-24β-ethyl-3β-ol (stigmasterol, ≥ 98%), 5α-cholestan-3β-ol (cholestanol, 95%) and 3-keto-5α-cholestane (cholestan-3-one, ≥ 98%). Cholesterol and cholestanol were purchased from Sigma Chemical (St. Louis, MO, USA). Stigmasterol and cholestan-3-one were bought from Steraloids Inc. (Newport, RI, USA).

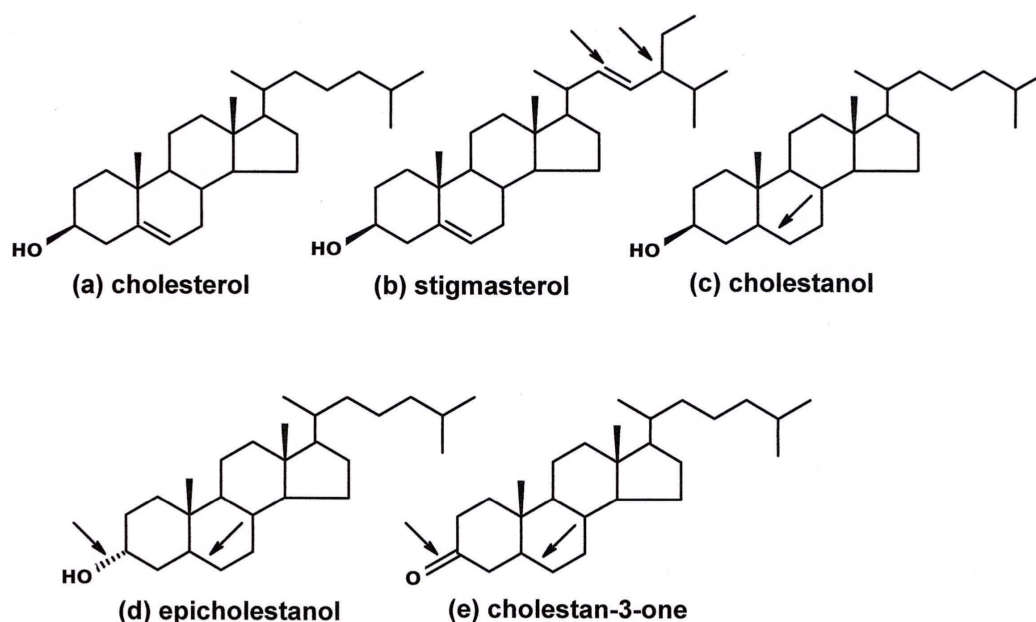


Fig. 5.1. Five sterols/steroids in this study. Cholesterol (a) is the dominant sterol in most insects, including those that feed on plants. Stigmasterol (b) is a common plant sterol, including in tobacco. It differs from cholesterol by having a C24 ethyl group, plus a C22 double bond. Cholestanol (c) and epi-cholestanol (d) are stanols; the latter being an isomer of the former (it has a 3α-hydroxyl group instead of 3β-hydroxyl). Both are similar to cholesterol, but lack a Δ5 double bond. Cholestan-3-one (e) is a keto-steroid. In contrast to cholesterol, this steroid has a C3 ketone instead of a C3 hydroxyl, and similarly to the stanols, it has no Δ5 double bond in the sterol nucleus.

In total four diets were made, each containing one of the four sterols/steroids listed above (at a concentration of 1 mg/g dry mass). These diets have been previously described in Chapter III.

5.2.3 Experimental design

Hatchlings were allocated for one of the four sterols/steroids treatments: cholesterol, stigmasterol, cholestanol and cholestan-3-one. Cholesterol was used as a control because it was the common sterol found in *H. zea*, and it supports good growth (without an biochemical modification). Upon hatching neonates were transferred individually to small rearing chambers (1 oz plastic condiment cups (Fabri-Kal)) containing approximately 10 ml of diet (109 neonates for cholesterol, 40 neonates for stigmasterol and cholestanol respectively, 63 neonates for cholestan-3-one). The insects that did not accept the diets initially were excluded from the experiment. All the insects were reared in a Percival incubator, Model # I66VLC8 (Percival Scientific, Inc) set at 27 °C with L : D 14 : 10. Insects were checked every day and sacrificed for the collection of midgut tissue upon reaching the 3rd instar. Midguts were dissected, and transferred to an autoclaved 1.5 ml eppendorf tube (VWR International, West Chester, PA) containing 0.5ml RNAlater. They were flash-freezed using liquid nitrogen, and then stored in a -80 °C freezer. Each tube contained 5 midguts as a biological repeat and 4 repeats were used for each treatment.

5.2.4 RNA extraction and Expressed Sequence Tag (EST) Project

Total RNA was extracted by using a TRIzol protocol from *H. zea* (Invitrogen Corporation, Carlsbad, CA) and quantifiedwith a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), while RNA quality was checked on 1% agarose gel. Turbo DNAse (Ambion) treatment was included to eliminate contaminating genomic DNA. The total RNA was further purified by using the RNeasy MinElute Clean up Kit (Qiagen) following the manufacturer's protocol. RNA integrity and quantity was verified on an Agilent 2100 Bioanalyzer, using RNA Nano chips (Agilent Technologies). Normalized full length-enriched cDNA libraries were generated using a combination of the SMART cDNA library construction kit and the Trimmer Direct cDNA normalization kit (Evrogen, Moscow, Russia), following the manufacturer's protocol. The normalization process enriches low abundance transcripts.

Single-pass sequencing of the 5' termini of cDNA library plasmid clones was carried out on an ABI 3730 xl automatic DNA sequencer (PE Applied Biosystems). Vector clipping, quality trimming and sequence assembly was done with the Lasergene software package (DNAStar Inc.). In total, 8 different cDNA libraries were generated from the tissues and developmental stages as described above and ~60,000 clones were sequenced. Additional sequencing was performed with a mixed cDNA pool on a Roche 454 FLX instrument, obtaining 274,607 high quality reads after trimming and quality filtering steps. The *H. armigera* ESTs generated and all publicly available Genbank sequences for this species were jointly assembled using Seqman NGen (Lasergene) and

clustered into 27,381 contigs (putative gene objects) subsequently used for microarray oligo probe design.

5.2.5 Microarray design, labeling, hybridization and data acquisition

In order to optimize our *H. armigera* microarray design and maximize the output of subsequent gene expression profiling experiments, a Pre Selection Strategy (PSS, Imagenes) approach was used to select high performing probes, based on initial test hybridizations. For the preliminary large array design, each gene was tiled by a maximum number of probes. A total of 231,399 oligos for the 27,381 contigs were designed, and a 244K Agilent microarray was hybridized with labeled complex total RNA mixture and genomic DNA. The best performing probes for each gene were selected, for the expressed genes based on the RNA hybridization, and for the non-expressed genes based on the DNA hybridization. A final condensed Agilent 4 x 44K array design based on the eArray platform (Agilent Technologies; https: // earray.chem.agilent.com/earray/) contains the few best performing probes of each gene (1-2 for each Gene Object), with a final number of 42446 non-control probe set and 1417 Agilent Technologies built in controls (structural and spike in).

There were four biological replicates for each treatment. Five freshly dissected midguts were flash-frozen in liquid nitrogen, and stored at -80 °C until RNA isolation. Total RNA was purified, quantified and quality tested as mentioned above. Agilent Technologies spike-in RNA was added to 500 ng of total RNA and labelled using the Low RNA Input Linear Amplification kit (Agilent Technologies). Treated RNA and

control samples were labelled with Cy5 and Cy3 dyes respectively, according to manufacturer instructions following a double reference dye-swap design. Labelled amplified cRNA samples were purified using Qiagen RNeasy® MinElute™ Cleanup kit and analysed on a Nanodrop spectrophotometer using the microarray function. Amplified cRNA samples were used for microarray hybridization only if the yield is >825 ng and the specific activity is >8.0 pmol Cy3 or Cy5 per µg cRNA. 825 ng each of cyanine 3 and cyanine5 labeled cRNA were used for each array. Hybridization was carried out at 65 °C for 17 hrs. Slides were washed in GE Wash Buffer 1 (Agilent Technologies) for 1 min at room temperature and a further minute in GE Wash Buffer 2 pre-warmed overnight to 37 °C. Slides were treated in stabilization and drying solution (Agilent Technologies), scanned with the Agilent Microarray Scanner, and data was extracted from the TIFF images with Agilent Feature Extraction software version 9.1. The initial technical validation included visual inspection of images to identify gross abnormalities or background. Prior to normalization the sensitivity of the array and relationship between RNA concentration and fluorescent signal was assessed by calculating the signal intensity generated by reporters complementary to 10 ‘alien’ synthetic RNA spikes introduced at known concentrations (from 1 pmole to 30 nmole prior to labeling).

5.2.6 Micorarray analysis

Expression profile of *H. zea* larval gut samples subjected to different sterol-containing diets was generated by normalizing fluorescence signals to the median

intensity, and log base 2-transformation of the normalized data. In order to determine the relationship between the samples per treatment, the clustering application (Euclidean distance, average linkage) and principal component analysis was applied and normalized to median, log-transformed, statistically significant data after ANOVA (unequal variance, no threshold, Benjamini and Hochberg false discovery rate (B&H FDR) multiple test correction, adjusted P with a cut-off < 0.001). This was done using the Geospiza GeneSifter genetic analysis software. Data was also filtered by pair-wise comparisons of each sterol-containing diet to the control diet by means of a Welch's t-test using Geospiza GeneSifter®. Only gene probes with corrected P-values less than 0.001 after B&H FDR were considered statistically differentially expressed and the genes having at least 2 fold changes to the control were reported (T-test; P cut off $<$ 0.01; B&H FDR multiple test correction).

5.2.7 Body sterol profile analysis

Several individual adults were homogenized as one sample, and for each treatment there were 3 independently pooled samples. Approximately 25% of each sample was removed, and weighed. A 0.5 ml of chloroform, a 0.5 ml of methanol, 5 μg of cholestane, and 2 glass beads (size 3, Kimble Kontes LLC) were then added to each sample. Each sample was shaken vigorously using a Pneumatic paint shaker (Central Pneumatic, stock No. 422) powered by a 3-gallon air compressor (Craftsman), for 30 mins. A 0.5 ml of H_2O was added to each tube, and vortexed; the solvent layers were then left to separate for 12 hrs. Following separation, the chloroform fraction (lower

layer) was removed and evaporated under nitrogen to a volume of 200 μl. Then the total steroids amount including free and esterified ones were measured following the same method used in Chapter IV.

5.2.8 Statistical analysis

Non-parametric survival analysis was used for larval development. The larval developmental time for those that died before the 3rd-intar was incorporated into the analysis as censored data. A Log-rank test was used instead of a Wilcoxon test in our analysis because the former tends to be more sensitive to distributional difference late in time, which is exactly the behavior of our data (Martinez and Naranjo, 2010). The larvae reaching 3rd-instar on day 6, and the success of eclosing adult from pupa, were binomial distributions, and thus analyzed using Likelihood Ratio Chi-Square statistics. The relative cholesterol amount was compared between treatments using Kruskal-Wallis Tests (Zar, 1999). Bonferroni corrections were used for the adjustment in multiple comparisons. All analyses were performed in SAS v. 9.2 (Cary, NC, USA).

5.3 Results

5.3.1 Larval and adult performance

Developmental time (from neonate to newly molted 3rd instar) on the different sterol/steroid treatments was significantly different ($\chi^2_3 = 253.6$, $P < 0.001$, Fig. 5.2a); and it was fastest on cholesterol, and slowest on the cholestan-3-one diet. Interestingly, developmental time on the stigmasterol diet, a common phytosterol found in plants, was

significantly slower compared to the cholesterol diet (although the difference was small). Developmental time on cholestanol was slower compared to the cholesterol and stigmasterol diets, but faster than the cholestan-3-one diet. The percentage of caterpillars surviving to the 3rd instar at day 6 was similar on the cholesterol, stigmasterol and cholestanol treatments, but was significantly lower on cholestan-3-one diet ($\chi^2_3 = 192.6$, $P < 0.001$, Fig. 5.2b).

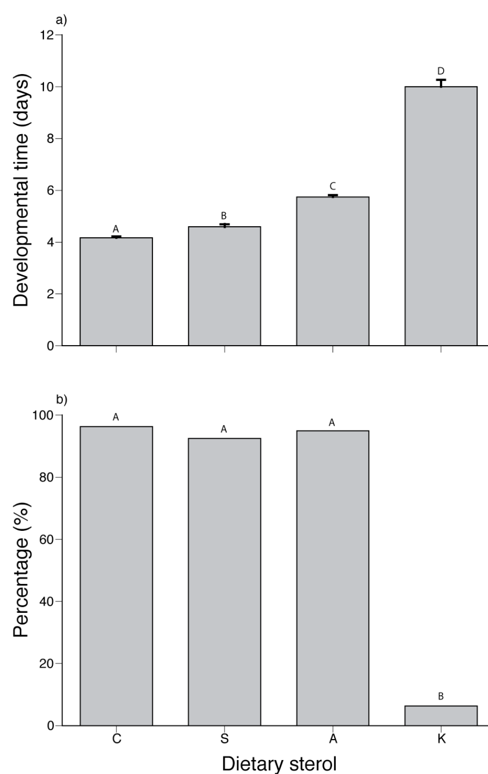
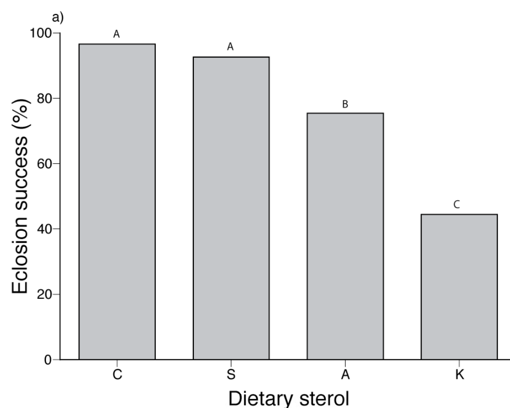


Fig. 5.2. Developmental time (a) and survival (b) of *H. zea* larvae, from hatch to the start of the 3rd-stadium. Larvae were reared on diets containing cholesterol (C), stigmasterol (S), cholestanol (A), and cholestan-3-one (K), at a concentration at a concentration of 1 mg/g (dry mass). Developmental time is reported as mean days (± SEM), while survival is reported as the percent larvae reaching the 3rd-instar by day 6. Different letters above the bars indicate statistically significant differences among the treatments.

Many adults on cholestan-3-one were deformed after eclosion (Fig. 5.3b), and these adults lost their ability to move or mate, so those individuals was regarded as a failure in the analysis. Across the different treatments there was a significant difference in eclosion success using this criteria ($\chi^2_3 = 37.4$, $P < 0.001$, Fig. 5.3a). The eclosion success on cholesterol and stigmasterol was similar, and significantly higher compared to the cholestanol treatment. Eclosion success rate on the cholestan-3-one treatment was significantly lower than that of the other three treatments.



b)

Fig. 5.3. Eclosion success of *H. zea* pupae reared on diets containing cholesterol (C), stigmasterol (S), cholestanol (A) and cholestan-3-one (K) at a concentration of 1 mg/g (dry mass). Eclosion success (a) was scored based on whether adults emerged with a “normal” or “deformed” appearance. Different letters above the bars indicate statistically significant differences among the treatments. (b) Left panel shows a successfully eclosed adult (typical for larvae reared on the cholesterol and stigmasterol diets), while right panel shows a deformed adult (observed most regularly on cholestan-3-one diets).

5.3.2 Body sterol profiles

Cholesterol was recovered in all samples, although its relative amount and percent differed among the four diet treatments (relative amount: $\chi^2_3 = 9.36$, $P = 0.025$; percent: $\chi^2_3 = 9.18$, $P = 0.027$; Table 5.1). On the cholesterol treatment, only cholesterol was recovered. On the stigmasterol treatment, cholesterol was the dominant sterol recovered, with only trace amounts of stigmasterol being detected. Cholestanol was the dominant sterol recovered from caterpillars fed the cholestanol diet, and the only other sterol recovered was cholesterol. Finally, for the cholestan-3-one fed caterpillars, cholestanol and epicholestanol were the two dominant sterols recovered (approximately 70% of the total), with cholesterol being the remaining sterol detected in these insects. Total steroids amounts were similar among the cholesterol, stigmasterol and cholestan-3-one treatments, but it was less when insects fed on cholestanol diet and only about 60% of that on the other three treatments.

5.3.3 Gene expression among different treatments

Different sterol/steroid diet treatments resulted in different patterns of gene expressions in the midgut (Fig. 5.4). In total, 1768, 2160 and 4484 genes on stigmasterol, cholestanol and cholestan-3-one diet respectively were differentially expressed relative to those on cholesterol. Of these, 1279 genes on the stigmasterol diet, 1505 genes on the cholestanol diet, and 2542 genes on the cholestan-3-one diet were up-regulated. In contrast, 489 genes on the stigmasterol diet, 655 genes on the cholestanol diet, and 1942 genes on the cholestan-3-one diet were down-regulated. Additionally,

Table 5.1 Sterol/steroid content in *H. zea* adults reared on cholesterol, stigmasterol, cholestanol, and cholestan-3-one (each diet had a sterol/steroid concentration of 1 mg/g (dry mass)). Tissue sterol/steroid is presented both as a concentration (µg/mg), and as a percent of the total sterol profile (shown in parenthesis); data are presented as medians ± MAD. Different letters indicate the significant difference for relative cholesterol amount between treatments.

	Body sterols/steroids					
Dietary sterols	Cholesterol	Stigmasterol	Cholestanol	Epicholestanol	Cholestan-3-one	Total body sterol/steroids
Cholesterol	1.27 ± 0.36a (100)	-	-	-	-	1.27 ± 0.36
Stigmasterol	1.29 ± 0.20a (100 ± 2.0)	trace	-	-	-	1.29 ± 0.23
Cholestanol	0.22 ± 0.05b (28.2 ± 2.7)	- -	0.56 ± 0.16 (71.2 ± 2.7)	- -	- -	0.78 ± 0.20
Cholestan-3-one	0.36 ± 0.05b (29.7 ± 0.5)	- -	0.69 ± 0.04 (47.6 ± 1.0)	0.31 ± 0.02 (22.8 ± 1.5)	- -	1.27 ± 0.12

some genes were only regulated by individual treatment. For example, 193 genes, 184 genes and 1248 genes were only up-regulated while 234 genes, 209 genes and 1466 genes were only down-regulated by stigmasterol, cholestanol and cholestan-3-one respectively.

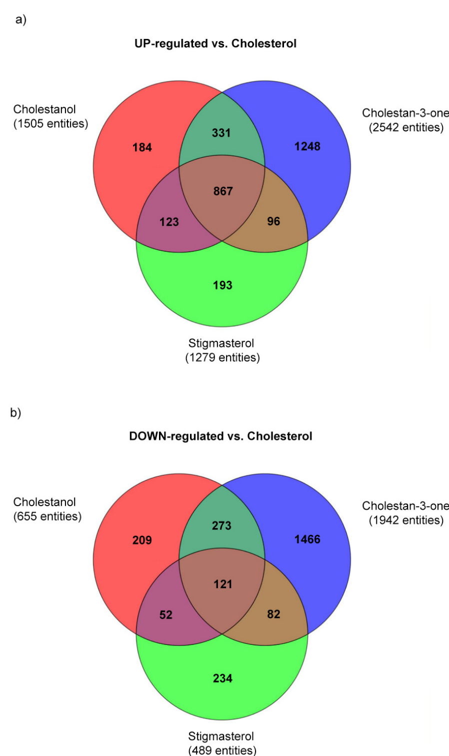


Fig. 5.4. Venn diagrams showing the number of overlapping and unique 3rd-instar larval midgut genes (a) induced, or (b) suppressed by different dietary sterols/steroids relative to the cholesterol diet. Green, stigmasterol; red, cholestanol, blue, cholestan-3-one. Results on gene expression profiles were generated from 4 biological replicates.

5.4 Discussion

The effects of variation in sterol/steroid structure on growth, development has been studied in a wide array of plant-feeding insects (reviewed in Behmer & Nes 2003), including *H. zea* (Nes et al. 1997). This study confirms that *H. zea* caterpillars grow and

develop well on diets containg stigmasterol, a typical phytosterol, and does poorly on foods that contain only stanols (i.e., cholestanol). This study firstly demonstrates caterpillar species have very poor growth and development on foods that contain 3-ketosteroids as the only dietary steroid and can convert 3-ketosteroids into stanols. This study is particularly unique because it is the first to use microarray techniques to study the gene regulation in insects fed different sterols/steroids. Interestingly, performance was generally correlated with gene numbers altered on a particular sterol, that is, insects had a good performance on stigmasterol which intrigued least gene expression difference from that on cholesterol while insects had a poor performance on cholestan-3-one which induced most gene expression difference from that on cholesterol.

H. zea had no problems in using stigmasterol. Its performance on this sterol was similar to that on cholesterol although the developmental time was slightly higher. Stigmasterol can support the development of all tested caterpillar species (Behmer and Nes, 2003) but grasshoppers have problem in using this sterol (Behmer and Elias, 2000). Sitosterol is good at supporting the development of grasshopper and the difference between sitosterol and stigmasterol is C22 double bond so the constrain of grasshoppers in removing this double bond may explain the poor performance on grasshoppers (Behmer and Elias, 1999a, b; Behmer et al., 1999b; Behmer and Nes, 2003). *H. zea* does not have this limit because almost all sterol was cholesterol in our experimental insects on stigmasterol and the relative amount was similar to that on cholesterol.

In spite of the similar structure between stigmasterol and cholesterol (Fig. 5.1), there were still so many genes were expressed differently on stigmasterol compared with

that on cholesterol. Four metabolites (5,22,24(28)-Trienes, 24,28-epoxides, 5,22,24-Triene, and desmosterol) appear sequentially in the conversion from stigmasterol to cholesterol (Ikekawa et al., 1993; Svoboda and Weirich, 1995), suggesting there are at least three types of enzymes (desaturases, oxidoreducases, and hydrolases) involved in the dealkylation process. Considering that many catalyses require energy, and that many byproducts (e.g., H_2O_2 and H_2O) need to be eliminated following metabolism by insects, it is perhaps not surprising that so many genes were differentially expressed on the stigmasterol diets (relative to cholesterol). The existence of Δ^{24} sterol reductase, an enzyme converting desmosterol into cholesterol by the removal of the C24 double bond, has been confirmed using inhibitors (Svoboda and Weirich, 1995; Svoboda et al., 1969b); the gene coding this reductase (DHCR24) was clarified in silkworm, *Bombyx mori*, recently (Ciufo et al., 2011). The protein expressed by this gene is an FAD-dependent oxidoreductase. However, there is still a big gap to fill, as other enzymes involved in the conversion were still unidentified. A possible list of genes involved in stigmasterol metabolism in *H. zea* is reported in Table 5.2. Interestingly, the gene that produces the best hit against the DHCR24 sequence was not up-regulated by stigmasterol. This might be due to the fact that it is not up-regulated much (i.e., 2 folds), or the microarray probe was suboptimal, leading to insufficient signals.

Developmental time of *H. zea* caterpillars, and eclosion success of the pupae on the cholestanol diet was both longer and poorer when compared to both the cholesterol- and stigmasterol-reared *H. zea*; a similar result was also found by Ritter and Nes (Ritter and Nes, 1981b). Cholestanol is a good “sparing” sterol, possibly only for structure

Table 5.2 Genes that are up-regulated 2-fold or more by the different sterol/steroid diet treatments relative to cholesterol diet The probe name, Genbank accession number, reference organism, possible function of the protein coded by the genes, Entrez geneID, and reference protein were reported. An asterisk indicates the homolog to the *Spodoptera littoralis* 3-dehydroecdysone 3α-reductase (Takeuchi et al., 2000).

Probe Name	Accession Number	Reference organism	Enzyme type	Entrez GeneID	Reference protein
Stigmasterol treatment					
Har_00003493	ACL36977	*Helicoverpa zea*	oxidase	v3ndG3105	NM_132754.3
Har_00003647	AAF81787	*Helicoverpa zea*	desaturase	v3ndA0003	NM_144474.1
Har_00011287	ACL36977	*Helicoverpa zea*	oxidase	v3ndA0458	NM_143209.1
Har_00019975	ACL36977	*Helicoverpa zea*	oxidase	Har_454C12878	NM_079814.2
Har_00021525	ACI32835	*Helicoverpa armigera*	oxidase	Har_454C13150	NM_135530.2
Har_00024977	YP_174250	*Bacillus clausii* KSM-K16	oxidoreductase	v3ndE0643	NM_134902.1
Har_00029119	ABF71570	*Bombyx mori*	hydrolase	v3ndE0375	NM_143134.1
Har_00032613	XP_001121337	*Apis mellifera*	oxidase	Har_454C15188	NM_001144134.1
Har_00034074	XP_002067032	*Drosophila willistoni*	oxidase	v3ndF1961	NM_135231.2
Har_00043432	XP_001657372	*Aedes aegypti*	oxidase peroxidase	v3ndB0243	NM_206501.2
Har_00046657	YP_002309658	*Shewanella piezotolerans* WP3	reductase	v3ndF1725	NM_134696.1
Har_00049832	EEC18865	*Ixodes scapularis*	reductase	v3ndB0367	NM_142460.2
Har_00052526	AAF70499	*Spodoptera littoralis*	reductase	v3ndF1655	NM_169085.1
Har_00058242	BAG30781	*Papilio xuthus*	aldo-keto reductase	v3ndD0221	NM_139582.2
Har_00060567	AAF70499	*Spodoptera littoralis*	reductase	Har_454C2780	NM_130519.2
Har_00067650	XP_001949485	*Acyrthosiphon pisum*	oxidase peroxidase	Har_454C3771	NM_206501.2
Har_00069635	NP_001037382	*Bombyx mori*	oxidase	v3ndE0070	NM_057431.3
Har_00073430	ABU98615	*Helicoverpa armigera*	hydrolase	v3ndB0079	NM_001103809.1
Har_00076173	AAF70499	*Spodoptera littoralis*	reductase	Har_454C4996	NM_141306.2
Har_00076361	BAG30781	*Papilio xuthus*	aldo-keto reductase	v3ndF1025	NM_140227.1
Har_00076386	XP_001648461	*Aedes aegypti*	aldo-keto reductase	Har_454C5021	NM_168467.1
Har_00098307	ACL36977	*Helicoverpa zea*	oxidase	v3ndG3111	NM_143209.1
Har_00120673	XP_002230203	*Branchiostoma floridae*	aldo-keto reductase	Har_454C27554	NM_168467.1
Cholestanol treatment					
Har_00012573	XP_312290	*Anopheles gambiae* str. PEST	abc transporter	Har_454C11496	NM_001038982.1
Har_00037915	NP_001037422	*Bombyx mori*	yellow e3-like protein	v3ndE0461	NM_137944.3
Har_00078553	XP_001650571	*Aedes aegypti*	abc transporter	v3ndE0692	NM_143371.1

Table 5.2 (*Continued*)

Probe Name	Accession Number	Reference organism	Enzyme type	Entrez GeneID	Reference protein
Cholestan-3-one treatment					
Har_00000388	AAR26515	*Mamestra brassicae*	reductase	v3ndE0074	NM_057810.3
Har_00005437	NP_001091765	*Bombyx mori*	oxidoreductase	Har_454C10196	NM_170227.2
Har_00005926	ABK29494	*Helicoverpa armigera*	reductase	v3ndA0440	NM_140347.2
Har_00007257	NP_001040364	*Bombyx mori*	reductase	v3ndF2253	NM_079361.2
Har_00007366	NP_001091765	*Bombyx mori*	oxidoreductase	v3ndF2204	NM_136593.2
Har_00009687	CAW30924	*Papilio dardanus*	aldo-keto reductase	v3ndF1447	NM_168501.2
Har_00011120	ABK29494	*Helicoverpa armigera*	reductase	Har_454C11223	NM_140347.2
Har_00013462	ABK25249	*Picea sitchensis*	reductase	v3ndA0332	NM_169083.1
Har_00016475	AAF70499	*Spodoptera littoralis*	3α-reductase	Har_454C12203	NM_169085.1
Har_00024607	ZP_01461313	*Stigmatella aurantiaca* DW4/3-1	reductase	Har_454C13707	NM_130690.1
Har_00024977	YP_174250	*Bacillus clausii* KSM-K16	oxidoreductase	v3ndE0643	NM_134902.1
Har_00028015	BAG30781	*Papilio xuthus*	aldo-keto reductase	v3ndD0221	NM_168467.1
Har_00030469	NP_001091765	*Bombyx mori*	oxidase	Har_454C14823	NM_167056.1
Har_00038192	XP_460493	*Debaryomyces hansenii*	aldo-keto reductase	Har_454C16080	NM_139583.3
Har_00044359	YP_001260814	*Sphingomonas wittichii* RW1	reductase	Har_454C17216	NM_130690.1
Har_00049832	EEC18865	*Ixodes scapularis*	reductase	v3ndB0367	NM_142460.2
Har_00052526	AAF70499	*Spodoptera littoralis*	3α-reductase	v3ndF1655	NM_169085.1
Har_00053893	XP_968650	*Tribolium castaneum*	aldo-keto reductase	v3ndE0373	NM_139583.3
Har_00057010	XP_623474	*Apis mellifera*	reductase	v3ndC0022	NM_137627.3
Har_00057575	XP_966520	*Tribolium castaneum*	reductase	v3ndD0213	NM_001169331.1
Har_00060567	AAF70499	*Spodoptera littoralis*	3α-reductase	Har_454C2780	NM_130519.2
Har_00076173	AAF70499	*Spodoptera littoralis*	3α-reductase	Har_454C4996	NM_141306.2
Har_00076386	XP_001648461	*Aedes aegypti*	aldo-keto reductase	Har_454C5021	NM_168467.1
Har_00078841	YP_174250	*Bacillus clausii* KSM-K16	oxidoreductase	v3ndG3230	NM_134902.1
Har_00079123	NP_001040432	*Bombyx mori*	reductase	Har_454C5436	NM_133105.2
Har_00087648	ABK25249	*Picea sitchensis*	reductase	Har_454C6609	NM_137627.3
Har_00100694	NP_001091765	*Bombyx mori*	oxidoreductase	v3ndE0151	NM_137070.2
Har_00101428	XP_969456	*Tribolium castaneum*	aldo-keto reductase	v3ndG2810	NM_140227.1
Har_00106752	AAF70499	*Spodoptera littoralis*	3α-reductase	Har_454C9281	NM_130519.2
Har_00111734	AAF70499	*Spodoptera littoralis*	3α-reductase	Har_454C9990	NM_130519.2
Har_00115379	XP_002051970	*Drosophila virilis*	reductase	Har_454C24330	NM_134840.2
Har_00120674	XP_002230203	*Branchiostoma floridae*	aldo-keto reductase	Har_454C27554	NM_168467.1

purpose, when a small amount of cholesterol is supplied (Clayton, 1964; Dupont, 1982; Kircher and Gray, 1978). There is no literature showing that insects are able to convert cholestanol into cholesterol, although cockroaches, *Eurycotis floridana* and *Blattella germanica*, can convert this sterol into Δ^7-cholestenol (Clayton and Edwards, 1963). However, no sterols other than cholesterol and cholestanol were found above detectable level by GC / MS (10 ng). Interestingly, there were still appreciable amount of cholesterol. This, however, likely comes from one or two sources: 1) very small sterol contamination in diet components (e.g., casein (see Chapter III), or 2) cholesterol content allocated by the mother. Female insects transfer significant quantities of cholesterol to their progeny (Kaplanis et al., 1960; Monroe et al., 1968), and the amount of sterols is high enough for the development of the progeny if there is a proper "sparing" sterol such as cholestanol in the diet (Kircher and Gray, 1978). It is, however, somewhat surprising that so many genes were regulated on cholestanol, given its structural similarity to cholesterol. Around 31% of them were metabolic enzymes coded genes, which indicated that *H. zea* might metabolize cholestanol. But this metabolism was very slowly if it did exsit. Interestingly, the total sterol/steriod amounts (indicated as a concentration) in *H. zea* was lowest on the cholestanol treatment. A similar phenomenon was also observed in other caterpillars reared on stanols (Chapter IV), so *H. zea* might be able to excrete cholestanol preferentially. ABC transporters are known to be involved in sterols excretion in mammals (Allayee et al., 2000; Berge et al., 2000) and we observed up-regulation of genes coding ABC transporters in *H. zea* reared on the cholestanol diets (Table 5.2).

Cholestan-3-one has many similar structures to 3-dehydroecdysone, a metabolite in steroid hormone synthesis pathway in insects. These include the four cycloalkane rings that attach adjacently, and a 3-keto group (Gilbert, 2004). Caterpillars reared on the cholestan-3-one diets had very slow development, and very poor eclosion success. Biochemically, caterpillars on the cholestan-3-one diets had total stanol levels that were equivalent to the cholestanol-reared caterpillars, but interestingly *H. zea* caterpillars reared on the cholestan-3-one diets produced two stanol isomers: 1) 5α-cholestan-3β-ol (cholestanol), and 2) 5α-cholestan-3α-ol (epicholestanol) (see Fig. 5.1). A similar metabolic response to cholestan-3-one has also been observed in two other Lepidoptera species, *Heliothis virescens* and *Maduca sexta* (Chapter IV) and one Diptera species, *Musca domestica* (Dutky et al., 1967). The enzymes mediating the metabolism of this steroid are possibly 3α-reductase and 3β-reductase, because in *Manduca sexta* they can convert 3-dehydroecdysone into 3α-ecdysone (3-epiecdysone) and 3β-ecdysone, respectively, and the midgut was the organ showing high activities of both enzymes (Gilbert, 2004; Weirich et al., 1993). The genes encoding both reductases in *Spodoptera littoralis* and the gene encoding 3β-reductase in *Bombyx mori* have been sequenced, and published, and they belong to aldo-keto reductase (AKR) superfamily, a group of NAD(P)H dependent oxidoreductases (Chen et al., 1999; Takeuchi et al., 2000; Yang et al., 2010). In the microarray analysis, one homologous sequence to *Spodoptera littoralis* 3-dehydroecdysone 3α-reductase, and many aldo-keto reductases, was found to be highly up-regulated (Table 5.2). Moreover, other possible enzymes involved in the metabolism of cholestan-3-one were also reported (Table 5.2).

It seems likely that cholestan-3-one may be regarded as an ecdysteroid agonist, and various pathways can be used by phytophagous insects to detoxifying ingested this steroid and its reduced products (Rharrabe et al., 2007). Additionally, the ingested cholestan-3-one may also disturb the normal physiological process regulated by ecdysone in *H. zea* such as molt, metamorphosis and other developmental process. Thus, it is not surprising that the gene express is more regulated on cholestan-3-one.

CHAPTER VI

CHOLESTEROL HOMEOSTASIS IN A GENERALIST GRASSHOPPER, *SCHISTOCERCA AMERICANA*

6.1 Introduction

Nutrient homeostasis is important for all organisms including humans and insects (Behmer, 2009; Simpson and Raubenheimer, 2009). Obesity or overweight, a common healthy problem in human caused by an imbalance of energy intake and energy expenditure, is associated with the increased incidence of many lethal diseases including cardiovascular disease, type 2 diabetes mellitus, stroke, etc. (Calle et al., 1999; Must et al., 1999; Simpson and Raubenheimer, 2005). Cholesterol homeostasis is important to human health because of its potential causal relationship to coronary heart disease, one type of cardiovascular disease (Link et al., 2007). More importantly, cholesterol is also the essential component of cellular membranes and the precursor to steroid hormones (Behmer et al., 2011). Humans can regulate cholesterol by coordinating dietary cholesterol intake, cholesterol synthesis and its absorption (Lecerf and de Lorgeril, 2011). However, the relationship among these three factors is very complicated. For example, cholesterol synthesis is not only regulated by the dietary cholesterol amount but also the available substrates, another stronger regulation factor (Jones, 1997). Moreover, the increase of exogenous cholesterol only reduces hepatic synthesis moderately but does not affect extrahepatic synthesis.

Cholesterol functions similarly in insects and, unlike humans, insects cannot synthesize cholesterol *de novo*, so exogenous sterols are the only sources for insects (Behmer and Nes, 2003). Dietary sterol concentration can affect insect performance significantly. Insects even fail to grow when the concentration is low, e.g., lower than 0.5 mg/g dry weight for *S. americana* (Behmer and Elias, 1999b), although no negative effects are found when it is relatively high (Dadd, 1960b; Ritter and Nes, 1981a). Carnivorous insects usually do not face the deficiency of cholesterol in their food but, in contrast, plant-feeding insects, e.g., grasshoppers and caterpillars, rarely encounter cholesterol in plants and must be able to convert phytosterols, a group of cholesterol derivatives having extra C24 alky group, into cholesterol (Behmer et al., 1999b; Ikekawa et al., 1993). Sitosterol (C24 ethyl) and campsterol (C24 methyl), two sterols only differing in C24 alky group, are the two most abundant phytosterols (Itoh et al., 1973), and most plant-feeding insects can develop well on them, even when mixed with a low percent of unsuitable sterols, e.g., less than 25% for a grasshopper, *Schistocerca americana* (Behmer and Elias, 1999b, 2000; Nes et al., 1997; Ritter and Nes, 1981b).

Insects have advantages over mammals and other animals in studying the dietary effects on cholesterol homeostasis. First, they cannot synthesize sterols, so the cholesterol profile is a direct reflection for the effect of dietary sterols. Second, it is much cheaper to conduct insect experiments, and the experimental period is shorter. Third, there is extensive information on sterol use in insects (reviewed by Behmer and Nes, 2003). Fourth, there are genomic sequences available for a few insect species, including *S. americana*, and this will open a wider window for the study of cholesterol

homeostasis. Last but not least, artificial diets are available for insects. For example, a nearly sterol-free artificial diet having defined chemical information is widely used for the study of nutrients regulation in grasshoppers (Behmer and Joern, 2008).

In this experiment, we used a grasshopper species, *S. americana*, to study cholesterol homeostasis. Different sterol concentration diets were fed to insects and the performance of insects, sterol profile at different stages, the amount of sterol eaten and excreted were recorded. Insects seem to keep cholesterol level constant and the failure of this regulation can lead to death.

6.2 Materials and Methods

6.2.1 Insects

A grosshopper species*, Schistocerca americana* (Drury) (Orthoptera: Acrididae) was used in this experiment and the stock colony was reared at 28° C with L : D 16 : 8 h on a diet of wheat seedlings cultured in greenhouse and wheat bran (purchased from HEB, TX). Different stadia of grasshoppers were separated into Bioquip cages (30 × 30 × 30 cm) and adults were allowed to mate and produce eggs in a 32 oz. deli cup filled with vermiculite (Sunshine, Sun Gro Horticulture, British Columbia, Canada). Third stadium grasshoppers within 12 hrs of having newly molted were used.

6.2.2 Sterol and diet

Sitosterol (5-cholesten-24β-ethyl-3β-ol) used in this experiment was bought from Steraloids Inc. (Newport, RI, USA). The purity of sitosterol in this product is 70.61%

and there are also 15.83% campesterol (5-cholesten-24α-methyl-3β-ol), 5.61% brassicasterol (5,22-cholestadien-24β-methyl-3β-ol) and 4.76% stigmasterol (5,22-cholestadien-24β-ethyl-3β-ol). These sterols widely exist in plants that grasshoppers encounter in nature and among all these sterols, the sum of sitosterol and campesterol accounted for 86.44%, so the grasshopper has no problem in using this sterol mixture (Behmer and Elias, 1999a, 2000; Behmer et al., 1999b).

The artificial diet in this experiment was similar to the one used by Behmer et al (Behmer and Elias, 1999b). All the artificial diets for different treatment contained identical nutrients except for sterol, and included 21% protein (a combination of casein, bacteriological peptone and egg albumen with the ratio of 3:1:1), 21% digestible carbohydrate (a combination of sucrose and white dextrin with the ratio of 1:1), 54% cellulose, 2.5% Wesson's salts, 0.55% linoleic acid, 0.23% ascorbic acid, 0.18% vitamin cocktail. The different concentration of sterols was added into the diet according to the treatments. Among all diet components, only albumen and casein contain cholesterol but the total concentration contributed from these two components was negligible (Behmer and Elias, 1999b) because the total cholesterol amount in these components is much less than that added into the treatment diets. All diets were kept at -20° C before use. In this experiment, 6 serial sterol concentrations were used: 1) 0, (only trace amount of sterol contamination, 0.287 μg/g, from albumen and casein); 2) 0.25 mg/g; 3) 0.5 mg/g; 4) 1 mg/g; 5) 2 mg/g; 6) 4 mg/g.

The grasshopper culture was checked twice a day and the newly molted (within 12 hrs) 3rd stadia grasshoppers were transferred into a Bioquip cage. After being

weighed, they were randomly distributed to each of the 6 treatments, and 20 males and 20 females were used for each treatment respectively. Insects were put individually into a clear plastic box (7 3/8 L × 5 1/4 W × 3 3/4 H inch) with the water supply in a deli cup (1 oz) delivered via a cotton wick. A piece of wire mesh (25 × 2 cm) was used for perch and molt, and the diet was given in a 5 cm Petri dish. All the diets in dishes were dried using desiccant for at least 24 hrs and weighed before they were put into an experimental arena. The experiment was conducted in a Percival incubator (Model # I66VLC8, Percival Scientific, Inc) at 30 °C with L : D 14:10. The insects were checked twice a day for molt or death. Water was checked every four days and refilled to make sure it was sufficient for the grasshoppers. After each molt, insects were weighed and frass was collected. The uneaten diets were dried as described above and then weighed to calculate how much diet each individual consumed. New dry diet was weighed and provided for the grasshoppers in the successive stadium. To prepare insects for sterol analysis, 5 insects for each sex were randomly selected and individually placed into a 1 oz solo cup containing a sterol-free diet (the trace sterol diet) for 24 hrs to make sure the old and sterol-containing diet in the alimentary tract was evacuated. Then the insect body and the corresponding frass were freeze-dried and stored at -20° C for the future sterol analysis. The experiments ran until all the insects reached the adult stage or died.

6.2.3 Sterol identification and quantification

Different amounts of solvent were used based on the size of sample. For 4th-stadium grasshoppers, each individual dry body was ground and weighed in a 15 ml

VWR centrifuge tube. A 1 ml volume of chloroform, 1 ml of methanol, 20 μg of cholestane and 4 glass beads (size 3, Kimble Kontes LLC) were added to each sample. For adults, each individual dry body was ground and weighed in a 15 ml VWR centrifuge tube. A 4 ml volume of chloroform, 4 ml of methanol, 80 μg of cholestane and 4 glass beads (size 3, Kimble Kontes LLC) were added to each sample. For grasshopper frass, all frass produced by each adult in all stadia was pooled and weighted in a 15 ml VWR centrifuge tube. A 4 ml volume of chloroform, 4 ml of methanol, 80 μg of cholestane and 4 glass beads (size 3, Kimble Kontes LLC) were added to each sample. The samples were shaken vigorously by using a Pneumatic paint shaker powered by a 3-gallon air compressor for 30 mins. A 350 μl volume of the extraction for the 4th-stadium grasshoppers, a 500 μl volume of the extraction for adults and a 500, 1000 or 1500 μl volume (depending on the mass of frass respectively) of the extraction for frass was transferred into a 2 ml glass bottle and a half volume of H_2O as that for the taken-out extraction was added into each tube. Each sample was vortexed and allowed to separate for 12 hrs. Following separation, the chloroform (lower layer) was removed and evaporated under nitrogen to a volume of 200 μl. The similar method was used to measure the total amount of sterols including base hydrolysable serol esters and free sterols (Chapter IV). Sterols/steroids were identified GC-MS and quantified by gas chromatography (GC).

6.2.4 Statistical analysis

Insect survival in each stadium was a binomial distribution and was analyzed using Likelihood Ratio Chi-Square statistics. No difference was found between males and females, so the two sets of data were combined for analysis. These data were presented by the percent of grasshoppers survived to a new stage. For the 4th-stadium grasshopper mass, most data fit the normal distribution well although the data for male on 0 and 2 mg/g diets showed slight lack of fit (Shaprio-Wilk test: male, 0, W = 0.89, P = 0.033; 0.25 mg/g, W = 0.97, P = 0.911; 0.5 mg/g, W = 0.97, P = 0.628; 1 mg/g, W = 0.96, P = 0.431; 2 mg/g, W = 0.89, P = 0.020; 4 mg/g, W = 0.96, P = 0.458; female: 0, W = 0.98, P = 0.972; 0.25 mg/g, W = 0.95, P = 0.394; 0.5 mg/g, W = 0.95, P = 0.354; 1 mg/g, W = 0.98, P = 0.937; 2 mg/g, W = 0.95, P = 0.363; 4 mg/g, W = 0.98, P = 0.869), and, more importantly, the data also showed equal variance between treatments (Brown-Forsythe test: male, $F_{5,123} = 0.93$, $P = 0.465$; female, $F_{5,116} = 0.83$, $P = 0.533$). Therefore, ANOVA is used for the analysis of 4th-stadium grasshopper mass. For other analysis, ANOVA was not proper because of the lack of fit to a normal distribution and unequal variance between treatments. Instead, a nonparametric analysis, Kruskal-Wallis test, was used for the analysis (Zar, 1999). These data were presented by median ± MAD. Bonfferoni correction was employed for the multiple comparison ($\alpha = 0.05$). For sterol ingestion and excretion, we also used linear regression analysis (least square analysis) to analyze the relationship between the measure and the sterol concentration in the diets. All the analysis was performed in JMP® 7.0.2 (Cary, NC, USA).

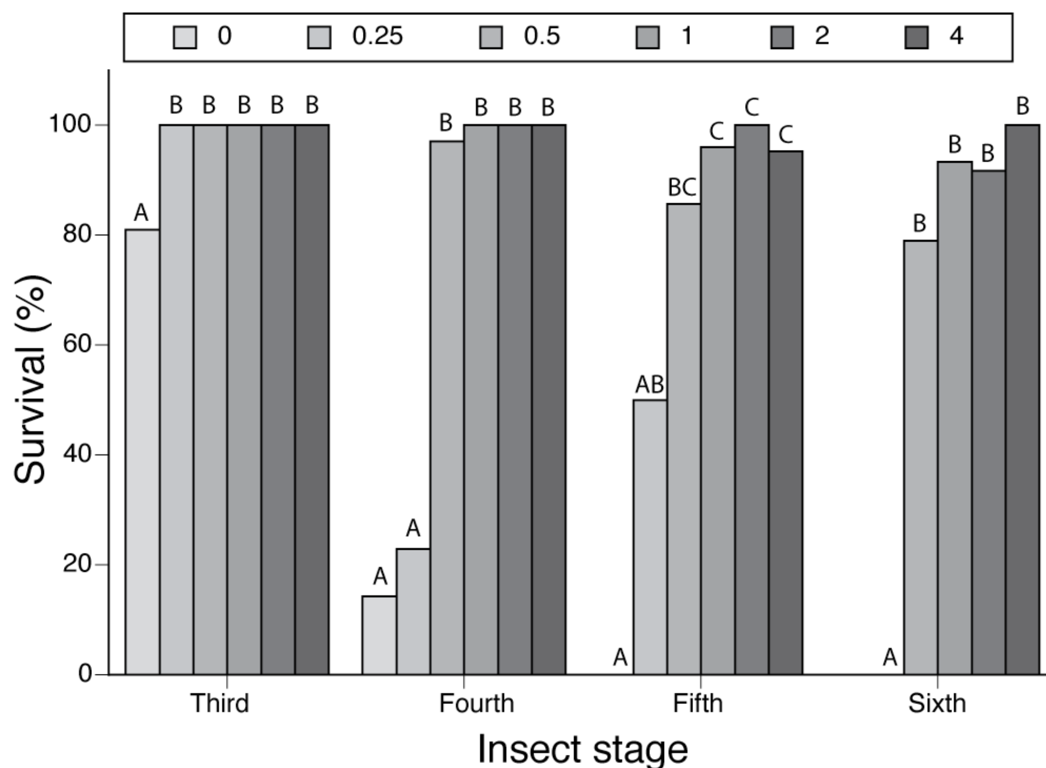


Fig. 6.1. The percent of insect survival at each of 4 stages on 6 sterol concentration diets, i.e., 0, 0.25 mg/g, 0.5 mg/g, 1 mg/g, 2mg/g and 4 mg/g. Survival at each stage represented the percent of insects survived to the next stage. The survival on different diets was compared within each stage and different letters indicated significant difference. The darkness of bars represented the concentration of the diets and a darker color indicated a higher concentration.

6.3 Results

6.3.1 Insect performance on different sterol concentration diets

6.3.1.1 Survival at each stage

The survival of grasshoppers at 4 stadia (3rd, 4th, 5th and 6th) on 6 sterol concentration diets was recorded (Fig. 6.1). During the 3rd stadium, the survival on 0 diet was significantly lower than that on other diets and there was no death on other diets ($\chi^2_5 = 42.84$, $P < 0.001$). In contrast, during the 4th stadium, the survival on 0.25 mg/g diet also reduced significantly compared with that on higher concentration diets and it

was similar to that on 0 diet (χ^2_5 = 133.82, P < 0.001). There was no survival on 0 diet at the 5th stadium. Interestingly, more percent insects on 0.25 mg/g diet survived to the 5th stadium reached to the 6th stadium comparing with those at the 4th stadium. The survival was even comparable to that on 0.5 mg/g diet, although it was still significantly lower than other higher concentration diets (χ^2_5 = 28.89, P < 0.001). At the final stadium, all insects on 0.25 mg/g diet died and there were no difference among other treatments (χ^2_5 = 13.04, P = 0.01).

6.3.1.2 Body mass on different diets

Newly molted insect was weighed for the 4th-stadium insects and adults. Fresh body mass differed between the sexes for both 4th-stadium grasshoppers (0, $|t_{27}|$ = 15.23, P = 0.040; 0.25 mg/g, $|t_{36}|$ = 11.63, P = 0.058; 0.5 mg/g, $|t_{38}|$ = 5.46, P = 0.315; 1 mg/g, $|t_{39}|$ = 21.63, P < 0.001; 2 mg/g, $|t_{40}|$ = 17.62, P = 0.001; 4 mg/g, $|t_{40}|$ = 15.92, P = 0.011) and adults (0.5 mg/g, χ^2_1 = 0.15, P = 0.699; 1 mg/g, χ^2_1 = 6.82, P = 0.009; 2 mg/g, χ^2_1 = 6.82, P = 0.009; 4 mg/g, χ^2_1 = 6.82, P = 0.009), so the analysis was conducted for the sexes separately. Both 4th-stadum insects and adults were lighter on low sterol concentration diets (4th stadium: male, $F_{5,116}$ = 2.14, P = 0.011; female, $F_{5,123}$ = 3.86, P = 0.003; Adult: male, χ^2_3 = 7.32, P = 0.062; female, χ^2_3 = 8.07, P = 0.045).

For 4th-stadium insects, the lowest mass was on 0 diet for both sexes and it was significantly lower than that on 0.5 mg/g, 1mg/g and 4 mg/g diets for male and that on 1 mg/g and 4 mg/g diets for female respectively. There was no difference among the non-zero sterol concentration diets for both sexes (Fig. 6.2A). No insects survived to adult

on 0 and 0.25 mg/g diets, so adult body mass was only analyzed among other 4 higher concentration diets. Body mass also reduced on the low concentration diets and it seemed that female body mass were more affected because a significant difference was found only for females (Fig. 6.2B).

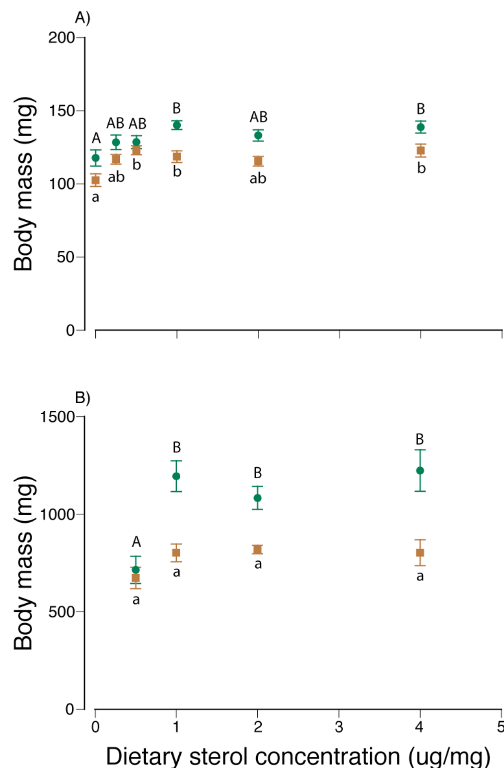


Fig. 6.2. Fresh body mass of male and female insects on different sterol concentration diets. A) Mean (± SEM) body mass of newly molted 4th-stadium grasshoppers on 6 different sterol concentration diets, i.e., 0, 0.25 mg/g, 0.5 mg/g, 1 mg/g, 2mg/g and 4 mg/g; B) Median (± MAD) body mass of newly molted adults on 4 sterol concentration diets, i.e., 0.5 mg/g, 1 mg/g, 2mg/g and 4 mg/g. Different statistical methods were used for the 4th-stadium insects and the adults (See method part for details). Green dot represented body mass for female and different uppercase letters in each panel indicated statistically significant difference among the treatments. Yellow square represented body mass for male and different lowercase letters in each panel indicated statistically significant difference among the treatments.

6.3.2 Sterol profile in grasshoppers on different sterol concentration diets

Cholesterol was mostly the only sterol found in insects, although stigmasterol was found in very low levels occasionally. Therefore, the sterol amount here represented only cholesterol amount in insects. No difference was found between females and males, so the data for two genders on each treatment were pooled for the analysis.

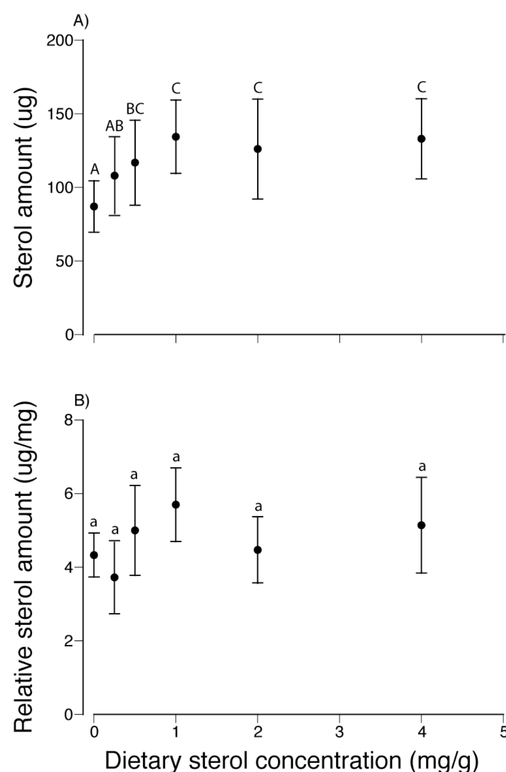


Fig. 6.3. Sterol profiles of newly molted 4th-stadium grasshoppers reared on different sterol concentration diets. A) Median (± MAD) total cholesterol amount in insects, B) Median (± MAD) relative cholesterol amount in insects. Only cholesterol was found in most insects and very low level of stigmasterol was found occasionally in some individuals so the sterol amount here represented cholesterol amount. Different letters in each panel indicated statistically significant differences among the treatments.

6.3.2.1 Sterol profile in the newly molted 4th-stadium grasshoppers

From 0 to 1 mg/g, the sterol amount in grasshoppers increased gradually but it was constant when higher sterol concentration was fed to insects ($\chi^2_5 = 14.78$, $P = 0.011$, Fig. 6.3A). Cholesterol amount on 0 diet was lowest among all treatments although it was similar to that on 0.25 mg/g diet. Cholesterol amount on 0 diet was significantly lower than that on other higher sterol concentration diets, while cholesterol amount on 0.25 mg/g was similar to that on 0.5 mg/g diet but significantly lower than that on 1 mg/g, 2mg/g and 4 mg/g diets. In contrast, there was no difference among all treatments for the relative sterol amount ($\chi^2_5 = 10.76$, $P = 0.056$; Fig. 6.3B).

6.3.2.2 Sterol profile in adults

No grasshoppers could reach the adult stage on 0 and 0.25 mg/g diets (Fig. 6.1) so only the data on other treatments, i.e., 0.5 mg/g, 1 mg/g, 2 mg/g and 4 mg/g were analyzed. Similar to that found in the 4th-stadium insects, a significant difference was only found for cholesterol amount ($\chi^2_3 = 10.94$, $P = 0.012$; Fig. 6.4A) but not for relative sterol amount ($\chi^2_3 = 7.40$, $P = 0.060$; Fig. 6.4B). The sterol amount on 0.5 mg/g diet was only significantly lower than that on 2 mg/g diet and there was no difference among other higher sterol concentration diets, i.e., 1 mg/g, 2 mg/g and 4 mg/g diets.

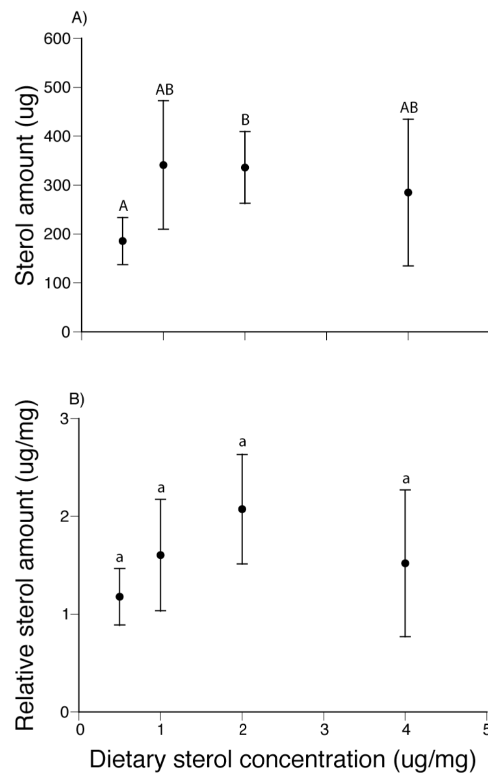


Fig 6.4. Sterol profiles of adults reared on different sterol concentration diets. No insects were able to survive to adult stage on 0 and 0.25 mg/g diets. A) Median (± MAD) total cholesterol amount in insects, B) Median (± MAD) relative cholesterol amount in insects. Only cholesterol was found in most insects and very low level of stigmasterol was found occasionally in some individuals so the sterol amount here represented cholesterol amount. Different letters in each panel indicated statistically significant differences among the treatments.

6.3.3 Non-sterol nutrients and sterol regulation in insects

For both daily non-sterol nutrients consumption and daily sterol consumption, significant difference was found between sexes except for 0.5 mg/g (Non-sterol: 0.5 mg/g, $\chi^2_1 = 0.15$, $P = 0.699$; 1 mg/g, $\chi^2_1 = 3.94$, $P = 0.047$; 2 mg/g, $\chi^2_1 = 6.82$, $P = 0.009$; 4 mg/g, $\chi^2_1 = 5.77$, $P = 0.016$; Sterol: 0.5 mg/g, $\chi^2_1 = 0.15$, $P = 0.699$; 1 mg/g, $\chi^2_1 = 3.94$, $P = 0.047$; 2 mg/g, $\chi^2_1 = 6.82$, $P = 0.009$; 4 mg/g, $\chi^2_1 = 5.77$, $P = 0.016$) so the data were analyzed for each sex.

There was no difference for daily non-sterol nutrients intake (Male: $\chi^2_3 = 2.53$, $P = 0.470$; Female: $\chi^2_3 = 4.45$, $P = 0.216$). In contrast, daily sterol intake increased proportionally with the increase of sterol concentration in the diets and there was significant difference among different diets (Male: $\chi^2_3 = 17.86$, $P = 0.001$; Female: $\chi^2_3 = 14.80$, $P = 0.002$; Fig. 6.5).

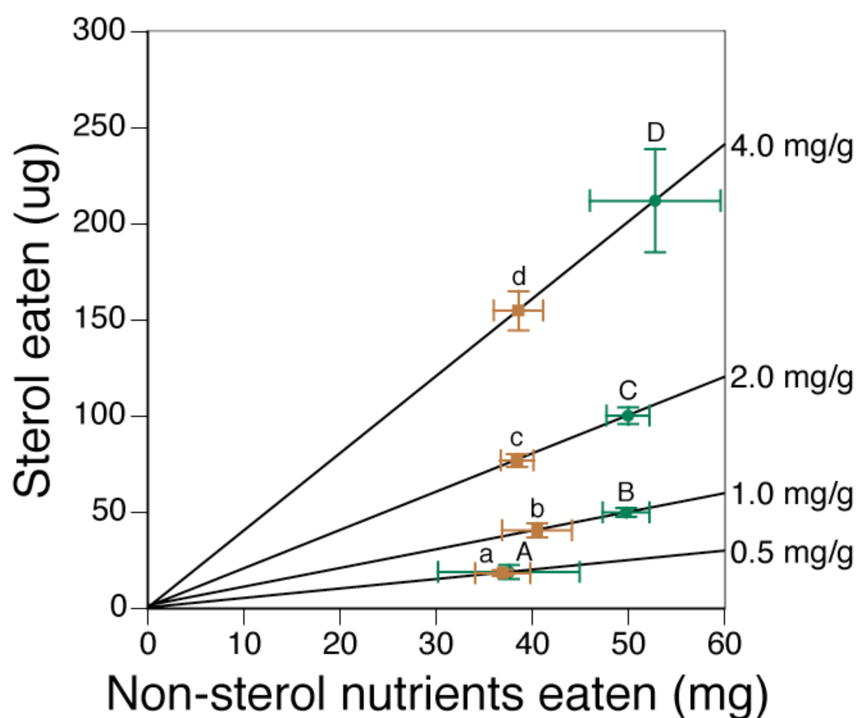


Fig. 6.5. Median (± MAD) non-sterol nutrients intake per day vs. sterol intake per day for male and female insects from 3rd-stadium grasshoppers to adults on different sterol concentration diets. Yellow square and green dot represented for male and female insects separately. No significant difference was found among non-sterol nutrients intake for both sexes so only multiple comparison was conducted for sterol intake. Different lowercase letters indicated statistically significant differences in sterol intake for male insects and different uppercase letters indicated statistically significant differences in sterol intake for female insects among the treatments.

6.3.4 Sterol ingestion and excretion

Sterol ingestion represents the total sterol amount eaten by each grasshopper reaching adult stage, the product of total diet consumption and its corresponding sterol concentration. Sterol excretion represents the total sterol amount in the frass produced by each grasshopper. There was significant difference between two sexes for sterol ingestion (0.5 mg/g, $\chi^2_1 = 3.75$, $P = 0.053$; 1 mg/g, $\chi^2_1 = 6.82$, $P = 0.009$; 2 mg/g, $\chi^2_1 = 5.77$, $P = 0.016$; 4 mg/g, $\chi^2_1 = 6.82$, $P = 0.009$) but not for sterol excretion frass (0.5 mg/g, $\chi^2_1 = 0.15$, $P = 0.699$; 1 mg/g, $\chi^2_1 = 0.96$, $P = 0.327$; 2 mg/g, $\chi^2_1 = 0.88$, $P = 0.347$; 4 mg/g, $\chi^2_1 = 2.45$, $P = 0.117$). For the convenience of comparison between sterol ingestion and excretion for each sex, the frass excretion was still analyzed separately by sex. Both sterol ingestion and the sterol excretion in frass were increased significantly along with the increase of sterol concentration in the diets (Ingestion: male, $\chi^2_3 = 17.86$, $P < 0.001$; female, $\chi^2_3 = 14.80$, $P = 0.002$; Excretion: male, $\chi^2_3 = 15.98$, $P = 0.001$; female, $\chi^2_3 = 14.80$, $P = 0.002$) and, furthermore, they were positively related to sterol concentration in the diets (Adjusted RSquare for sterol ingestion: $r^2_{male} = 0.99$, P < 0.001, $r^2_{female} = 0.94$, P < 0.001; Adjusted RSquare for sterol excretion: $r^2_{male} = 0.97$, P < 0.001, $r^2_{female} = 0.91$, P < 0.001) although the increase in frass sterol from 0.5 mg/g diet to 1 mg/g diet was a little less than that in ingested sterol, which was more significant for male grasshoppers (Fig. 6.6).

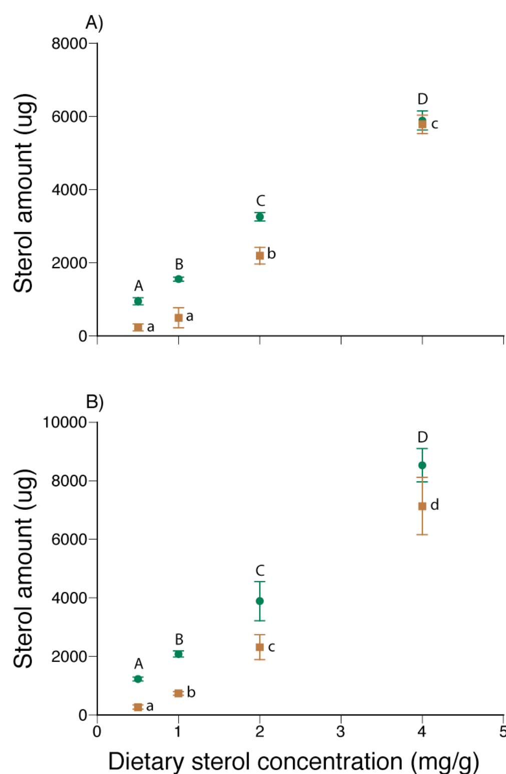


Fig. 6.6. Total amount of sterol ingested and total amount of sterol excreted in frass on different sterol concentration diets. A) Median (± MAD) sterol ingestion and sterol excretion in frass for male insects, B) Median (± MAD) sterol ingestion and sterol excretion in frass for female insects. Green dot represented the ingested sterol amount and different uppercase letters in each panel indicated statistically significant differences among the treatments. Yellow square represented the sterol amount in frass and different lowercase letters in each panel indicated statistically significant differences among the treatments.

6.4 Discussion

6.4.1 Insect performance on sterol diets of different concentration

The survival of *S. americana* increased as the sterol concentration in the diets increased. 0.5 mg/g was the threshold for them to have normal survival and no negative effects were found with increased sterol concentrations. Behmer and Elias also found that the newly molted 4th-stadium *S. americana* could only survive to adult stage when the sterol concentration was higher than 0.5 mg/g (Behmer and Elias, 1999b). This threshold may vary among different species. For example, caterpillar species, *Heliothis*

zea, could have a normal development and growth when the concentration was not less than 0.1 mg/g (Ritter and Nes, 1981a). The reason that insects cannot extract enough sterols from a low sterol concentration diet is because sterols are passively absorbed across intestine (Behmer and Elias, 1999b; Jouni et al., 2002; Turunen and Crailsheim, 1996).

Interestingly, the survival on 0.25 mg/g is identical to that on other higher concentration diets and the survival on 0 diet is still high (> 80%) during the 3rd stadium and both of them began to drop significantly from the 4th stadium. In contrast, the newly molted 4th-stadium insect body mass was almost twice as that of the newly molted 3rd ones (data not shown). Two possible mechanisms can explain this: 1) there must be a sterol reservoir in insects; the fat body is reported to be able to store cholesterol (Yun et al., 2002); 2) insects can bear a certain level of cholesterol dilution in their cells. However, obviously these two mechanisms only solve the sterol deficiency for a certain period.

6.4.2 Sterol homeostasis

To have a normal development or, at least, to survive, insects must maintain a certain amount of cholesterol. Otherwise, high death rate may occur. For example, the 4th-stadium grasshoppers on 0 and 0.25 mg/g diet contained significantly less sterols than others and their death rate was higher. Insects could compensate for sterol deficiency by reducing their body size, which would reduce the required amount of cholesterol, a cellular membrane component, correspondingly because the 4th-stadium

grasshoppers on 0 diet and adults on 0.5 mg/g diet, were lighter than others insects respectively but their relative cholesterol amount was similar to those on the higher sterol concentration diets. Therefore, the hypothesis of the cholesterol dilution in cells we proposed above is not accurate or, at least, not significantly important considering the similar relative cholesterol amount among treatments. Furthermore, this compensation ability is limited because insects require a minimal body material accumulation, i.e., body mass, for the next stage (Nijhout, 2003). The body cholesterol amount on 0.5 mg/g was probably the minimum sterol amount for this compensation because insects had a comparable survival but a lower mass on this diet compared with other higher sterol concentration diets.

Insects are able to regulate body sterol content partially by storing cholesterol in fat bodies (Jouni et al., 2002) but this ability is limited because the total sterol amount in insects did not increase when the dietary sterol concentration was higher than 1 mg/g. The fat body is also the principle organ for storing other nutrients including proteins, carbohydrates, lipid, etc. (Chapman, 1998) so the space for storing cholesterol is constrained.

6.4.3 No regulation was found for sterol intake and excretion

Insect herbivores can regulate the intake of multiple nutrients simultaneously (Raubenheimer, 1992; Raubenheimer and Simpson, 1990). The importance of the geometric framework, which can investigate the regulation of multiple nutrients simultaneously, was established in studying insect herbivore nutrient regulation

(Behmer, 2009). When insects eat diluted carbohydrate-protein food, they will ingest more food to compensate for this dilution and have a normal development and growth. In this study, the intake of dietary sterols was only positively related to the sterol concentration in the diet while the intake of other nutrients was constant across all treatment (Fig. 6.5). Additionally, sterol amount in frass also changed proportionally with dietary sterol concentration (Fig. 6.6). Both evidence indicated that this species did not regulate sterol intake.

Insects cannot regulate all the nutrients in their food because of the energy cost and the increased predation risk of separated attention (Bernays, 2001). Although sterols are essential nutrients in insects, the required amount of this nutrient is low compared with other nutrients such as carbohydrates and proteins and, more importantly, an appreciable amount of this nutrient exists in plants (Nes, 1977). In other words, dietary plants providing insect herbivores non-sterol nutrients generally also provide sufficient sterols. Therefore, insect herbivores have no reason to be able to sense this nutrient and, further to regulate it.

Obesity has become an important healthy issue in modern society. Obesity is always coupled with the increase of cholesterol amount in body (Krause and Hartman, 1984; Yu, 2009) and cholesterol imbalance, e.g., overload and altered distribution in tissue, and is related to insulin resistance, coronary heart disease and other obese diseases (Dugail et al., 2003). However, how much dietary cholesterol contributes to body cholesterol is always confounded with the *in situ* synthesis of cholesterol. For example, Angel and Farkas found that cholesterol accumulation still increased when

cholesterol-free diet was fed to rats (Angel and Farkas, 1974). Our research indicates that dietary sterol has limited effect in improving cholesterol amount in insects. This finding has potential implication in explaining the effect of dietary cholesterol on obesity.

CHAPTER VII

CONCLUSION

Caterpillar species can convert stigmasterol into cholesterol and, at the same time, they can also convert other common phytosterols into cholesterol with similar enzymatic activities (Ikekawa et al., 1993). Our gene expression data shed light on the possible enzymes involved in this dealkylation process. It is important to verify what kind of enzymes are involved in dealkylation because this will not only fill in the gap in this area but also help to find inhibitors for these enzymes, and effective inhibitors could have great potential for controlling caterpillar pests. Firstly, according to previous research, dietary unconverted phytosterols, i.e., stigmasterol, may have significant negative effects on insect herbivores, i.e., grasshoppers, even mixed with appreciable amounts of convertible sterols. So it is reasonable to infer that the interruption of the conversion of common phytosterols, which account for more than 90% of total phytosterols, may significantly reduce their performance (Itoh et al., 1973). Secondly, these inhibitors will have little effect on the sterol physiological process in insect herbivore predators or parasitoids directly because the sterol in their food is usually different from phytosterols (Behmer and Nes, 2003). However, to confirm the real function of these genes and their coding enzymes, more work is necessary such as confirming that those genes are induced, expressing these enzymes and verifying their functions.

The ratio of unsuitable sterols does affect insect performance but insects can reduce the negative effect of cholestan-3-one by sterol metabolism, especially those preferentially convert cholestan-3-one into cholestanol instead of epicholestanol, because of epicholestanol is not as good as cholestanol in replacing cholesterol (Clayton, 1964). Other structural modifications, especially changes in the sterol nucleus, could enhance the negative effects of unsuitable sterols, possibly because of the inability of insects to convert them into cholesterol, or other "good" sparing sterols (Costet et al., 1987; Nes et al., 1997). Therefore, more sterols/steroids should be included into the screen of effective sterol agents in reducing insect performance, and secondly, the method of expressing these agents in plants should be explored extensively. Without a doubt, the research in transgenic sterol modification in crops for pest resistance is a promising area.

The finding that cholestanol can reduce the total sterol content is very novel in insects and we also report several genes that may be involved in this process. It is surprising that this phenomenon has not been previously documented. Rather, earlier work has tended to focus on why cholestanol was a good "sparing" sterol, i.e., the incorporation of cholestanol into insect tissue, but sterol quantification was not emphasized, so the total amount of steroids was neglected (Kuthiala and Ritter, 1988; Lasser et al., 1966). The other possibility is that the technology used in these earlier studies limited researchers. If the same, or similar, sterol lowering mechanism known in mammals can be confirmed in insects, insect will be a very good model in studying sterol absorption and regulation, because compared with mammals sterol *in situ*

synthesis is not a confounding factors. This means results can be explained without the confounding effects provided by endogenously produced sterols (i.e., cholesterol), as is the case in human.

A strong result from the work presented in this dissertation is that there was a much stronger negative effects of unsuitable dietary sterols in the second generation, which suggests sterols in eggs are important for insect performance. However, egg sterol profiles have received limited attention from previous researchers, probably because it was believed that the small sterol amount in an egg would not contribute too much to insect performance (Ritter, 1984). The inconvenience in examining low sterol amount in eggs is possibly another reason for the deficiency of related studies in this area. But since the egg sterol profile is so important for investigating sterol utilization in insect, more data on egg sterol content and profile, especially as it relates to dietary sterols and steroids, is urgently needed. Two ideas are proposed for examining sterol profile in eggs: 1) collect enough egg material from several mating pairs and then estimate sterol content in each egg by average, and 2) compare insect parents sterol profile before and after they produce eggs, to estimate the sterol allocation to eggs, and its affect on insect longevity and lifetime reproduction.

REFERENCES

Allayee, H., Laffitte, B.A., Lusis, A.J., 2000. An absorbing study of cholesterol. Science 290, 1709-1711.

Angel, A., Farkas, J., 1974. Regulation of cholesterol storage in adipose tissue. Journal of Lipid Research 15, 491-499.

Behere, G., Tay, W., Russell, D., Heckel, D., Appleton, B., Kranthi, K., Batterham, P., 2007. Mitochondrial DNA analysis of field populations of *Helicoverpa armigera* (Lepidoptera : Noctuidae) and of its relationship to *H. zea*. BMC Evolutionary Biology 7: 117.

Behmer, S., 2009. Insect herbivore nutrient regulation. Annual Review of Entomology 54, 165-187.

Behmer, S.T., Elias, D.O., 1999a. Phytosterol structure and its impact on feeding behaviour in the generalist grasshopper *Schistocerca americana*. Physiological Entomology 24, 18-27.

Behmer, S.T., Elias, D.O., 1999b. The nutritional significance of sterol metabolic constraints in the generalist grasshopper *Schistocerca americana*. Journal of Insect Physiology 45, 339-348.

Behmer, S.T., Elias, D.O., 2000. Sterol metabolic constraints as a factor contributing to the maintenance of diet mixing in grasshoppers (Orthoptera : Acrididae). Physiological and Biochemical Zoology 73, 219-230.

Behmer, S.T., Elias, D.O., Bernays, E.A., 1999a. Post-ingestive feedbacks and associative learning regulate the intake of unsuitable sterols in a generalist grasshopper. Journal of Experimental Biology 202, 739-748.

Behmer, S.T., Elias, D.O., Grebenok, R.J., 1999b. Phytosterol metabolism and absorption in the generalist grasshopper, *Schistocerca americana* (Orthoptera : Acrididae). Archives of Insect Biochemistry and Physiology 42, 13-25.

Behmer, S.T., Grebenok, R.J., 1998. Impact of dietary sterols on life-history traits of a caterpillar. Physiological Entomology 23, 165-175.

Behmer, S.T., Grebenok, R.J., Douglas, A.E., 2011. Plant sterols and host plant suitability for a phloem-feeding insect. Functional Ecology 25, 484-491.

Behmer, S.T., Joern, A., 2008. Coexisting generalist herbivores occupy unique nutritional feeding niches. Proc. Natl. Acad. Sci. USA 105, 1977-1982.

Behmer, S.T., Nes, W.D., 2003. Insect sterol nutrition and physiology: a global overview, in: Advances in Insect Physiology, Vol 31. Academic Press Ltd, London, pp. 1-72.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289-300.

Berge, K.E., Tian, H., Graf, G.A., Yu, L., Grishin, N.V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R., Hobbs, H.H., 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science 290, 1771-1775.

Bernays, E.A., 2001. Neural limitations in phytophagous insects: implications for diet breadth and evolution of host affiliation. Annual Review of Entomology 46, 703-727.

Blanco, C.A., Portilla, M., Abel, C.A., Winters, H., Ford, R., Streett, D., 2009. Soybean flour and wheat germ proportions in artificial diet and their effect on the growth rates of the tobacco budworm, *Heliothis virescens*. Journal of Insect Science 9, 1-9.

Bloem, K.A., Kelley, K.C., Duffey, S.S., 1989. Differential effect of tomatine and its alleviation by cholesterol on larval growth and efficiency of food utilization in *Heliothis zea* and *Spodoptera exigua*. Journal of Chemical Ecology 15, 387-398.

Briscoe, J., Therond, P., 2005. Hedgehog signaling: from the *Drosophila* cuticle to anti-cancer drugs. Developmental Cell 8, 143-151.

Calle, E.E., Thun, M.J., Petrelli, J.M., Rodriguez, C., Heath, C.W., 1999. Body-mass index and mortality in a prospective cohort of US adults. New England Journal of Medicine 341, 1097-1105.

Canavoso, L.E., Jouni, Z.E., Karnas, K.J., Pennington, J.E., Wells, M.A., 2001. Fat metabolism in insects. Annual Review of Nutrition 21, 23-46.

Champagne, D.E., Bernays, E.A., 1991. Phytosterol unsuitability as a factor mediating food aversion learning in the grasshopper *Schistocerca americana*. Physiological Entomology 16, 391-400.

Chapman, R.F., 1998. The Insects: Structure and Function. Cambridge University Press, Cambridge.

Charlesworth, D., Charlesworth, B., 1987. Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics 18, 237-268.

Chen, J.H., Turner, P.C., Rees, H.H., 1999. Molecular cloning and characterization of hemolymph 3-dehydroecdysone 3β-reductase from the cotton leafworm, *Spodoptera littoralis* - A new member of the third superfamily of oxidoreductases. Journal of Biological Chemistry 274, 10551-10556.

Ciufo, L., Murray, P., Thompson, A., Rigden, D., Rees, H., 2011. Characterisation of a desmosterol reductase involved in phytosterol dealkylation in the silkworm, *Bombyx mori*. PLoS ONE 6, e21316.

Clark, A.J., Bloch, K., 1959a. Absence of sterol synthesis in insects. Journal of Biological Chemistry 234, 2578-2582.

Clark, A.J., Bloch, K., 1959b. Function of sterols in *Dermestes vulpinus*. Journal of Biological Chemistry 234, 2583-2588.

Clayton, R.B., 1964. The utilization of sterols by insects. Journal of Lipid Research 5, 3-19.

Clayton, R.B., Bloch, K., 1963. Sterol utilization in hide beetle, *Dermestes vulpinus*. Journal of Biological Chemistry 238, 586-591.

Clayton, R.B., Edwards, A.M., 1961. Essential cholesterol requirement of roach *Eurycotis floridana*. Biochemical and Biophysical Research Communications 6, 281-284.

Clayton, R.B., Edwards, A.M., 1963. Conversion of 5α-cholestan-3β-ol to Δ^7-5α-cholesten-3β-ol in cockroaches. Journal of Biological Chemistry 238, 1966-1972.

Clayton, R.B., Edwards, A.M., Hinkle, P.C., Smith, D.A., 1964. Intestinal absorption of cholesterol, its esters and some related sterols and analogues in the roach, *Eurycotis floridana*. Comparative Biochemistry and Physiology 11, 333-350.

Cohen, A.C., 2004. Insect Diets: Science and Technology. CRC Press LLC, Boca Raton, FL.

Connor, W.E., Wang, Y.M., Green, M., Lin, D.S., 2006. Effects of diet and metamorphosis upon the sterol composition of the butterfly *Morpho peleides*. Journal of Lipid Research 47, 1444-1448.

Cooke, D.T., Burden, R.S., James, C.S., Seco, T., Sierra, B., 1994. Influence of sterols on plasma-membrane proton-pumping atpase activity and membrane fluidity in oat shoots. Plant Physiology and Biochemistry 32, 769-773.

Corbin, D.R., Grebenok, R.J., Ohnmeiss, T.E., Greenplate, J.T., Purcell, J.P., 2001. Expression and chloroplast targeting of cholesterol oxidase in transgenic tobacco plants. Plant Physiology 126, 1116-1128.

Costet, M.F., Charlet, M., Benveniste, P., Hoffman, J., 1989. Metabolism of dietary Δ^8-sterols and 9β,19-cyclopropyl sterols by *Locusta migratoria*. Archives of Insect Biochemistry and Physiology 11, 47-62.

Costet, M.F., Elachouri, M., Charlet, M., Lanot, R., Benveniste, P., Hoffmann, J.A., 1987. Ecdysteroid biosynthesis and embryonic-development are disturbed in insects (*Locusta migratoria*) reared on plant diet (*Triticum sativum*) with a selectively modified sterol profile. Proc. Natl. Acad. Sci. USA 84, 643-647.

Dadd, R.H., 1957. Ascorbic acid and carotene in the nutrition of the desert locust, *Schistocerca gregaria* Forsk. Nature 179, 427-428.

Dadd, R.H., 1960a. The nutritional requirements of locusts - I. Development of synthetic diets and lipid requirements. Journal of Insect Physiology 4, 319-347.

Dadd, R.H., 1960b. The nutritional requirements of locusts - II. Utilization of sterols. Journal of Insect Physiology 5, 161-168.

Demel, R.A., De Kruyff, B., 1976. The function of sterols in membranes. Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes 457, 109-132.

Dong, S.Z., Ye, G.Y., Guo, J.Y., Hu, C., 2009. Roles of ecdysteroid and juvenile hormone in vitellogenesis in an endoparasitic wasp, *Pteromalus puparum* (Hymenoptera : Pteromalidae). General and Comparative Endocrinology 160, 102-108.

Dugail, I., Le Lay, S., Varret, M., Le Liepvre, X., Dagher, G., Ferre, P., 2003. New insights into how adipocytes sense their triglyceride stores. Is cholesterol a signal? Hormone and Metabolic Research 35, 204-210.

Dupont, J., 1982. Cholesterol Systems in Insects and Animals. CRC Press LLC, Boca Raton, FL.

Dutky, R.C., Robbins, W.E., Shortino, T.J., Kaplanis, J.N., Vroman, H.E., 1967. Conversion of cholestanone to cholestanol by housefly *Musca domestica* L. . Journal of Insect Physiology 13, 1501-1510.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. Evolution 18, 586-608.

Ewart, G.D., Howells, A.J., Suresh V. Ambudkar, M.M.G., 1998. ABC transporters involved in transport of eye pigment precursors in *Drosophila melanogaster*, in: Methods in Enzymology. Academic Press, pp. 213-224.

Feldlaufer, M.F., Svoboda, J.A., 1988. Sterol metabolism and utilization in insects. ISI Atlas of Science-Animal & Plant Sciences 1, 9-14.

Gilbert, L.I., 2004. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. Molecular and Cellular Endocrinology 215, 1-10.

Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. Annual Review of Entomology 47, 883-916.

Grieneisen, M.L., 1994. Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. Insect Biochemistry and Molecular Biology 24, 115-132.

Halaweish, F.T., Tallamy, D.W., Santana, E., 1999. Cucurbitacins: a role in cucumber beetle steroid nutrition? Journal of Chemical Ecology 25, 2373-2383.

Harano, T., 2011. Inbreeding depression in development, survival, and reproduction in the adzuki bean beetle (*Callosobruchus chinensis*). Ecological Research 26, 327-332.

Heyer, J., Parker, B., Becker, D., Ruffino, J., Fordyce, A., De Witt, M., Bedard, M., Grebenok, R., 2004. Steroid profiles of transgenic tobacco expressing an *Actinomyces* 3-hydroxysteroid oxidase gene. Phytochemistry 65, 2967-2976.

Hobson, R.P., 1935. On a fat-soluble growth factor required by blow-fly larvae. I. Distribution and properties. Biochemical Journal 29, 1292-1296.

Igel, M., Giesa, U., Lutjohann, D., von Bergmann, K., 2003. Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. Journal of Lipid Research 44, 533-538.

Ikeda, I., Tanaka, K., Sugano, M., Vahouny, G.V., Gallo, L.L., 1988. Inhibition of cholesterol absorption in rats by plant sterols Journal of Lipid Research 29, 1573-1582.

Ikekawa, N., Morisaki, M., Fujimoto, Y., 1993. Sterol metabolism in insects: dealkylation of phytosterol to cholesterol. Accounts of Chemical Research 26, 139-146.

Itoh, T., Tamura, T., Matsumoto, T., 1973. Sterol composition of 19 vegetable oils. Journal of the American Oil Chemists' Society 50, 122-125.

Janson, E., Grebenok, R., Behmer, S., Abbot, P., 2009. Same host-plant, different sterols: variation in sterol metabolism in an insect herbivore community. Journal of Chemical Ecology 35, 1309-1319.

Jones, P.J.H., 1997. Regulation of cholesterol biosynthesis by diet in humans. The American Journal of Clinical Nutrition 66, 438-446.

Joshi, M., Agarwal, H.C., 1977. Site of cholesterol absorption in some insects. Journal of Insect Physiology 23, 403-404.

Jouni, Z.E., Zamora, J., Wells, M.A., 2002. Absorption and tissue distribution of cholesterol in *Manduca sexta*. Archives of Insect Biochemistry and Physiology 49, 167-175.

Kaplanis, J.N., Robbins, W.E., Tabor, L.A., Shortino, T.J., 1960. The untilization and metabolism of 4-C^{14}-cholesterol by the adult house fly. Annals of the Entomological Society of America 53, 260-264.

Kinsella, J.E., Smyth Jr, T., 1966. Lipid metabolism of *Periplaneta americana* L. during embryogenesis. Comparative Biochemistry and Physiology 17, 237-244.

Kircher, H.W., Gray, M.A., 1978. Cholestanol-cholesterol utilization by axenic *Drosophila melanogaster*. Journal of Insect Physiology 24, 555-559.

Klowden, M.J., 2007. Physiological Systems in Insects, 2nd ed. Academic Press, Boston, MA.

Kodicek, E., Levinson, Z.H., 1960. Metabolism of β-sitosterol and other lipids in the presence of acetate-2-^{14}C by blowfly larvae. Nature 188, 1023-1025.

Komoto, N., Quan, G., Sezutsu, H., Tamura, T., 2009. A single-base deletion in an ABC transporter gene causes white eyes, white eggs, and translucent larval skin in the silkworm w-3(oe) mutant. Insect Biochemistry and Molecular Biology 39, 152-156.

Krause, B.R., Hartman, A.D., 1984. Adipose tissue and cholesterol metabolism. Journal of Lipid Research 25, 97-110.

Kuthiala, A., Ritter, K.S., 1988. Uptake of cholesterol and cholestanol by the intestine, hemolymph, and fat body of *Heliothis zea*. Archives of Insect Biochemistry and Physiology 7, 225-236.

Lafont, R., Dauphin-Villemant, C., Warren, J.T., Rees, H., 2005. Ecdysteroid Chemistry and Biochemistry, in: Gilbert, L.I., latrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science. Elsevier, Amsterdam, The Netherlands, pp. 125-195.

Lasser, N.L., Edwards, A.M., Clayton, R.B., 1966. Distribution and dynamic state of sterols and steroids in the tissues of an insect, the roach *Eurycotis floridana*. Journal of Lipid Research 7, 403-412.

Lecerf, J.M., de Lorgeril, M., 2011. Dietary cholesterol: from physiology to cardiovascular risk. British Journal of Nutrition 106, 6-14.

Link, J.J., Rohatgi, A., de Lemos, J.A., 2007. HDL cholesterol: physiology, pathophysiology, and management. Current Problems in Cardiology 32, 268-314.

Lönnfors, M., Doux, J.P.F., Killian, J.A., Nyholm, T.K.M., Slotte, J.P., 2011. Sterols have higher affinity for sphingomyelin than for phosphatidylcholine bilayers even at equal acyl-chain order. Biophysical Journal 100, 2633-2641.

Macdonald, D.L., Ritter, K.S., 1988. Effect of host sterols on the sterol composition and virulence of a nuclear polyhedrosis virus of *Heliothis zea*. Lipids 23, 1107-1113.

Martinez, R.L.M.C., Naranjo, J.D., 2010. A pretest for choosing between logrank and Wilcoxon tests in the two-sample problem. Metron - International Journal of Statistics, 111-125.

Monroe, R.E., 1959. Role of cholesterol in house fly reproduction. Nature 184, 1513-1513.

Monroe, R.E., Polityka, C.S., Lamb, N.J., 1968. Utilization of larval cholesterol-4-C^{14} for reproduction in house flies fed unlabeled cholesterol in the adult diet. Annals of the Entomological Society of America 61, 292-296.

Moreau, R.A., Whitaker, B.D., Hicks, K.B., 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research 41, 457-500.

Must, A., Spadano, J., Coakley, E.H., Field, A.E., Colditz, G., 1999. The disease burden associated with overweight and obesity. JAMA 282, 1523-1529.

Nasir, H., Noda, H., 2003. Yeast-like symbiotes as a sterol source in anobiid beetles (Coleoptera, Anobiidae): possible metabolic pathways from fungal sterols to 7-dehydrocholesterol. Archives of Insect Biochemistry and Physiology 52, 175-182.

Nes, W.D., Lopez, M., Zhou, W., Guo, D.A., Dowd, P.F., Norton, R.A., 1997. Sterol utilization and metabolism by *Heliothis zea*. Lipids 32, 1317-1323.

Nes, W.R., 1977. The biochemistry of plant sterols. Advances in Lipid Research 15, 233-324.

Nes, W.R., Krevitz, K., Joseph, J., Nes, W.D., Harris, B., 1977. Phylogenetic distribution of sterols in tracheophytes. Lipids 12, 511-527.

Nijhout, H., 1994. Insect Hormones. Princeton University Press, Princeton, NJ.

Nijhout, H., 2003. The control of body size in insects. Developmental Biology 261, 1-9.

Noland, J.L., 1954. Sterol metabolism in insects. I. Utilization of cholesterol derivatives by the cockroach *Blattella germanica* L. Archives of Biochemistry and Biophysics 48, 370-379.

Omoloye, A.A., Vidal, S., 2007. Abundance of 24-methylenecholesterol in traditional African rice as an indicator of resistance to the African rice gall midge, *Orseolia oryzivora* Harris & Gagne. Entomological Science 10, 249-257.

Piironen, V., Lindsay, D.G., Miettinen, T.A., Toivo, J., Lampi, A.M., 2000. Plant sterols: biosynthesis, biological function and their importance to human nutrition. Journal of the Science of Food and Agriculture 80, 939-966.

Pogue, M.G., 2004. A new synonym of *Helicoverpa zea* (Boddie) and differentiation of adult males of *H. zea* and *H. armigera* (Hubner) (Lepidoptera : Noctuidae : Heliothinae). Annals of the Entomological Society of America 97, 1222-1226.

Popp, B., Schmid, A., Benz, R., 1995. Role of sterols in the functional reconstitution of water-soluble mitochondrial forins from different organisms. Biochemistry 34, 3352-3361.

Porter, J.A., Young, K.E., Beachy, P.A., 1996. Cholesterol modification of hedgehog signaling proteins in animal development. Science 274, 255-259.

Raubenheimer, D., 1992. Tannic acid, protein, and digestible carbohydrate: dietary imbalance and nutritional compensation in locusts. Ecology 73, 1012-1027.

Raubenheimer, D., Simpson, S.J., 1990. The effects of simultaneous variation in protein, digestible carbohydrate and tannic-acid on the feeding-behavior of larval *Locusta migratoria* (L.) and *Schistocerca gregaria* (Forskal). 1. short term studies. Physiological Entomology 15, 219-233.

Rharrabe, K., Alla, S., Maria, A., Sayah, F., Lafont, R., 2007. Diversity of detoxification pathways of ingested ecdysteroids among phytophagous insects. Archives of Insect Biochemistry and Physiology 65, 65-73.

Ripa, P.V., Adler, J.H., 1987. Sterol conjugates of two phenotypically different calli of *Beta vulgaris*. Plant Cell Reports 6, 219-222.

Ritter, K.S., 1984. Metabolism of Δ^0-sterol, Δ^5-sterol, and Δ^7-sterol by larvae of *Heliothis zea*. Archives of Insect Biochemistry and Physiology 1, 281-296.

Ritter, K.S., Nes, W.R., 1981a. The effects of cholesterol on the development of *Heliothis zea*. Journal of Insect Physiology 27, 175-181.

Ritter, K.S., Nes, W.R., 1981b. The effects of the structure of sterols on the development of *Heliothis zea*. Journal of Insect Physiology 27, 419-424.

Robbins, W.E., Kaplanis, J.N., Svoboda, J.A., Thompson, M.J., 1971. Steroid metabolism in insects. Annual Review of Entomology 16, 53-72.

Roeder, K.A., Kuriachan, I., Vinson, S.B., Behmer, S.T., 2010. Evaluation of a microbial inhibitor in artificial diets of a generalist caterpillar, *Heliothis virescens*. Journal of Insect Science 10, 1-12.

Salt, T.A., Xu, S.H., Patterson, G.W., Adler, J.H., 1991. Diversity of sterol biosynthetic capacity in the Caryophyllales. Lipids 26, 604-613.

Schoonhoven, L.M., Loon, J.J.A., Dicke, M., 2005. Insect-Plant Biology, 2nd ed. Oxford University Press Inc., New York.

Shaaya, E., Shirk, P.D., Zimowska, G., Plotkin, S., Young, N.J., Rees, H.H., Silhacek, D.L., 1993. Declining ecdysteroid levels are temporally correlated with the initiation of vitellogenesis during pharate adult development in the Indianmeal moth, *Plodia interpunctella*. Insect Biochemistry and Molecular Biology 23, 153-158.

Shelton, A.M., Zhao, J.Z., Roush, R.T., 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annual Review of Entomology 47, 845-881.

Shirk, P.D., Bean, D.W., Brookes, V.J., 1990. Ecdysteroids control vitellogenesis and egg maturation in pharate adult females of the indianmeal moth, *Plodia interpunctella* Archives of Insect Biochemistry and Physiology 15, 183-199.

Simons, K., Ikonen, E., 2000. How cells handle cholesterol. Science 290, 1721-1726.

Simpson, S., Raubenheimer, D., 2009. Nutritional pharmecology: doses, nutrients, toxins, and medicines. Integrative and Comparative Biology 49, 329-337.

Simpson, S.J., Raubenheimer, D., 2005. Obesity: the protein leverage hypothesis. Obesity Reviews 6, 133-142.

Svoboda, J., Thompson, M., Robbins, W., 1972. 24-Methylenecholesterol: isolation and identification as an intermediate in the conversion of campesterol to cholesterol in the tobacco hornworm. Lipids 7, 156-158.

Svoboda, J.A., 1967. Desmosterol, an intermediate in dealkylation of β-sitosterol in the tobacco hornworm. Life science 6, 395-404.

Svoboda, J.A., 1968. Desmosterol as a common intermediate in the conversion of a number of C28 and C29 plant sterols to cholesterol by the tobacco hornworm. Experientia 24, 1131-1132.

Svoboda, J.A., 1999. Variability of metabolism and function of sterols in insects. Critical Reviews in Biochemistry and Molecular Biology 34, 49-57.

Svoboda, J.A., Hutchins, R.F.N., Thompson, M.J., Robbins, W.E., 1969a. 22-trans-cholesta-5,22,24-trien-3β-ol -- An intermediate in the conversion of stigmasterol to cholesterol in the tobacco hornworm, *Manduca sexta* (Johannson). Steroids 14, 469-476.

Svoboda, J.A., Rees, H.H., Thompson, M.J., Hoggard, N., 1988. Intermediates of stigmasterol metabolism in *Spodoptera littoralis*. Steroids 53, 329-343.

Svoboda, J.A., Thompson, M.J., Robbins, W.E., Kaplanis, J.N., 1978. Insect steroid metabolism. Lipids 13, 742-753.

Svoboda, J.A., Weirich, G.F., 1995. Sterol metabolism in the tobacco hornworm, *Manduca sexta* - A reveiw. Lipids 30, 263-267.

Svoboda, J.A., Womack, M., Thompson, M.J., Robbins, W.E., 1969b. Comparative studies on activity of 3β-hydroxy-Δ^5-norcholenic acid on Δ^{24}-sterol reductase enzyme(s) in an insect and rat. Comparative Biochemistry and Physiology 30, 541-549.

Takeuchi, H., Chen, J.H., O'Reilly, D.R., Rees, H.H., Turner, P.C., 2000. Regulation of ecdysteroid signalling: molecular cloning, characterization and expression of 3-dehydroecdysone 3α-reductase, a novel eukaryotic member of the short-chain dehydrogenases/reductases superfamily from the cotton leafworm, *Spodoptera littoralis*. Biochemical Journal 349, 239-245.

Thompson, M.J., Svoboda, J.A., Kaplanis, J.N., Robbins, W.E., 1972. Metabolic pathways of steroids in insects. Proceedings of the Royal Society of London. Series B, Biological Sciences 180, 203-221.

Truman, J.W., Riddiford, L.M., 2002. Endocrine insights into the evolution of metamorphosis in insects. Annual Review of Entomology 47, 467-500.

Turunen, S., Chippendale, G.M., 1977. Lipid absorption and transport: sectional analysis of larval midgut of corn borer, *Diatraea grandiosella*. Insect Biochemistry 7, 203-208.

Turunen, S., Crailsheim, K., 1996. Lipid in sugar absorption, in: Lehane, M., Billingsley, P. (Eds.), Biology of the Insect Midgut, 1st ed. Chapman and Hall, London, UK, pp. 293-323.

Weirich, G.F., Feldlaufer, M.F., Svoboda, J.A., 1993. Ecdysone oxidase and 3-oxoecdysteroid reductases in *Manduca sexta*: developmental changes and tissue distribution. Archives of Insect Biochemistry and Physiology 23, 199-211.

Wolfenbarger, L.L., Phifer, P.R., 2000. Biotechnology and ecology - The ecological risks and benefits of genetically engineered plants. Science 290, 2088-2093.

Yang, H., Liu, X., Zhou, F., Hu, J., Bhaskar, R., Li, X., Sun, C., Malik, F.A., Niu, Y., Miao, Y., 2010. Cloning and expression pattern of 3-dehydroecdysone 3β-reductase (3DE 3β-reductase) from the silkworm, *Bombyx mori* L. Archives of Insect Biochemistry and Physiology 76, 55-66.

Yu, B.L., 2009. Cholesterol imbalance in adipocytes: a possible mechanism of adipocytes dysfunction in obesity. Obesity Reviews 11, 560-567.

Yun, H.K., Jouni, Z.E., Wells, M.A., 2002. Characterization of cholesterol transport from midgut to fat body in *Manduca sexta* larvae Insect Biochemistry and Molecular Biology 32, 1151-1158.

Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall, Upper Saddle River, NJ.

APPENDIX

A listing of ingredients used in ritter's diet, and modifications to the ritter diet for the current study (major differences are highlighted using bold text). Ingredients are measured by mass (gram) unless otherwise mentioned; the amounts presented are sufficient to prepare 100 g diet. Ingredients are listed in the order they are mixed; a total of 7 steps and needed to make this diet.

Ingredient	Manufacturer	Experimental diet	Ritter's diet
Step 1 (casein and cellulose):			
Casein	Sigma-Aldrich	6.00E+00	6.00E+00
Cellulose	Sigma-Aldrich	5.00E+00	5.00E+00
Step 2 (sterols/steroids):			
Sterol (mg)	Sigma-Aldrich	2.00E-02	2.00E-02
Chloroform (ml)	Mallinckrodt Baker	7.00E+00	7.00E+00
Step 3 (other main ingredients):			
Wesson's salt	MP Biomedicals	1.00E+00	1.00E+00
Sucrose	Sigma-Aldrich	2.00E+00	2.00E+00
Cysteine HCl	Sigma-Aldrich	1.00E-01	1.00E-01
Choline chloride	Sigma-Aldrich	1.00E-01	1.00E-01
Myo-inositol	Sigma-Aldrich	4.00E-02	4.00E-02
Torula yeast	MP Biomedicals	1.00E+00	-
Non-fat dry milk	Hill Country	7.50E-01	-
Vitamin mix	Bio-Serv	1.88E-01	-
Step 4 (antibiotics):			
l-ascorbic acid	Sigma-Aldrich	4.00E-01	4.00E-01
37% formaldehyde (ml)	Mallinckrodt Baker	7.30E-02	7.30E-02
Methyl 4-hydroxybenzoate	Sigma-Aldrich	1.95E-01	1.95E-01
Chlorotetracycline HCl	Sigma-Aldrich	3.47E-03	3.47E-03
Streptomycin sulphate	Sigma-Aldrich	3.47E-03	3.47E-03
Sorbic acid	Sigma-Aldrich	1.00E-01	-
Step 5 (ethanol-soluble components):			
dl-alpha-tocopherol acetate (ml)	Sigma-Aldrich	1.00E-02	1.00E-02
Cholecalciferol	Sigma-Aldrich	5.00E-04	5.00E-04
Menadione	Sigma-Aldrich	5.00E-04	5.00E-04
Linoleic acid (ml)	Sigma-Aldrich	5.50E-02	5.50E-02
alpha-linolenic acid (ml)	Sigma-Aldrich	2.70E-02	2.70E-02
100% ethanol (ml)	Pharmco-Aaper	1.00E+00	1.00E+00

Ingredient	Manufacturer	Experimental diet	Ritter's diet
Step 6 (water-soluble components):			
Nicotinic acid amide	Sigma-Aldrich	1.00E-03	1.00E-03
Calcium pantothenate	Sigma-Aldrich	1.00E-03	1.00E-03
Thiamine HCl	Sigma-Aldrich	2.50E-04	2.50E-04
Riboflavin	Sigma-Aldrich	5.00E-04	5.00E-04
Pyridoxine HCl	Sigma-Aldrich	2.50E-04	2.50E-04
Folic acid	Sigma-Aldrich	2.50E-04	2.50E-04
Biotin	Sigma-Aldrich	2.00E-05	2.00E-05
Vitamin B12	Sigma-Aldrich	2.00E-06	2.00E-06
Zinc acetat	Sigma-Aldrich	5.00E-04	5.00E-04
Cobalt chloride	Sigma-Aldrich	2.50E-04	2.50E-04
Sodium molybdate	Sigma-Aldrich	2.50E-04	2.50E-04
H_2O (ml)	-	6.00E+00	6.00E+00
Step 7 (setting the diet with agar):			
H_2O (ml)	-	7.35E+01	7.54E+01
Agar	Sigma-Aldrich	2.50E+00	2.50E+00

VITA

Name:	Xiangfeng Jing
Address:	507A, Minnie Belle Heep Building, TAMU, College Station, TX 77843-2475
Email Address:	jxf_zb@yahoo.com
Education:	B.A., Agronomy, Shandong Agricultural University, 2001 M.S., Zoology, Huazhong Agricultural University, 2004 Ph.D., Entomology, Texas A&M University, 2011