THE EFFECTS OF STEROLS ON *DROSOPHILA MELANOGASTER*: PHYSIOLOGY AND BIOCHEMISTRY

A Thesis

by

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

MASTER OF SCIENCE

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August 2015

Major Subject: Entomology

ABSTRACT

Sterols are essential components of cellular membranes and are required precursors for important hormones regulating growth and development. Unlike most animals, insects lack the ability to synthesize sterols *de novo* and they must acquire sterols from their food. Cholesterol is the typical sterol recovered from animals, including most insects. Plant and fungal sterols differ structurally from cholesterol, mostly in side chain configuration and the number and position of double bonds. In the lab, *Drosophila* are reared on diets that contain 4 different sterols – cholesterol (animal sterol), sitosterol and stigmasterol (plant sterols), plus ergosterol (fungal sterol); ergosterol comprises nearly 75% of the dietary sterol content. Like vertebrates, *Drosophila* requires cholesterol for membrane structure and hormone production. However, their inability to synthesize sterols *de novo* makes them a model organism to study sterol use and metabolism. Two experiments were performed. First, using a recently developed holidic diet, larvae were individually reared (from hatch) on each of the 4 different sterols in standard *Drosophila* diet, each at a range of different concentrations. When individual sterols are incorporated at different concentrations into a holidic diet, performance and overall survival are significantly affected. Individuals reared on cholesterol only diets exhibited significantly faster developmental times to pupation and also to eclosion from pupation; additionally, overall survival to pupation and eclosion was significantly increased compared to sitosterol, stigmasterol, and ergosterol. In the absence of sterols, individuals arrested development. As an individual sterol ergosterol minimally supported adult survival at

low concentrations and failed to support survival at high concentrations. Next, the extent to which sparing occurs in *Drosophila melanogaster* was examined using different cholesterol and ergosterol ratios in the diet. When ergosterol was supplemented with cholesterol in different ratios, survival was dramatically improved and in some instances exceeded that of only cholesterol. Survival to pupation was significantly reduced as the ratio of cholesterol increased. Collectively the results show that slight variations in sterol structure have pronounced effects on *Drosophila* growth and development, and that a small amount of dietary cholesterol, likely for metabolic purposes, is required.

DEDICATION

This thesis is dedicated to my parents for their love and support in all my endeavors; my grandparents who helped raise me and have supported me in every possible way; and to Adam for motivating and inspiring me.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. Spencer Behmer for giving me the opportunity to work in his laboratory. He has been very influential and I would like to thank him for his guidance and patience during my research. I would also like to thank the members of my committee: Dr. Aaron Tarone and Dr. Robert Chapkin for their advice, guidance, and encouragement during my graduate studies.

Thank you to Jonathon Parrot for helping me establish a colony for my experiments and teaching me how to maintain them. Thank you to Dr. Robert Grebenok for assisting in my research. Many thanks to all the past and present members of the Behmer lab but especially Rebecca Clark, Carrie Deans, Marion Le Gall, Paul Lenhart, Richelle Marquess, Trevor Steele, and Harriet Thwinda for their encouragement and friendship during my graduate studies. A special thank you to the EEB group and all the friends I've made.

I want to thank my parents and grandparents for their unconditional love and support. Finally, I would like to thank Adam for his constant support and encouragement during the course of my graduate study.

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CHAPTER I

INTRODUCTION

Sterols are essential components of cellular membranes responsible for biological functions including: growth and development, membrane permeability, signal transduction, and hormone production in eukaryotes. Insects lack the ability to synthesize sterols *de novo* (Clark and Block 1959) and must acquire sterols through a dietary source (Hobson 1935, Clayton 1964, Svoboda and Thompson 1985). Hobson (1935) first demonstrated the need for dietary sterols in the blowfly, *Lucilia sericata*, followed by Van't Hoog (1936) in the fruit fly, *Drosophila melanogaster.* The dietary need for sterols has subsequently been demonstrated in Orthoptera, Blattaria, Hemiptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera (Svoboda et al. 1994, Behmer and Nes 2003). Sterols are characterized by a tetracyclic ring, carbon side chain, and 3β-hydroxyl group. Cholesterol is the primary sterol produced by vertebrates and is present at high concentrations in eukaryotic plasma membranes (Cavalier-Smith 1987). Both plant and fungal sterols differ structurally from cholesterol in regards to side chain atoms as well as the number and position of double bonds (Figure 1.1). For example, the phytosterol sitosterol differs from cholesterol by the addition of an ethyl group at C-24. Stigmasterol, another phytosterol has an ethyl group at C-24 and an additional double bond at C-22. Ergosterol, the dominant fungal sterol differs from cholesterol by the addition of a C-24 methyl group and double bonds at C-22 and C-7.

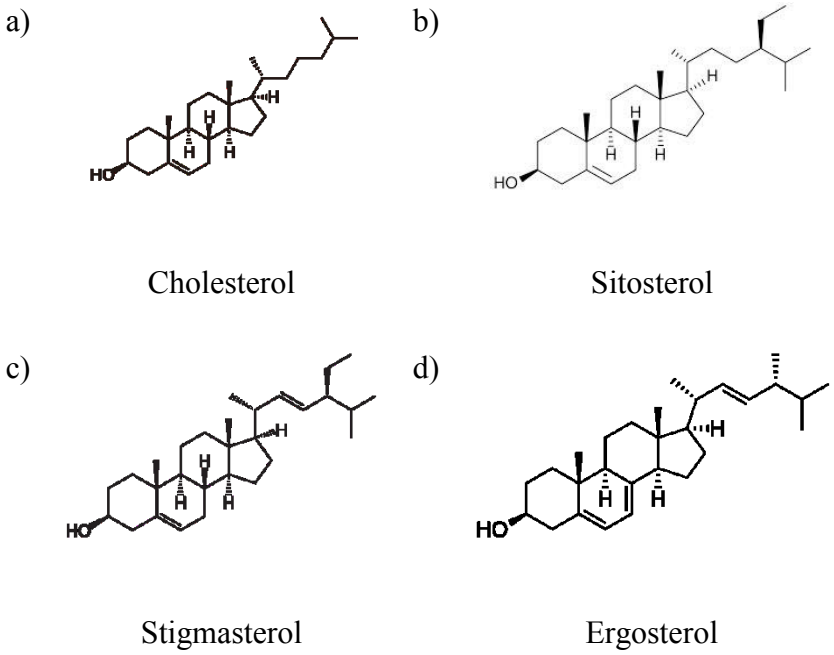


Figure 1.1. Important sterols used in these studies. Cholesterol (a) is the primary sterol found in animals. It is characterized by a 3β hydroxyl group, tetracyclic ring structure, and carbon side chain. Sitosterol (b) and Stigmasterol (c) are plant sterols. Sitosterol differs from cholesterol by the addition of a C-24 ethyl group. Stigmasterol differs from cholesterol by the addition of a C-24 ethyl group and a C-22 double bond. Ergosterol (d) is a fungal sterol, differing from cholesterol by the addition of a C-24 methyl group, C-22 double bond, and a C-7 double bond.

The amount of sterol needed to support growth and development is influenced by the size of the insect (Behmer and Nes 2003), and cholesterol is the essential precursor to insect molting hormone that is responsible for development, metabolism and reproduction. In grasshoppers, if sterol levels drop beneath the required threshold, growth and embryogenesis are compromised (Costet et al. 1987). In *Drosophila,* development does not progress from first to second-instar on cholesterol-free medium (Parkin and Burnet 1986) or low cholesterol medium (Carvalho et al. 2010). When cholesterol is absent from the diet, normal hormone pulses regulating molting between

larval instars is disrupted. Ecdysone is produced and released by the prothoracic gland of the ring gland located in the brain and ecdysone pulses are required for multiple aspects of development including embryogenesis, hatching, first instar development, larval molting, pupation, and eclosion. Pupation is initiated by an ecdysone pulse at the end of the third larval instar (Riddiford 1993). A series of ecdysone pulses drives development throughout the pupal stage and is essential for the differentiation of imaginal discs to adult structures (D'Avino and Thummel 1998, Hall and Thummel 1998, D'Avino and Thummel 2000, Zheng et al. 2003).

Cholesterol is also used in membranes, but some insects can substitute other sterols in place of cholesterol if cholesterol is lacking in the diet (reviewed by Behmer and Nes 2003). The concept of "sparing" sterols was introduced by Clayton (1964). Fruit flies can complete development on cholesterol-rich diets and diets containing mostly cholestanol with trace cholesterol but not 100% cholestanol or trace cholesterol diets (Kircher and Gray 1978). The idea here is that a small amount of cholesterol is required for metabolic purposes (i.e. as a precursor for molting hormone), but that there is less specificity for structural purposes (i.e. in membranes). The ratio of "good" sterols or sterols metabolized to cholesterol, to "bad" sterols or sterols not metabolized to cholesterol, has been shown to impact growth and development in grasshoppers (Behmer and Elias 1999, Behmer and Elias 2000). Key questions for *Drosophila* are 1) how much sterol do they need? and 2) how does the ratio of "good" to "bad" dietary sterol affect growth and development? The importance of dietary sterols is that for many insects is their ability to convert dietary sterols into cholesterol is limited. For example, sterol

metabolism differs between diptera species (Feldlaufer et al. 1995). In *Aedes aegypti*, larvae have retained the ability to dealkylate phytosterols to cholesterol (Svoboda et al. 1982). However, derived diptera (Brachycera) have lost the ability to dealkylate (Maddison et al. 2001, Behmer and Nes 2003).

Early literature concluded *D. melanogaster* dealkylated phytosterols to produce cholesterol (Cooke and Sang 1970). However, later studies show *Drosophila* is incapable of dealkylating phytosterols to cholesterol (Kircher et al. 1984, Maddison et al. 2001) but that *D. melanogaster* is capable of dealkylating ergosterol to cholesterol (Redfern 1984). More recently, Carvalho et al. (2010) showed larval arrest in the first and second instar of individually-reared *Drosophila* larvae on a lipid-depleted medium. They also reported that stigmasterol and sitosterol were capable of supporting development of *Drosophila* larvae to adulthood, but ergosterol did not and development arrested in the third larval instar. In these studies it is difficult to determine the extent to which cholesterol was removed from the "cholesterol-free" diets. In some studies, trace amounts of cholesterol were found in diets but deemed insignificant due to the amount of diet that would need to be consumed (Feldlaufer et al. 1995) and other studies lacked valid analytical analyses altogether (Cooke and Sang 1970). The development of a holidic diet medium (Piper et al. 2014) has allowed for easy manipulation and control of sterols and sterol concentrations while all other dietary components remain constant. The diet is capable of supporting development for multiple generations but at a reduced rate. Using this diet, it is possible to ensure no other sterols are present, unlike past methods which used lipid extraction techniques that left trace amounts of sterol in the diet.

Drosophila melanogaster has been used as a model organism to study developmental and physiological processes for over a century (Arias 2008). Like vertebrates, *Drosophila* requires cholesterol for membrane structure and hormone production. However, their inability to synthesize sterols *de novo* makes them a model organism to study sterol use and metabolism. In this thesis, I use the holidic diet developed by Piper et al. (2014) to determine which sterols in the *Drosophila* diet are capable of supporting growth, development, and survival in *D. melanogaster,* and also the amount of sterol required for these functions. I then examine the extent to which sparing occurs in *D. melanogaster* by examining the effects different cholesterol and ergosterol ratios in the diet have on the larval and pupal stages.

CHAPTER II

THE EFFECTS OF STEROLS ON THE GROWTH AND DEVELOPMENT OF *DROSOPHILA MELANOGASTER*

2.1 Introduction

Sterols are important components of cellular membranes responsible for membrane structure and hormone production in eukaryotes. Insects lack the ability to synthesize sterols *de novo* (Clark and Block 1959) and require a dietary source of sterols (Hobson 1935, Clayton 1964, Svoboda and Thompson 1985). Cholesterol is the primary sterol produced by vertebrates and is present at high concentrations in eukaryotic plasma membranes (Cavalier-Smith 1987). Cholesterol is an essential precursor to steroid hormones including ecdysone and 20-hydroxyecdysone which are important hormones involved in development, metabolism and reproduction. The absence of cholesterol, the necessary precursor for ecdysteroid production, interferes with normal hormone pulses regulating molting between larval instars (Warren et al. 2002, Spindler et al. 2009) In addition to steroid hormone requirements, *Drosophila* requires membrane sterols for development (Carvalho et al. 2010).

In the lab *Drosophila* are reared on diets containing animal, plant and fungal sterols. Sterols are characterized by a tetracyclic ring, carbon side chain, and 3β-hydroxyl group. Both plant and fungal sterols differ structurally from cholesterol in regards to side chain atoms as well as the number and position of double bonds (Figure 2.1). For example, the phytosterols sitosterol and stigmasterol differ from cholesterol by

the addition of an ethyl group at C-24. Stigmasterol also has an additional double bond at C-22. Ergosterol, the dominant fungal sterol differs from cholesterol by the addition of a C-24 methyl group and two additional double bonds at C-22 and C-7.

Like vertebrates, *Drosophila melanogaster* requires cholesterol for membrane structure and hormone production. However, their inability to synthesize sterols *de novo* makes them an ideal model organism to study sterol use and metabolism. Previous *Drosophila* studies utilized lipid extraction techniques that left trace amounts of sterol in the diet (Goodnight and Kircher 1971, Kircher and Gray 1978, Svoboda et al. 1989, Carvalho et al. 2010). The development of a holidic diet medium (Piper et al. 2014) has allowed for easy manipulation and control of sterols and sterol concentrations while all other dietary components remain constant. Flies were also mass reared in test tubes allowing potential sterol accumulation through active scavenging and cannibalism. By rearing individual larvae within an individual well of a 48-well plate identical nutrition is ensured for all individuals and the effects of active scavenging and cannibalism (Vijendravarma et al. 2013) are eliminated.

The purpose of this experiment is to (1) determine which sterols in the *Drosophila* diet are capable of supporting growth, development, and survival in *D. melanogaster* and (2) the amount of sterol required for these functions. This is tested by adding each of the individual sterols (Figure 2.1) in different concentrations to artificial diets. This experiment demonstrates how dietary sterol content can affect *D.*

melanogaster at both larval and pupal stages. Using the artificial diet medium ensures the sitosterol, stigmasterol, and ergosterol-based diets are completely void of cholesterol. The sterol profile of pupae reared on each diet is also analyzed.

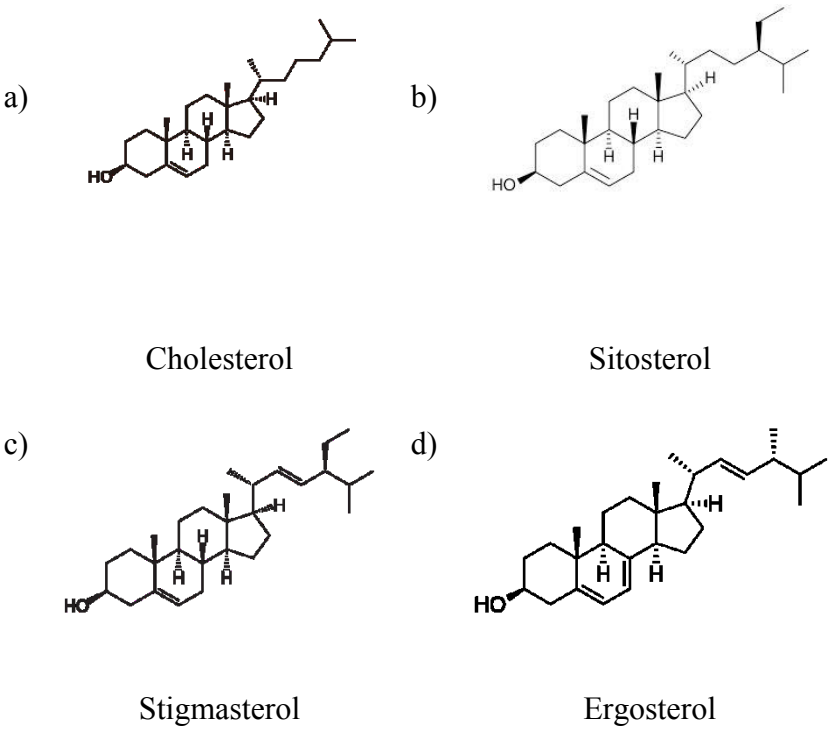


Figure 2.1: The four sterols used in this study. Cholesterol (a) is the primary sterol found in animals. It is characterized by a 3β hydroxyl group, tetracyclic ring structure, and carbon side chain. Sitosterol (b) and Stigmasterol (c) are plant sterols. Sitosterol differs from cholesterol by the addition of a C-24 ethyl group. Stigmasterol differs from cholesterol by the addition of a C-24 ethyl group and a C-22 double bond. Ergosterol (d) is a fungal sterol, differing from cholesterol by the addition of a C-24 methyl group, C-22 double bond, and a C-7 double bond.

2.2 Materials and Methods

2.2.1 Drosophila strains and maintenance

Wild-type Canton-S obtained from stocks maintained in the Tarone laboratory in the Department of Entomology at Texas A&M University were used for this experiment. *Drosophila* stocks were maintained at room temperature (23°C) and cultured on standard cornmeal-yeast medium (Appendix A).

2.2.2 Experimental diets

A complete chemically defined (holidic) diet, developed by Piper et al. (2014) was used in this experiment with slight modifications (Appendix B). This diet allowed complete control of dietary sterol type and concentration, while ensuring all other dietary components remained constant. Chloroform was used to solubilize all sterols instead of the ethanol and allowed to evaporate for 24 hours before mixing the diet. The vitamin solution was quadrupled from the original recipe. The four sterols used in this study were (1) Cholesterol (Sigma, ≥95%); (2) Sitosterol (Sigma, ≥70%; impurities: campesterol and B-sitostanol, residual); (3) Stigmasterol (Sigma, ≥95%); and (4) Ergosterol (Sigma, ≥95%). For each sterol, a range of concentrations was tested (based on pilot studies). The concentrations selected were: 0.2 g/L, 0.4 g/L, 0.8 g/L, 1.6 g/L, and 3.2 g/L. A diet that lacked sterol was also tested.

2.2.3 Experimental design

Embryos were collected from apple juice/agar plates with yeast paste for 4 hours in the

afternoon during two consecutive days. Individual embryos were transferred to a 48-well plate, with each well containing ~60µl of diet. Plates were then transferred to an incubator (Model # I-66VL, Percival Scientific, Inc Perry, IA, USA) and maintained at 25±1°C with a 12h:12h light-dark cycle. Individual rearing prevented cannibalism, scavenging, and ensured identical nutrition for each individual.

Two plates were established for each experimental diet. One of these plates was used to collect data on pupation (including pupation success, time to pupation, and pupal mass). Once data was recorded for the larvae that successfully pupated, they were frozen and set aside for sterol analysis (see below). The second plate contained individuals that were monitored for eclosion success and time to eclosion. Upon pupation, these individuals were removed from the well plates and transferred to 1.5mL Eppendorf tubes and monitored for eclosion. Over the first 48 hours of the experiment each well on each plate was checked to determine the hatching success of the embryos; wells with embryos that did not hatch were excluded from the analysis. Thereafter larvae were observed daily to record mortality, pupation and eclosion.

2.2.4 Sterol analysis

The free sterol and esterified sterol profile of pupae were analyzed in the Grebenok lab. Free sterols have an underivatized C3-OH group on the A ring of the sterol nucleus while in esterified sterols, the C3-OH group is covalently bound to another constituent. EtOH was added to each pulverized sample and incubated at room temperature in the dark for 12 hours. 50-micrograms of 5α-cholestane was added to each sample as an

internal standard to ensure proper extraction and quantification. The ethanol solution was divided into two samples to analyze free sterols (FS) and esterified sterols (ES).

The FS sample was evaporated and resuspended in 70% methanol and partitioned against water equilibrated hexane three times. The hexane fraction was evaporated, overblown with nitrogen, sealed, wrapped with foil and stored away from light at 4°C. The samples were resuspended in a known volume of hexane, filtered and analyzed for free sterols using GC-fid/GC-MS and HPLC techniques.

The ES sample was hydrolyzed by incubating the ethanol sample in a 7:3 alkaline ethanol: water solution at 50°C to yield free sterol. After incubating, the solution was washed three times with water-equilibrated hexane. The hexane fraction was backwashed with water to remove KOH and neutralize pH. The sample was then treated as a FS sample as described above.

2.2.5 Data analysis

Analysis was made in regard to: survival to pupation, survival through eclosion, time to pupation, time to eclosion, pupal mass, and sterol composition. Survival to both pupation and eclosion was scored as a percent of individuals hatched on each diet and analyzed using Logistic Regression. Developmental time to pupation and eclosion was analyzed using Survival Analysis. ANOVA was used in conjunction with Tukey post-hoc tests to analyze pupal mass and growth rate, between comparisons.

Sterol profiles of pupae were visually inspected across treatments to compare sterol levels within the different diet treatments. Total sterol was calculated and percent

body sterol was determined by dividing the total sterol by the pupal mass. Total sterol and percent body sterol were analyzed using ANOVA. Sterol profiles (calculated both as absolute amounts and proportion of total body sterol) were analyzed using MANOVA. All analysis was performed in JMP v 11 (SAS Institute Inc.).

2.3 Results

2.3.1 Larval performance

Four larval performance traits were measured across diets: 1) survival to pupation, 2) developmental time to pupation, 3) pupal mass, and 4) growth rate. Only one individual pupated on the no sterol diet (n=270) and was therefore excluded from analysis.

Pupation success was significantly affected by sterol type. Pupation success was highest on cholesterol and significantly reduced on sitosterol, stigmasterol, and ergosterol (Fig. 2.2a, Table 2.1a). There was also a sterol-by-concentration interaction. At low concentrations, pupation success was similar among sterol treatments. As concentrations approached 0.8 g/L and higher, pupation success on cholesterol significantly increased while sitosterol, stigmasterol, and ergosterol decreased. Individuals reared on 1.6 g/L and 3.2 g/L cholesterol diets had over 50% more individuals pupate than those on sitosterol, stigmasterol, and ergosterol diets. Pupal success was lowest on ergosterol for all treatments except 0.2 g/L.

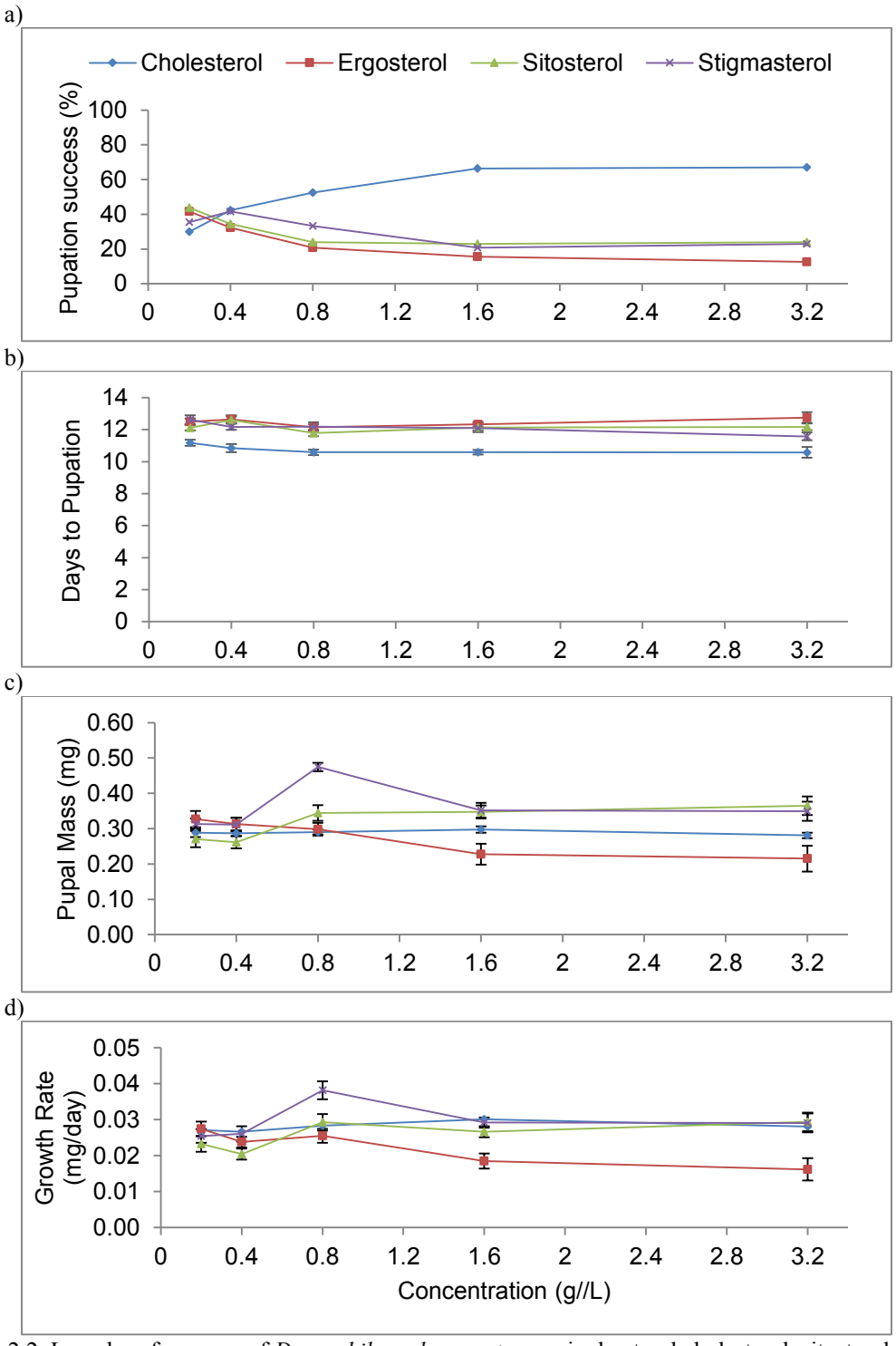


Figure 2.2. Larval performance of *Drosophila melanogaster* on single-sterol cholesterol, sitosterol, stigmasterol, and ergosterol based diets at five concentrations. Pupation success (a), developmental time to pupation (b), pupal mass (c), and growth rate (d) were recorded for each experiment. Pupation success is presented as percent of total. Developmental time, pupal mass, and growth rate are presented as the mean (±SEM). See Table 2.1 for statistical output.

Table 2.1. Statistical analysis for larval traits on single sterol diets.

(a) Pupation success

Source of Variation	df	F-ratio	P-value
Sterol	3	33.04	**<0.001**
Concentration	1	0.49	0.484
Sterol x Concentration	3	83.17	**<0.001**

(b) Time to pupation

Source of Variation	df	X^2-value	P-value
Sterol	3	175.92	**<0.001**
Concentration	1	1.07	0.300
Sterol x Concentration	3	3.25	0.355

(c) Pupal mass

Source of Variation	df	F-ratio	P-value
Sterol	3	11.81	**<0.001**
Concentration	1	0.01	0.837
Sterol x Concentration	3	7.49	**<0.001**

(d) Pupal growth rate

Source of Variation	df	F-ratio	P-value
Sterol	3	8.32	**<0.001**
Concentration	1	0.01	0.880
Sterol x Concentration	3	4.40	**0.002**

Larval developmental time was significantly affected by sterol type, but there was no concentration effect or sterol-by-concentration interaction. Developmental time was fastest on cholesterol but equally slow on sitosterol, stigmasterol, and ergosterol (Fig. 2.2b, Table 2.1b).

Pupal mass was affected by sterol type. Pupal mass was significantly higher on stigmasterol-based diets (Fig. 2.2c, Table 2.1c). There was also a sterol-by-concentration interaction. Pupal mass on cholesterol was consistent across all concentrations. Ergosterol began higher and showed a decrease as concentrations increased. In contrast sitosterol and stigmasterol began low and increased as the concentration increased.

Growth rate, calculated as mass (mg) divided by larval developmental time (days) was also calculated. Growth rate was significantly affected by sterol type. Growth rate on cholesterol and stigmasterol were equally high and were significantly greater than sitosterol and ergosterol diets (Fig. 2.2d, Table 2.1d). There was also a sterol-by-concentration interaction. For cholesterol growth rate gradually increased as concentration increased in contrast to ergosterol which decreased as concentration increased. For stigmasterol, growth rate gradually increased until 0.8g/L and then declined, leveling off to be similar to cholesterol at the highest concentrations. The growth rate of individuals reared on cholesterol were significantly different from individuals reared on both sitosterol and ergosterol diets.

2.3.2 Pupal performance

Adult performance was measured by four traits: 1) eclosion success from hatch, 2) eclosion success from pupation, 3) developmental time from hatch to eclosion, and 4) developmental time from pupation to eclosion.

Eclosion success from hatch was significantly affected by sterol type. Eclosion success was highest on cholesterol and significantly reduced on sitosterol, stigmasterol, and ergosterol (Fig. 2.3a, Table 2.2.a). There was also a sterol-by-concentration interaction. At 0.2 g/L, eclosion success was similar among cholesterol, sitosterol, and stigmasterol but as concentrations increased, eclosion success on cholesterol increased significantly and all non-cholesterol diets decreased. Individuals reared on 1.6 g/L and 3.2 g/L cholesterol diets had over 30% more individuals eclose than those on sitosterol, stigmasterol, and ergosterol diets. Eclosion success was significantly reduced on all ergosterol based diets and no pupae eclosed on ergosterol at 1.6 g/L or 3.2 g/L.

Developmental time from hatch was significantly affected by sterol type, but there was no concentration effect or sterol-by-concentration interaction. Developmental time was fastest on cholesterol but equally slow on sitosterol, stigmasterol, and ergosterol (Fig. 2.3b, Table 2.2b). Developmental data is unavailable for individuals on the 1.6 g/L and 3.2 g/L ergosterol diets because no pupae successfully eclosed on these diets.

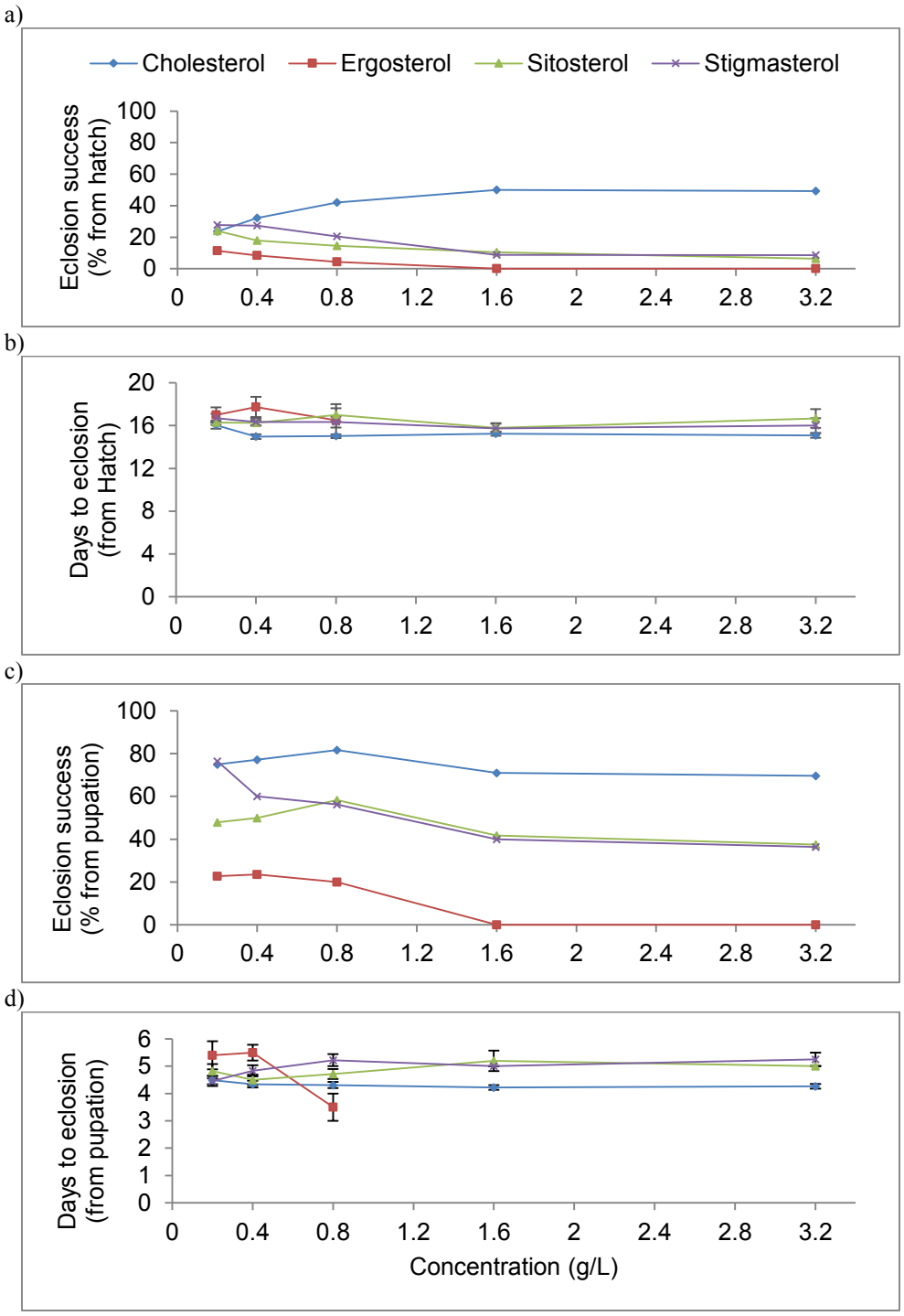


Figure 2.3. Pupal performance of *Drosophila melanogaster* on single-sterol cholesterol, sitosterol, stigmasterol, and ergosterol based diets at five concentrations. Eclosion success from hatch (a), developmental time from hatch (b), eclosion success from pupation (c), and developmental time from pupation (d) were recorded for each experiment. Eclosion success (a, b) is presented as percent of total. Developmental times (c, d) are presented as the mean (±SEM). See Table 2.2 for statistical output.

Table 2.2. Statistical analysis for pupal traits on single sterol diets.

(a) Eclosion success (from hatch)

Source of Variation	df	F-ratio	P-value
Sterol	3	34.54	**<0.001**
Concentration	1	8.37	0.084
Sterol x Concentration	3	1.09	**<0.001**

(b) Time to eclosion (from hatch)

Source of Variation	df	X^2-value	P-value
Sterol	3	9.19	**0.027**
Concentration	1	0.07	0.791
Sterol x Concentration	3	0.82	0.844

(c) Eclosion success (from pupation)

Source of Variation	df	F-ratio	P-value
Sterol	3	34.54	**<0.001**
Concentration	1	8.37	0.084
Sterol x Concentration	3	1.09	**<0.001**

(d) Time to eclosion (from pupation)

Source of Variation	df	X^2-value	P-value
Sterol	3	11.95	**<0.001**
Concentration	1	6.37	**0.013**
Sterol x Concentration	3	4.46	**0.016**

Eclosion success from pupation was significantly affected by sterol type. Eclosion success was highest on cholesterol and significantly reduced on sitosterol, stigmasterol and ergosterol diets (Fig. 2.3c, Table 2.2c). There was also a sterol-by-concentration interaction. At all concentrations eclosion success was highest on cholesterol and lowest on ergosterol. At low concentrations stigmasterol had a higher eclosion success than sitosterol but as concentrations increased sitosterol and stigmasterol exhibited similar eclosion success. Eclosion success was significantly reduced on all ergosterol based diets.

Developmental time to eclosion from pupation was significantly affected by sterol type and concentration (Fig. 2.3d, Table 2.2d). Individuals reared on cholesterol were significantly different than stigmasterol and sitosterol. Individuals reared on stigmasterol were also significantly different than ergosterol. There was also a sterol-by-concentration interaction. Developmental time on cholesterol was consistent at all concentrations. At low concentrations (<0.8 g/L) ergosterol demonstrated an increased developmental time.

2.3.3 Sterol Profiles

The relative amount of total body sterol and percent body sterol varied among treatments (Fig 2.4a-d). The total sterol content and percent body sterol was consistent across all concentrations on both cholesterol (Fig. 2.4a, Table 2.3a) and ergosterol (Fig. 2.4d, Table 2.3a) The relative amount of total sterol content of individuals reared on sitosterol were significantly affected by concentration (Table 2.3a) and increased as concentration

increased (Fig. 2.4b) however the percent body sterol was consistent across concentrations (Fig. 2.4b, Table 2.3b). The relative amount of total sterol recovered from individuals reared on stigmasterol was consistent across concentrations, with the exception of 0.4g/L (Fig. 2.4c, Table 2.3a) however the percent body sterol remained consistent across concentrations (Fig 2.4c, Table 2.3b).

Cholesterol was recovered from all samples (Figure 2.4e-h). On the cholesterol treatment, cholesterol was the dominant sterol recovered at all concentrations (Fig. 2.4e). At low concentrations of cholesterol, stigmasterol and sitosterol were recovered in addition to cholesterol. As concentrations increased, only trace amounts of phytosterols were recovered and at the highest concentration only cholesterol was recovered (Fig 2.4e, Table 2.3c). Similarly, on sitosterol and stigmasterol the dominant sterol recovered at all concentrations was sitosterol (Fig. 2.4f) and stigmasterol (Fig. 2.4g), respectively. Concentration was significantly affected by the relative sterol profile for individuals reared on sitosterol (Table 2.3c). The relative amount of sterols recovered at 0.2g/L was significantly less than that recovered at higher concentrations. Individuals on stigmasterol were also significantly affected by concentration (Table 2.3c). A decline in the relative sterol profile was observed at 0.4g/L. There was a significant concentration effect for the relative body sterol profile of individuals reared on ergosterol (Table 2.3c). Cholesterol was recovered at all concentrations but ergosterol was undetectable at 0.2g/L and only trace amounts were observed at 0.8g/L (Fig. 2.4h). At 3.2g/L nearly equal amounts of cholesterol and ergosterol were present in the diet at values more than double that of the lower concentrations.

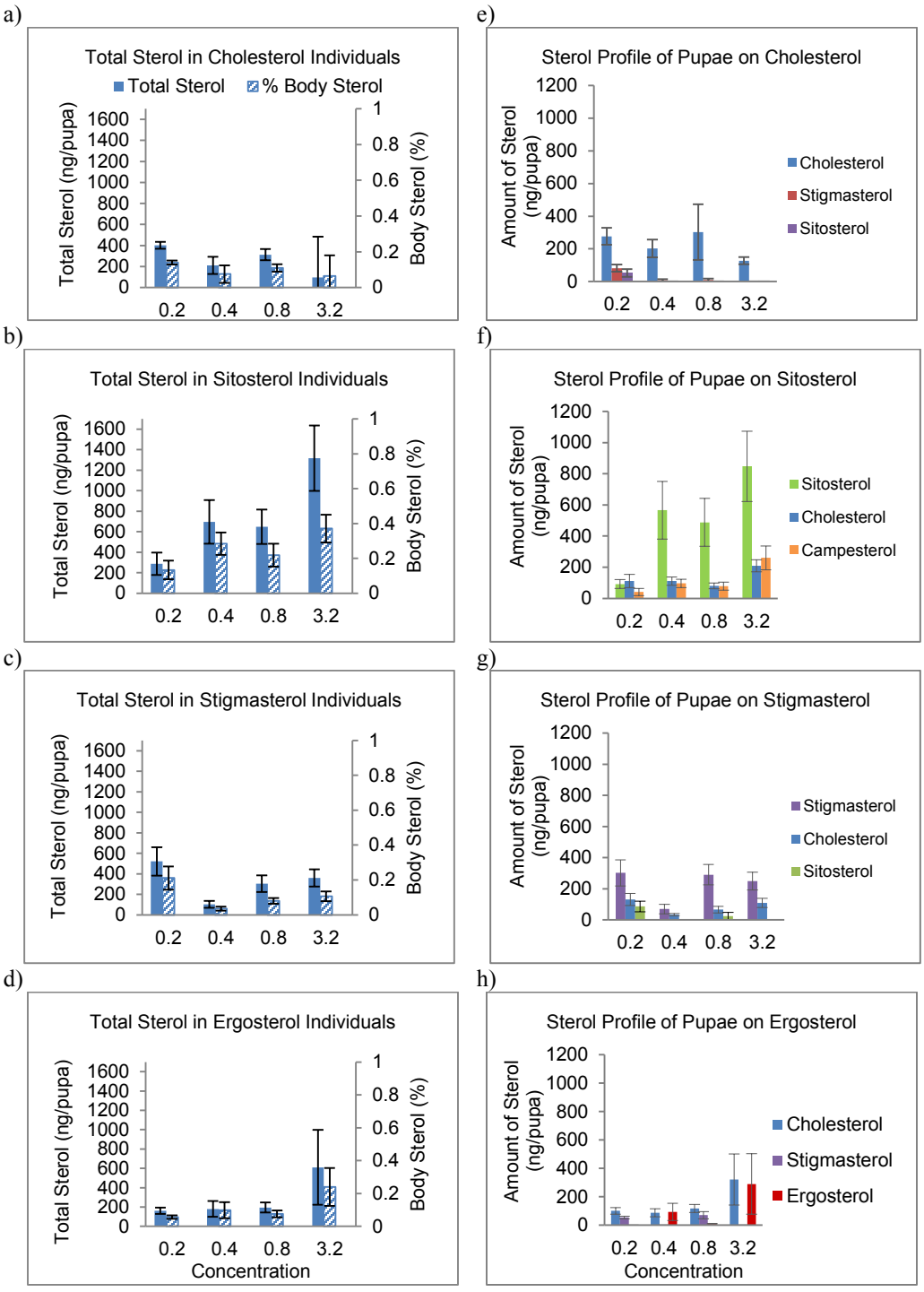


Figure 2.4. Mean (±SEM) total body sterol of *D. melanogaster* pupae that had been reared as larvae on single-sterol cholesterol, sitosterol, stigmasterol, and ergosterol diets at four different concentrations. Panels a-d show the sum of all sterols found in *Drosophila* pupae as well as the percent of their body that is sterol. See Table 2.3a,b for statistical output. Panels e-h show the total amount (ng) of the three dominant sterols found in *Drosophila* pupae for a given diet. See Table 2.3 for statistical output.

Table 2.3. Statistical analysis for sterol profile.

(a) Total sterol

Sterol	df	F-ratio	P-value
Cholesterol	3	1.41	0.257
Sitosterol	3	2.91	**0.049**
Stigmasterol	3	4.08	**0.015**
Ergosterol	3	1.74	0.183

(b) Percent body sterol

Sterol	df	F-ratio	P-value
Cholesterol	3	1.01	0.400
Sitosterol	3	2.16	0.112
Stigmasterol	3	3.79	**0.020**
Ergosterol	3	2.71	0.065

(c) Relative body sterol profile

Sterol	df	F-ratio	P-value
Cholesterol	9	2.36	**0.019**
Sitosterol	9	2.29	**0.023**
Stigmasterol	9	2.80	**0.006**
Ergosterol	9	2.30	**0.023**

(d) Percent sterol profile

Sterol	df	F-ratio	P-value
Cholesterol	9	4.99	**<0.001**
Sitosterol	9	5.80	<**0.001**
Stigmasterol	9	3.02	**0.015**
Ergosterol	9	4.53	<**0.001**

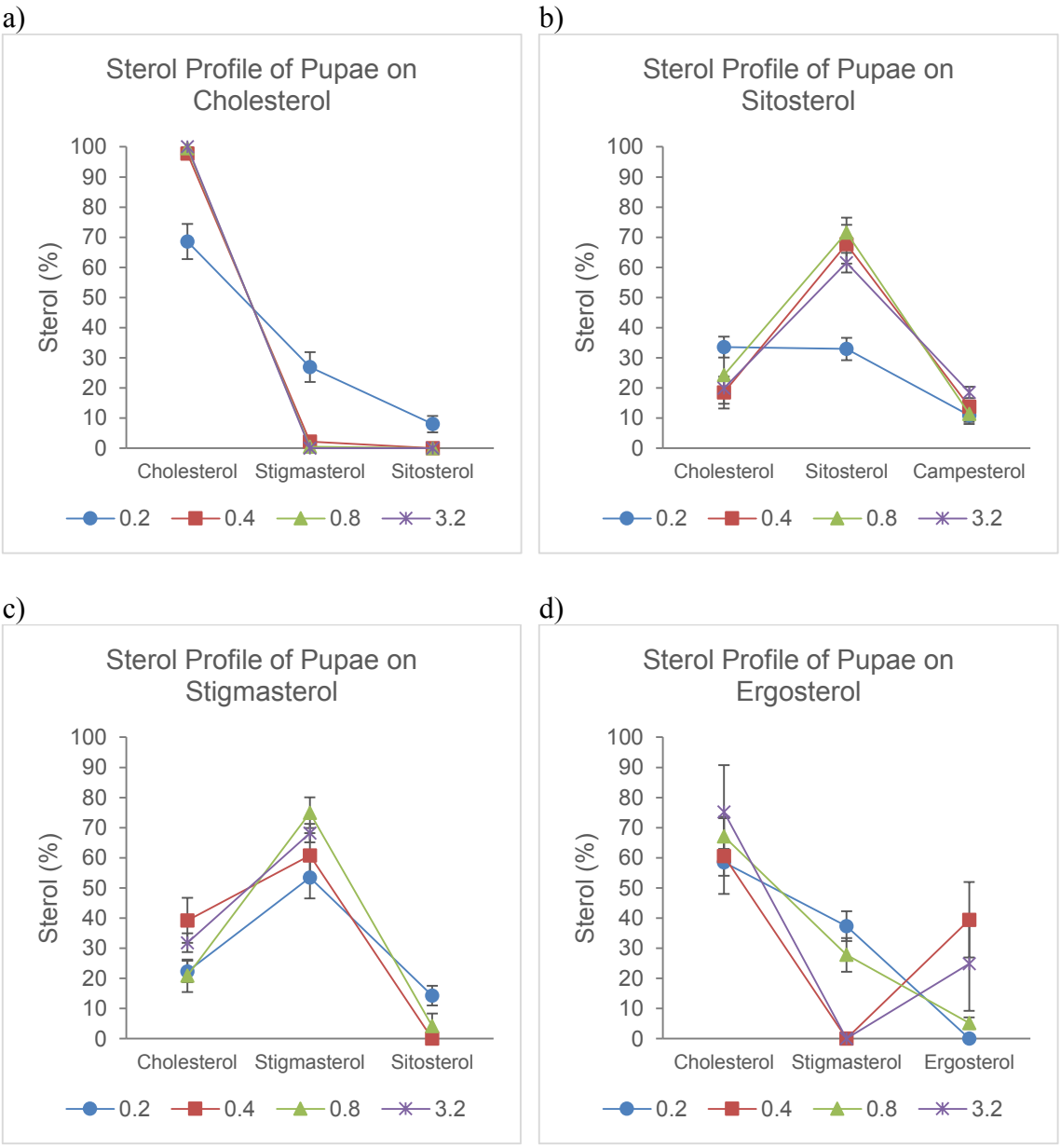


Figure 2.5. Mean (±SEM) sterol profiles of *D. melanogaster* pupae that were reared as larvae on single-sterol cholesterol (a), sitosterol (b), stigmasterol (c), and ergosterol (d) diets at four different concentrations. Data are expressed as the percent of each sterol found in pupae at each concentration. See Table 2.3 for statistical output.

The percent sterol profile of each concentration was also determined for each of the dietary sterols (Fig. 2.5a-d). Percent body sterol, calculated as amount of sterol divided by the pupal mass, was determined for the three major sterols recovered. The percent of cholesterol in the diet increased as the concentration of cholesterol increased (Fig. 2.5a, Table 2.3d). There was a significant concentration effect for individuals reared on sitosterol (Table 2.3d). At the lowest concentration, cholesterol and sitosterol were both present at approximately 32%. As concentrations increased the percent body cholesterol decreased to around 20% and the concentration of sitosterol increased to more than 60% (Fig. 2.5b). Individuals reared on stigmasterol were also significantly affected by concentration (Table 2.3d). At the lowest concentration, sitosterol was reported at 15% in the sterol profile. As concentrations increased sitosterol was reduced to less than 5%. As concentrations increased, individuals began accumulating more stigmasterol (Fig. 2.5c). Individuals reared on ergosterol diets were also significantly affected by concentration (Table 2.3d). Cholesterol was the dominant sterol reported in the profile at all concentrations and increased as concentration increased (Fig. 2.5d). Ergosterol was only detected at concentrations of 0.4g/L and higher.

2.4 Discussion

In nature, *Drosophila* larvae develop on rotting fruit that contains a mixture of plant and fungal sterols. Additionally, they have access to some animal sterols because *Drosophila* larvae are known to eat conspecifics that die during development (Vijendravarma et al. 2013). In the lab, *Drosophila* larvae have access to this same suite of sterols. Standard

Drosophila medium contains a mixture of four sterols: cholesterol, ergosterol, stigmasterol, and sitosterol. Approximately 75% of dietary sterol is ergosterol while less than 1% is cholesterol. Phytosterols constitute the remaining dietary sterol with approximately 19% sitosterol and 5% stigmasterol. Until recently, variability in *Drosophila* diets and mass-rearing techniques made it challenging to study sterols in *Drosophila.* Previous studies with *D. melanogaster* used lipid extraction techniques to extract lipids from the diet medium (Goodnight and Kircher 1971, Kircher and Gray 1978, Kircher et al. 1984, Svoboda et al. 1989, Feldlaufer et al. 1995, Carvalho et al. 2010). In Carvalho et al. (2010) lipids were extracted but non-sterol lipids were not reconstituted into the diet as described in Behmer and Grebenok (1998). The introduction of a synthetic diet by Piper et al. (2014) has allowed for precise control of dietary sterols and concentration of sterols in the diet. Additionally, Carvalho et al. (2010) introduced individual rearing to eliminate sterol acquisition through cannibalism and ensure identical nutrition for each individual.

Our use of the Piper holidic diet confirmed the requirement of sterols for *Drosophila*, as only 1 of the 270 eggs that hatched on the no sterol treatment pupated. This individual did not, however, eclose. The remaining individuals arrested development with the first and second instars as seen in Carvalho et al. (2010). The absence of cholesterol, the necessary precursor for Ecdysteroid production, interferes with normal hormone pulses regulating molting between larval instars (Gilbert et al. 2002, Warren et al. 2002). Sterol analysis of the diets confirmed the sterol content of the diets truly matched the sterol treatments. The one exception was the sitosterol treatment,

which contained sitosterol and campesterol (90% and 10%, respectively). However, this was expected due to the purity of sitosterol (<70%, purchased from Sigma Chem).

While less than 1% of dietary sterol in standard *Drosophila* medium is cholesterol, performance was best on cholesterol only diets. Individuals reared on cholesterol had greater pupal survival and quicker developmental times. As the concentration of dietary cholesterol increased, so did the number of individuals surviving to pupation. At the lowest concentrations, less than 25% of individuals reached pupation. As concentration reached 0.8g/L, survival increased to 50%. At the highest concentrations survival to pupation approached 70%. Increased concentrations of dietary cholesterol also reduced the developmental time required to reach pupation. Developmental time to pupation was significantly faster for cholesterol compared to sitosterol, stigmasterol and ergosterol treatments, however at low concentrations development was delayed and the time to pupation increased. Pupal mass remained consistent across all concentrations of cholesterol, however growth rate demonstrated a slight upward trend as the concentration of dietary cholesterol increased.

Based on the number of individuals to hatch, survival to eclosion peaked at 50% on cholesterol diets. As the concentration of dietary cholesterol increased, so did the number of individuals surviving to eclosion. At low concentrations, eclosion success was similar among cholesterol and the phytosterols but as concentrations increased so did the number of individuals eclosing. Insects, including *Drosophila* have a metabolic and structural need for sterols. Cholesterol is the known precursor to steroid molting hormones including Ecdysone and 20-hydroxyecdysone (Nation 2008) that are important

for *Drosophila* larval molting and metamorphosis (Warren et al. 2002, Spindler et al. 2009) Since larvae can arrest development before pupation, eclosion success was also calculated from the percent of individuals that pupated. The pupal stage is a highly active metabolic period in which no additional feeding occurs. Pupation is initiated by an ecdysone pulse at the end of the third larval instar (Riddiford 1993) and a series of ecdysone pulses drive development throughout the pupal stage (Figure 2.6). The trend demonstrated by individuals eclosing after pupation is different than the trend seen based on the hatching success. As the concentration of dietary cholesterol increased from 0.2g/L to 0.8g/L, so did the percentage of individuals surviving to eclosion. Survival to eclosion (from pupation) peaked at 80% at 0.8g/L. As concentrations increased from 0.8g/L to 3.2g/L survival to eclosion decreased nearly 10%. Survival was reduced from the almost 100% survival for individuals reared on cholesterol seen in Carvalho et al. (2010). However, given the reduced survival reported by Piper et al. (2014) on *Drosophila* reared on the holidic diet, this was reduction in survival was to be expected.

In addition to steroid hormone requirements, *Drosophila* also requires bulk membrane sterols for development (Carvalho et al. 2010). Cholesterol is the simplest sterol in the *Drosophila* diet with no additional double bonds or alkyl group side chains. Variation in sterol structure can impact membrane packing and have a negative effect on growth and development (Dufourc 2008). Cholesterol has been shown to promote good growth and development in Orthoptera, Blattaria, Hemiptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera (Behmer and Nes 2003).

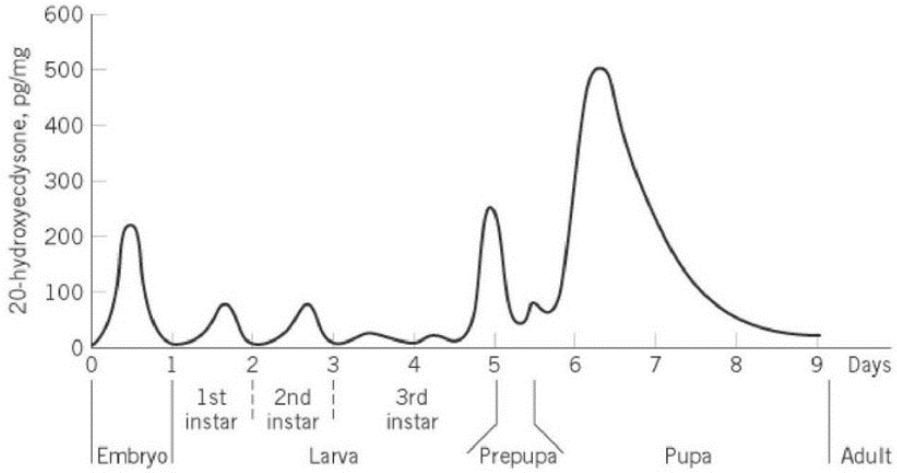


Figure 2.6. Series of ecdysone pulses observed in *Drosophila* during the developmental stages. Adapted from Riddiford, 1993).

The requirement for bulk membrane sterol can be fulfilled by sterols other than cholesterol. Sitosterol and stigmasterol both differ from cholesterol by the addition of an ethyl group at C-24 and stigmasterol differs from cholesterol and sitosterol by the addition of a double bond at C-22. Sitosterol and stigmasterol were found to support *Drosophila* development but to a lesser extent than cholesterol. Survival to pupation was reduced by almost 50% on phytosterols compared to cholesterol at all concentrations except 0.2g/L where survival on the phytosterols was comparable to that of cholesterol. As the concentration of phytosterols in the diet increased, the number of individuals surviving to pupation decreased. Additionally, developmental time was also increased by more than a day compared to individuals reared on cholesterol diets. In general, individuals reared on phytosterols were heavier than individuals reared on cholesterol and pupal mass increased as the concentration of dietary sterol increased. Survival to

eclosion from both hatch and pupation was reduced by 50% or more on phytosterols compared to cholesterol at all concentrations except 0.2g/L, where survival to eclosion on the phytosterols was comparable to that of cholesterol. Interestingly, Carvalho et al. (2010) reported almost 100% survival on individuals fed sitosterol and stigmasterol. Similarly, Cooke and Sang (1970) reported survival was 'as good as, or better' on sitosterol and stigmasterol compared to cholesterol. The results from my study, using a more precise diet, suggest for the first time that common phytosterols (i.e., sitosterol and stigmasterol) are not equivalent to cholesterol in terms of supporting growth and development. Svoboda et al. (1989) previously reported that *Drosophila* were incapable of dealkylating and converting sitosterol to cholesterol.

Even though 75% of dietary sterol profile in the fly diet (Bloomington; Appendix A) is ergosterol, my study demonstrated that as an individual sterol, ergosterol failed to support adult development when present at high concentrations, and only minimally supported adult development at low concentrations. Ergosterol differs from cholesterol by the addition of a methyl group at C-24 and a double bonds at C-22 and C-7. Less than 30% of the individuals reared on ergosterol treatments pupated and less than 5% eclosed. At the lowest concentrations, survival to pupation was similar to individuals reared on cholesterol. In contrast to cholesterol, as the concentration of ergosterol in the diet increased, the number of individuals surviving to pupation decreased. Similar to the phytosterols, increasing concentration of ergosterol increased the developmental time to pupation. In contrast to the other dietary sterols, the pupal mass and growth rate of individuals reared on ergosterol decreased as dietary sterol concentration increased. The

majority of individuals reared on ergosterol arrested development in the third larval instar as seen in Carvalho et al (2010). In contrast, Cooke and Sang (1970) reported development and survival on ergosterol as being 'as good as, or better than' cholesterol. The inability to define the diet in Cooke and Sang (1970) questions the extent to which cholesterol was truly absent from these diets. Survival to eclosion from both hatch and pupation was significantly reduced on ergosterol from both cholesterol and the phytosterols. At low concentrations (≤0.8g/L) approximately 20% of the individuals that pupated, eclosed. While pupation was reduced at high concentrations, none of the individuals reared on 1.6g/L or 3.2g/L ergosterol eclosed. The inability of ergosterol to support development at high concentrations questions whether it is a structural or metabolic interference. Additional studies by Carvalho et al. (2010) suggest a metabolic interference given that when ergosterol is supplemented with minimal amounts of cholesterol 84% survival is observed.

Cholesterol was found in the body sterol profile of all individuals, even those reared on single sterol sitosterol, stigmasterol, and ergosterol diets. The relative and percent body sterol was consistent across concentrations of cholesterol. However, at low concentrations peaks of sitosterol and stigmasterol were also reported even though the diet contained only cholesterol. Similarly, Feldlaufer et al. (1995) also reported traces of sitosterol in addition to traces of campesterol in pupal sterols from individuals reared only on cholesterol. In the current experiment, embryos were collected from *Drosophila* reared on stock diets containing the aforementioned combination of sterols: ergosterol, sitosterol, stigmasterol, and cholesterol which favor support for a supply of sterols

deposited maternally as reported by Niwa and Niwa (2011). However as concentrations increased only small amounts of these phytosterols were recovered from select individuals and at 3.2g/L only cholesterol was recovered.

For individuals reared on sitosterol and stigmasterol, that phytosterol was generally the dominant sterol found in the body sterol profile. Cholesterol was present at all concentrations and at low concentrations there is support for a maternal deposit of sterols but at higher concentrations this could suggest the potential for conversion of phytosterols to other metabolites in small quantities undetected by GC-MS. The amount of cholesterol recovered from pupae reared on 3.2g/L sitosterol was nearly double that of the lower concentrations. Svoboda et al. (1989) reported less than 1% of the sitosterol ingested was converted to cholesterol. The relative and percent body sterol profile for individuals was consistent across 0.2g/l, 0.8g/L and 1.6g/L but for unexplainable reasons, both the relative and percent body sterol of individuals reared on 0.4g/L stigmasterol were reduced compared to the other concentrations. It is surprising that with such consistency across concentrations that such a decline in survival to both pupation and eclosion was observed as concentration increased on stigmasterol treatments.

Individuals reared on ergosterol were unique in that ergosterol was minimally detectable on the low concentrations. It is interesting to note that the percent body sterol of individuals to pupate was lowest at 0.2g/L when survival to both pupation and eclosion were the highest. As the amount of total sterol began accumulating in the body, survival to pupation was reduced and at the highest concentrations eclosion was unsuccessful. Ergosterol began accumulating at concentrations of 0.4g/L and higher, at

which a decline in survival was observed. When the amount of ergosterol in the body sterol profile was highest at 3.2g/L with an average of 290ng, few individuals pupated and no individuals eclosed.

In conclusion, this study has demonstrated the extent to which 1) variation in sterol structure and 2) dietary sterol concentration affects *Drosophila* growth, development, and survival when individually reared on a holidic diet. Sterol profile analysis of pupae revealed cholesterol was present at all concentrations regardless of dietary sterol. Overall performance was far superior on single-sterol cholesterol diets– survival to both pupation and cholesterol were greater and developmental time was reduced. Performance was significantly reduced on ergosterol diets and with increased developmental time to pupation and decreased pupation and eclosion success. This experiment also confirms the dietary need for sterols to promote growth and survival, as only 1 out of 270 individuals pupated in the absence of dietary sterol.

CHAPTER III

THE EFFECTS OF MIXED STEROL DIETS ON THE GROWTH AND DEVELOPMENT OF *DROSOPHILA MELANOGASTER*

3.1 Introduction

Sterols serve important structural and metabolic roles in all eukaryotes including insects. Sterols are important components of cellular membranes and precursors to hormones regulating insect molting. Insects are incapable of synthesizing sterols *de novo* (Clark and Block 1959) and must obtain sterols through a dietary source (Hobson 1935, Clayton 1964, Svoboda and Thompson 1985). Cholesterol has been shown to promote good growth and development in Orthoptera, Coleoptera, Dictyoptera, Diptera, Siphonaptera, Lepidoptera, and Hymenoptera (Behmer and Nes, 2003). Cholesterol is the dominant tissue sterol produced in vertebrates and invertebrates, however its limited production in plants means that plant feeding insects must metabolize phytosterols to cholesterol. *Drosophila* feed on rotting fruits, which contain both plant sterols and fungal sterols. However, *Drosophila* are incapable of dealkylating the common phytosterol sitosterol to cholesterol (Svoboda et al. 1989).

Standard *Drosophila* diet (Bloomington; Appendix A) contains a mixture of four sterols: cholesterol, ergosterol, stigmasterol, and sitosterol all with slightly varying structural configurations (Figure 3.1). Approximately 75% of dietary sterol is ergosterol (fungal in origin) while less than 1% is cholesterol (Figure 3.2a). Results from a previous experiment (Chapter II) revealed that as an individual sterol, ergosterol failed to

support good growth and development whereas single-sterol cholesterol diets promoted good growth and development. The sterol profile of pupae fed on the Bloomington medium contained approximately 4% cholesterol but only 44% ergosterol (Figure 3.2b). This suggests some phytosterol (likely sitosterol) is converted to cholesterol. Additionally, the presence of ergost-7,22 dien and desmosterol in the sterol profile *Drosophila* suggests significant amounts of ergosterol are metabolized.

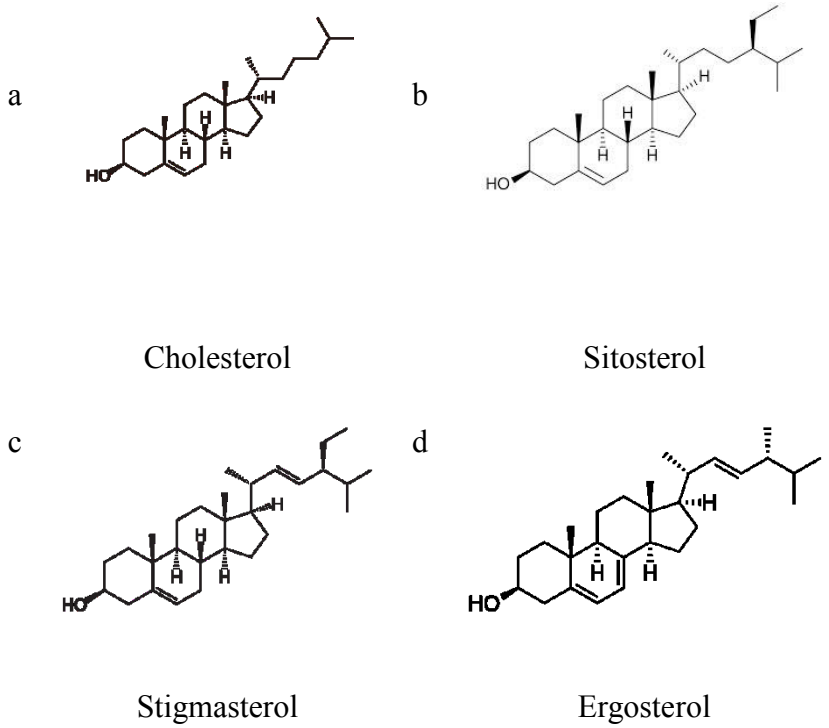
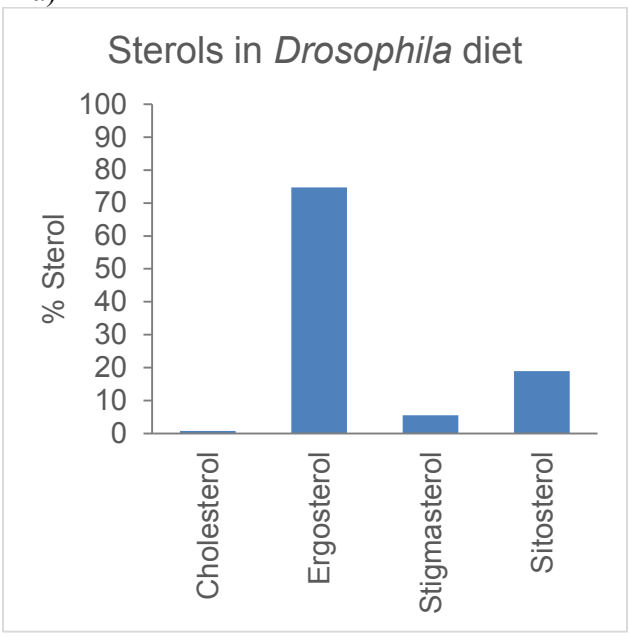


Figure 3.1. The four major sterols found in standard *Drosophila* diet. Cholesterol (a) is the primary sterol found in animals. It is characterized by a 3β hydroxyl group, tetracyclic ring structure, and carbon side chain. Sitosterol (b) and Stigmasterol (c) are plant sterols. Sitosterol differs from cholesterol by the addition of a C-24 ethyl group. Stigmasterol differs from cholesterol by the addition of a C-24 ethyl group and a C-22 double bond. Ergosterol (d) is a fungal sterol, differing from cholesterol by the addition of a C-24 methyl group, C-22 double bond, and a C-7 double bond.

a)



b)

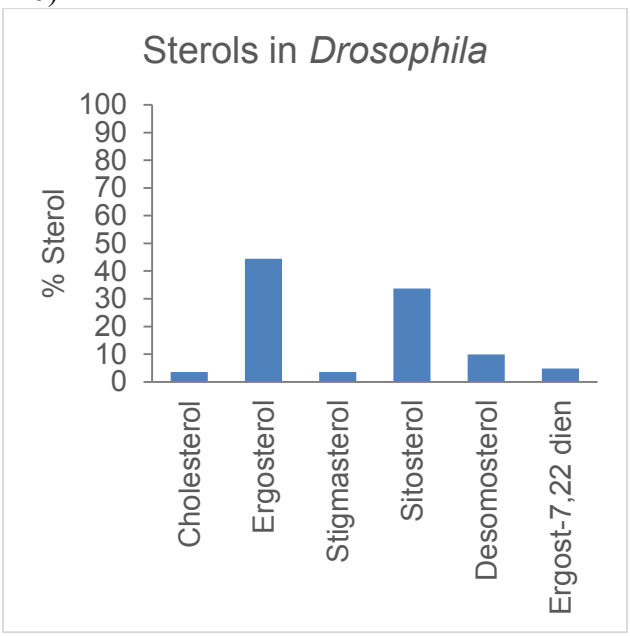


Figure 3.2: Sterol profiles of *Drosophila.* a) Sterols found in standard diet (Appendix A) for *Drosophila*. b) Sterols found in *Drosophila* pupae reared on traditional stock diet.

Insects have both a metabolic and structural requirement for sterols. Cholesterol is a necessary precursor to 20-OH ecdysone, a hormone required for development; however some insects can substitute other sterols in place of cholesterol if cholesterol is lacking in the diet (reviewed by Behmer and Nes 2003). The concept of "sparing" sterols was introduced by Clayton (1964). *D. melanogaster* can complete development on cholesterol-rich diets and diets containing mostly cholestanol with trace cholesterol but not 100% cholestanol or trace cholesterol diets (Kircher and Gray 1978). The ratio of "good" sterols or sterols metabolized to cholesterol, to "bad" sterols or sterols not metabolized to cholesterol, has been shown to impact growth and development in grasshoppers (Behmer and Elias 1999, 2000).Key questions for *Drosophila* are 1) how much sterol do they need? and 2) how does the ratio of "good" to "bad" dietary sterol affect growth and development?

The inability of *Drosophila* to synthesize sterols *de novo* makes them a model organism for studying sterol use and metabolism. The purpose of these experiments is to determine the extent in which sparing occurs in *Drosophila* using the holidic diet medium developed by Piper et al. (2014). The holidic diet medium ensures the diet is void of contaminating sterols and that only the sterols of interest are included in the diet. These experiments will determine how much cholesterol is necessary to support normal growth, development, and survival in *D. melanogaster* when ergosterol is the dominant sterol. The first experiment examines a sterol ratio similar to that found in the standard *Drosophila* medium (Fig. 3.2). Until now, the holidic diet medium has only been used to look at individual sterols at different concentrations. This experiment is the first to

demonstrate individual survival on a mixed sterol diet. Using the holidic diet medium, the second experiment will test different ratios of cholesterol and ergosterol in the diet. This experiment will demonstrate how sterol ratios can affect *D. melanogaster* at both larval and pupal stages.

3.2 Materials and Methods

3.2.1 Drosophila strains and maintenance

Wild-type Canton-S obtained from stocks maintained in the Tarone laboratory in the Department of Entomology at Texas A&M University were used for these experiments. *Drosophila* stocks were maintained at room temperature (23 °C) and cultured on standard cornmeal-yeast medium (Appendix A).

3.2.2 Experimental diets

The holidic diet developed by Piper et al. (2014) was used for these experiments with slight modifications (Appendix B). This diet allowed complete control of dietary sterol type and concentration while ensuring all other dietary components remained constant. Chloroform was used to solubilize all sterols instead of the ethanol and allowed to evaporate for 24 hours before mixing the diet. The vitamin solution was quadrupled from the original recipe. The four sterols used were (1) Cholesterol (Sigma, ≥95%); (2) Sitosterol (Sigma, ≥70%; impurities: campesterol and B-sitostanol, residual); (3) Stigmasterol (Sigma, ≥95%); and (4) Ergosterol (Sigma, ≥95%).

For the first experiment, a diet containing all four sterols was created to replicate the sterol profile of the standard *Drosophila* medium. The diet designed to replicate the standard *Drosophila* medium contained ergosterol (E), sitosterol (S), stigmasterol (T), and cholesterol (C) in a ratio of 75E:19S:5T:1C added at a total concentration of 1g/L. The standard *Drosophila* medium was also tested in this experiment.

For the second experiment, five mixed sterol diets were tested containing cholesterol (C) and ergosterol (E) at various ratios: (1) 25C:75E, (2) 5C:95E, (3) 1C:99E, (4) 0.5C:99.5E, and (5) 0.01C:99.9E added at a total concentration of 1g/L. The next two diets contained only a single sterol, either cholesterol (100C) or ergosterol (100E) added at a concentration of 1g/L to serve as the positive and negative controls, respectively.

3.2.3 Experimental design

Embryos were collected on apple juice/agar plates with yeast paste for 4 hours over two consecutive days. Individual embryos were transferred to a 48-well plate, with each well containing ~60µl of diet and maintained in an incubator (Model # I-66VL, Percival Scientific, Inc Perry, IA, USA) at 25±1°C with a 12h:12h light-dark cycle.

Two plates were established for each experimental diet with three replicates. One of these plates was used to collect data on pupation (including pupation success, time to pupation, and pupal mass). Once data was recorded for the larvae that successfully pupated, they were frozen and set aside for sterol analysis (see below). The second plate contained individuals that were monitored for eclosion success and time to eclosion.

Upon pupation, these individuals were removed from the well plates and transferred to 1.5mL Eppendorf tubes and monitored for eclosion. Over the first 48 hours of the experiment each well on each plate was checked to determine the hatching success of the embryos; wells with embryos that did not hatch were excluded from the analysis. Thereafter larvae were observed daily to record mortality, pupation and eclosion.

3.2.4 Data analysis

Analysis was made in regard to: survival to pupation, survival through eclosion, time to pupation, time to eclosion, pupal mass, and sterol composition. Survival to both pupation and eclosion was scored as a percent of individuals hatched on each diet and analyzed using Logistic Regression. Developmental time to pupation and eclosion was analyzed using Survival Analysis. ANOVA was used in conjunction with Tukey post-hoc tests to analyze pupal mass and growth rate between comparisons. All analysis was performed in JMP v 11 (SAS Institute Inc.).

3.3 Results

Experiment 1 compared the traditional stock diet (Bloomington) and a holidic diet (Piper) designed to replicate sterol ratios found in the stock diet. Experiment 2 compared five mixed sterol diets that contained cholesterol (C) and ergosterol (E) at various ratios: (1) 25C:75E, (2) 5C:95E, (3) 1C:99E, (4) 0.5C:99.5E and (5) 0.01C:99.9E along with single sterol cholesterol and ergosterol diets. The Piper diet from Experiment 1 was included in the analysis for Experiment 2.

3.3.1. Larval performance

Four larval performance traits were measured across diets: 1) survival to pupation, 2) developmental time to pupation, 3) pupal mass, and 4) growth rate. All analyses have been adjusted to exclude individuals that did not hatch.

Pupation success was significantly affected by diet in both Experiment 1 and 2. In Experiment 1 pupation success was highest on the Bloomington diet and significantly reduced on Piper (Fig. 3.3a, Table 3.1a). Pupation success on Bloomington was nearly 20% greater than Piper. In Experiment 2, pupation success was highest on 25C:75E and significantly different than 100C, 5C:95E, 1C:99E, 0.5C:99.5E, 0.01C:99.9E, and 100E (Fig. 3.4a, Table 3.1a). There was only a 10% difference between Piper and 25C:75E. There was no significant difference between Piper and 100C. Pupation success was significantly reduced on the single sterol ergosterol diet. Pupation success on ergosterol was reduced by more than 50% from 25C:75E and nearly 40% from the single sterol cholesterol diet and Piper. In general, as the ratio of cholesterol in the diet increased from 0.1g/L to 0.25g/L pupation increased from 40% at 0.1g/L to almost 80%.

Larval developmental time was significantly affected by diet in both Experiments 1 and 2. In Experiment 1, the average day of pupation was 4 days faster on the Bloomington diet than on Piper (Fig. 3.3b, Table 3.1b). In Experiment 2, developmental time to pupation was on average fastest on Piper and equally delayed on 100C, 25C:75E, 5C:95E, 1C:99E, 0.5C:99.5E, 0.01C:99.9E. Time to pupation was longest on 100E and delayed from Piper by 2 days (Fig. 3.4b, Table 3.1b).

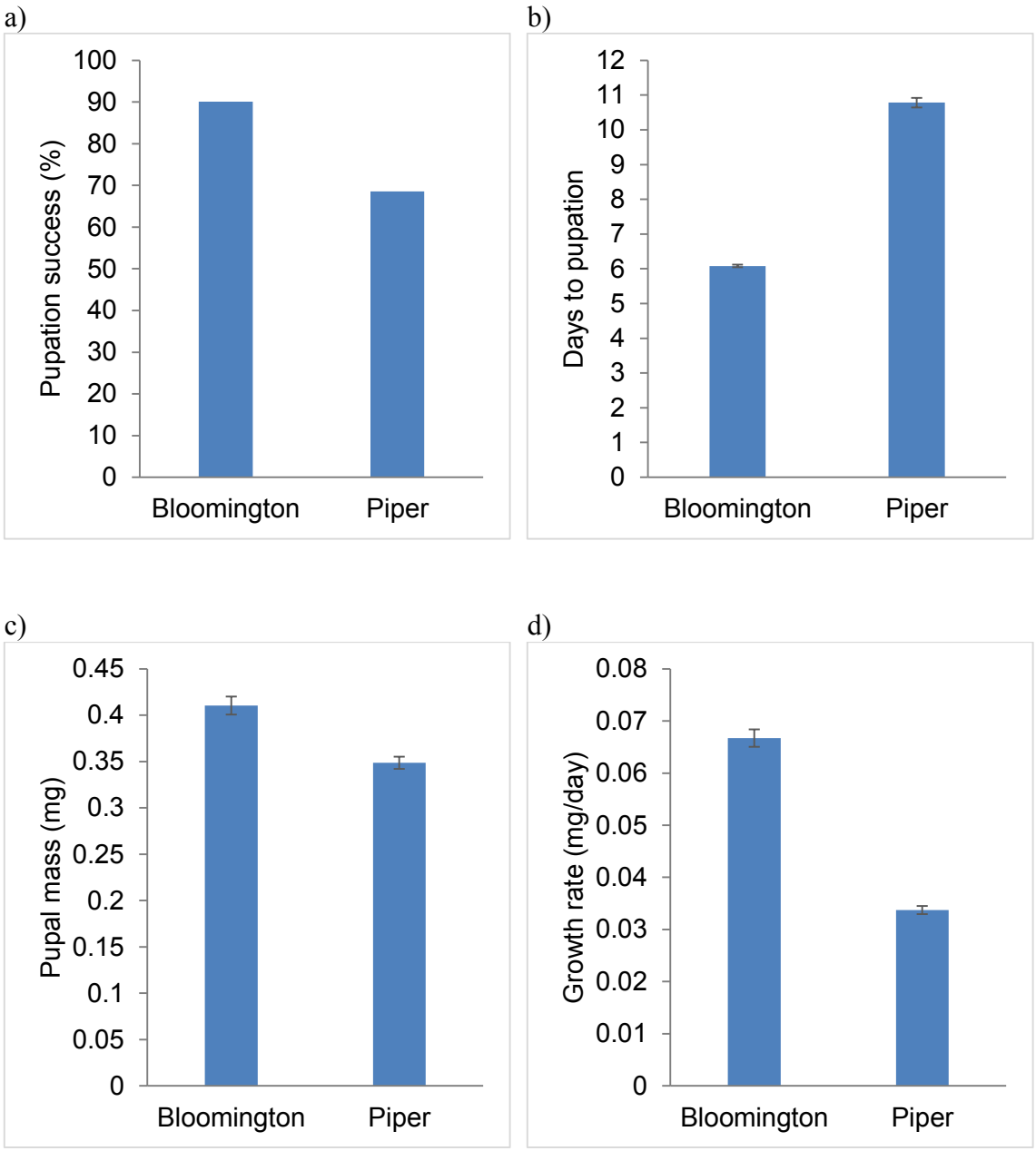


Figure 3.3. Experiment 1 larval performance of *Drosophila melanogaster* on standard stock diet (Bloomington) and a holidic diet mixture replicating sterol ratios found in the stock diet (Piper). Pupation success (a), developmental time to pupation (b), pupal mass (c), and growth rate (d) were recorded for each experiment. Pupation success (a) is presented as percent of total. Developmental time (b), pupal mass (c), and growth rate (d) are presented as the mean (±SEM). See Table 3.1 (Experiment 1) for statistical output.

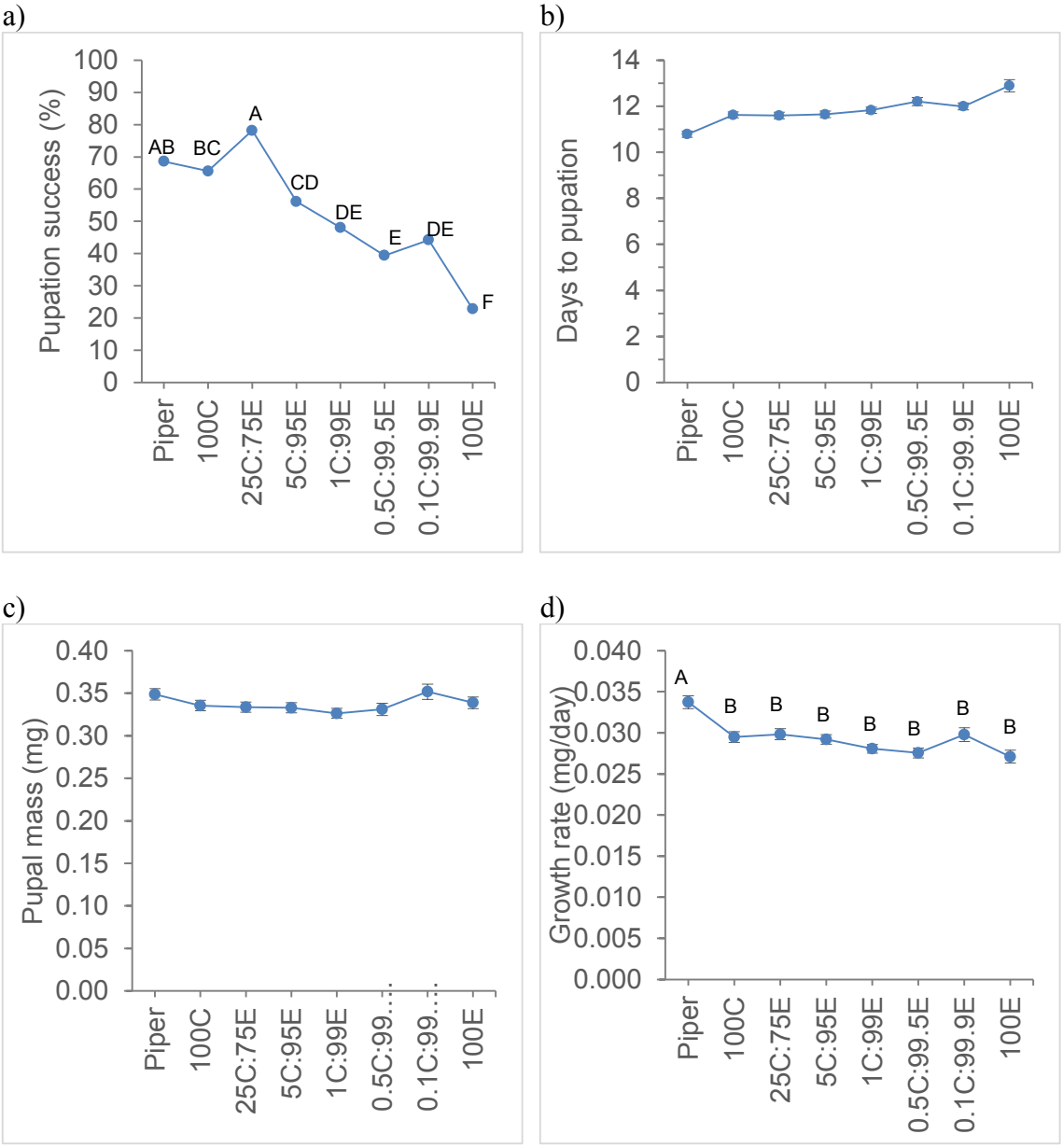


Figure 3.4. Experiment 2 larval performance of *Drosophila melanogaster* on mixed sterol diets. Five different ratios of ergosterol and cholesterol were tested along with single-sterol cholesterol and ergosterol diets. The holidic diet mixture replicating sterol ratios found in stock diets (Piper) was included for comparison. Pupation success (a), developmental time to pupation (b), pupal mass (c), and growth rate (d) were recorded for each experiment. Pupation success (a) is presented as percent of total. Developmental time (b), pupal mass (c), and growth rate (d) are presented as the mean (±SEM). Different letters indicate statistically significant differences between the sterols (α=0.05). See Table 3.1 (Experiment 2) for statistical output.

Table 3.1. Statistical analysis for larval traits on mixed sterol diets.

	Experiment 1			Experiment 2	
(a) Pupation success					
df	F-ratio	P-value	df	F-ratio	P-value
1	42.30	**<0.001**	7	39.81	**<0.001**
(b) Time to pupation					
df	X^2-value	P-value	df	X^2-value	P-value
1	750.02	**<0.001**	7	67.51	**<0.001**
(c) Pupal mass					
df	F-ratio	P-value	df	F-ratio	P-value
1	23.06	**<0.001**	7	1.58	0.138
(d) Larval growth rate					
df	F-ratio	P-value	df	F-ratio	P-value
1	245.58	**<0.001**	7	8.46	**<0.001**

Pupal mass was significantly affected by diet in Experiment 1. Pupal mass was significantly greater on the Bloomington diet than Piper (Fig. 3.3c, Table 3.1c). Individuals on the Bloomington diet were on average 0.05mg heavier than those reared on Piper. Pupal mass was not significantly affected by diet in Experiment 2 (Fig. 3.4c, Table 3.1c).

Growth rate, calculated as mass (mg) divided by larval developmental time (days) was also calculated. Growth rate was significantly affected by diet in both Experiments 1 and 2. In Experiment 1, growth rate was significantly greater on the Bloomington diet. Individuals reared on the stock diet gained nearly twice as much weight per day than individuals on Piper (Fig. 3.3d, Table 3.1d). In Experiment 2, growth rate was significantly greater on Piper (Fig. 3.4d, Table 3.1d). Individuals reared on Piper gained an average of 0.005mg more per day than individuals on all other diets.

3.3.2 Pupal performance

Adult performance was measured by four traits: 1) eclosion success from hatch, 2) developmental time from hatch to eclosion, 3) eclosion success from pupation, and 4) developmental time from pupation to eclosion.

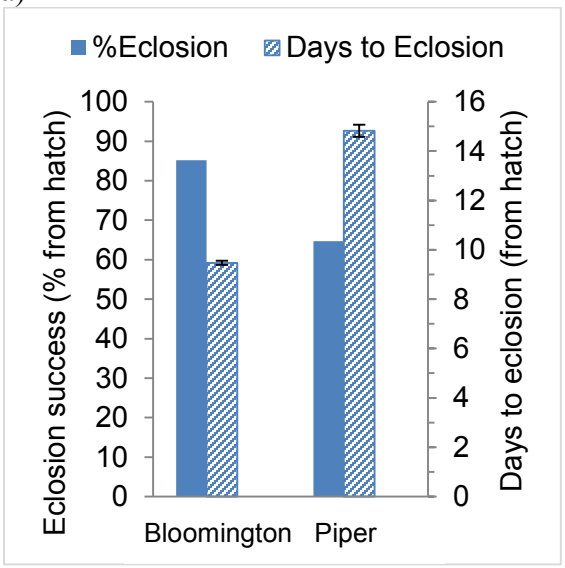
Eclosion success from hatch was significantly affected by diet in both Experiments 1 and 2. In Experiment 1, eclosion success was significantly greater on the Bloomington diet (Fig. 3.5a, Table 3.2a). Eclosion success was reduced on Piper by more than 20%. In Experiment 2, eclosion success was significantly greater on Piper, 100C, and 25C:75E than all other diets (Figure 3.6a, Table 3.2a). As the ratio of dietary

cholesterol increased from 99.9E:0.1C to 75:E25C, so did eclosion success, peaking at 65% on 75E:25C. Eclosion success was slightly reduced on 100C from Piper and 25C:75E, although not significantly different. Eclosion success was significantly reduced on Ergosterol with only 10% successfully eclosing.

Eclosion success from pupation was significantly affected by diet in both Experiments 1 and 2. In Experiment 1, eclosion success from pupation was significantly greater on the Bloomington diet (Fig. 3.5b, Table 3.2c). However, eclosion success on Piper was reduced by less than 10%. In Experiment 2, eclosion success from pupation was highest on Piper. In general, as the amount of dietary cholesterol decreased from 25C:75E to 0.1C:99.9E, eclosion success decreased (Fig. 3.6c, Table 3.2c). Eclosion success was significantly reduced on ergosterol from Piper, 100C, and 25C:75E with only 50% of the individuals reared on the single sterol ergosterol diet eclosing.

Developmental time to eclosion from pupation was significantly affected by diet in both Experiments 1 and 2. In Experiment 1, developmental time to eclosion from pupation was statistically significantly greater on the Piper diet but only differed from Bloomington by an average of 0.2 days (Fig. 3.5d, Table 3.1d). In Experiment 2, developmental time to eclosion from pupation was greatest on single-sterol ergosterol treatments (Fig. 3.6d, Table 3.1d).

a)



b)

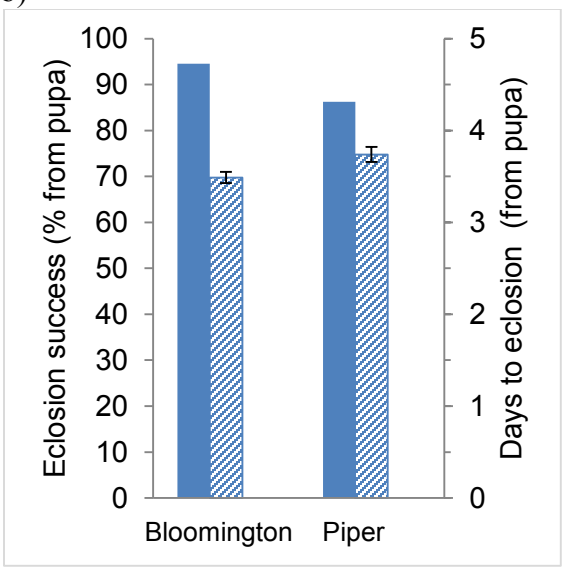


Figure 3.5. Experiment 1 pupal performance of *Drosophila melanogaster* on standard stock diet (Bloomington) and a holidic diet mixture replicating sterol ratios found in the stock diet (Piper). Eclosion success from hatch and developmental time from hatch (a) along with eclosion success from pupation and developmental time from pupation (b) were recorded for each experiment. Eclosion success presented as percent of total. Developmental times are presented as the mean (±SEM). See Table 3.2 (Experiment 1) for statistical output

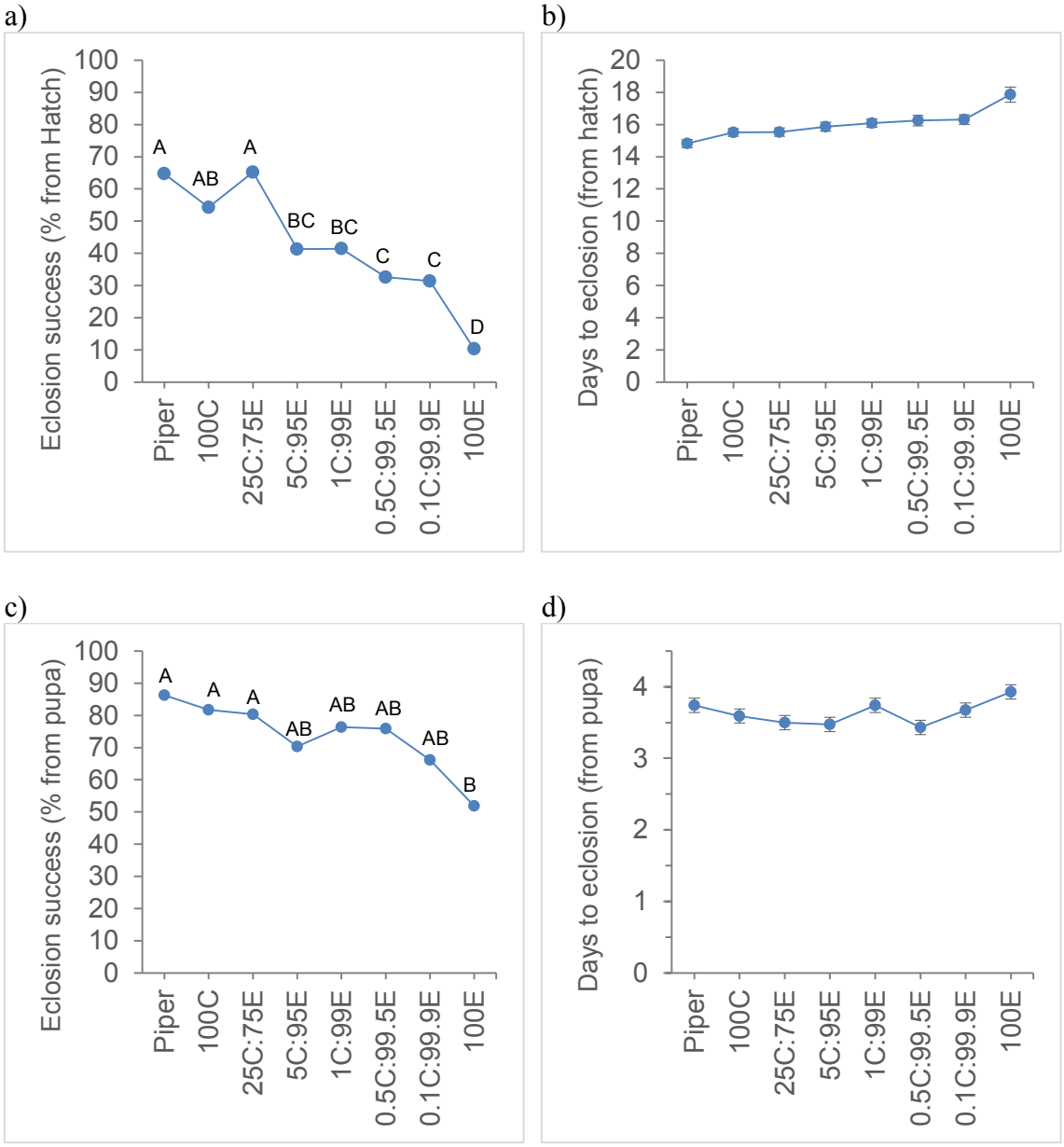


Figure 3.6. Experiment 2 pupal performance of *Drosophila melanogaster* on mixed sterol diets. Five different ratios of ergosterol and cholesterol were tested along with single-sterol cholesterol and ergosterol diets. The holidic diet mixture (1C:5T:19S:75E) replicating sterol ratios found in stock diets was included for comparison. Eclosion success from hatch (a), eclosion success from pupation (b), developmental time from hatch (c), and developmental time from pupation (d) were recorded for each experiment. Eclosion success (a, b) is presented as percent of total. Developmental times (c, d) are presented as the mean (±SEM). Different letters indicate statistically significant differences between the sterols. See Table 3.2 (Experiment 2) for statistical output.

Table 3.2. Statistical analysis for pupal traits on mixed sterol diets.

	Experiment 1			Experiment 2	
(a) Eclosion success (from hatch)					
df	F-ratio	P-value	df	F-ratio	P-value
1	16.47	**<0.001**	7	21.82	**<0.001**
(b) Developmental time to eclosion (from hatch)					
df	F-ratio	P-value	df	F-ratio	P-value
1	332.09	<**0.001**	7	25.00	**<0.001**
(c) Eclosion success (from pupation)					
df	X^2-value	P-value	df	X^2-value	P-value
1	4.72	**0.031**	7	3.38	**0.002**
(d) Developmental time to eclosion (from pupation)					
df	F-ratio	P-value	df	F-ratio	P-value
1	10.57	**0.001**	7	22.22	**0.002**

3.4 Discussion

Drosophila is a model genetic organism but standard rearing approaches hinder our ability to use it as a model for understanding sterol physiology. However, the availability of a new holidic medium for *Drosophila* (Piper et al. 2014) now makes this possible, especially when combined with individual rearing approaches. In Experiment 1 a diet was created (henceforth referred to as the Piper diet) that replicated the sterol profile of the Bloomington diet. Previously the Piper diet has only examined individual sterols, either cholesterol (Piper et al. 2014), or as in Chapter II, a selection of individual sterols; in most cases these individual sterols have be examined at different concentrations. Performance was generally quite good on the Piper diet, but compared to the Bloomington diet, pupal survival was reduced, larval and pupal developmental times were longer, and eclosion success was reduced. Most dramatically, individuals reared on the Piper diet took nearly twice as long to develop and their growth rate was reduced by half compared to individuals reared on the Bloomington diet. However, the percent of individuals eclosing from pupation on the Piper diet was within 10% of the Bloomington diet, indicating the majority of arrest occurred during the larval stage. It is important to know how the mixed sterols in Piper compared to the Bloomington diet to get a baseline for expected survival in subsequent mixed sterol experiments. Piper et al. (2014) reported reduced survival on the holidic diet from standard yeast medium when only cholesterol was present in the diet, so a similar reduction in survival was to be expected. My data, combined with the earlier work comparing the Bloomington diet to the Piper

diet containing cholesterol as the only sterol, suggests that reduced performance is likely the outcome of some non-sterol dietary factor missing from the Piper diet.

Experiment 2 was designed to determine the extent to which sparing occurs in *Drosophila* on diets that contained different ratios of cholesterol and ergosterol. When "good" sterols are limiting, insects may utilize a sterol sparing mechanism. My results indicate when cholesterol is less than 1% of the diet (i.e. 0.5C, 0.1C, and 100E) pupation success is compromised as less than 50% of the individuals on 0.5C and 0.1C pupated and fewer than 25% of the individuals on 100E pupated. Even though ergosterol comprises 75% of the dietary sterol profile in the standard fly diet, as an individual sterol, ergosterol failed to support adult development when present at high concentrations, and only minimally supported adult development at low concentrations (Chapter II). Similarly, these results from Experiment 2 show significantly poorer performance on ergosterol when cholesterol was absent from the diet. However, when ergosterol was combined with cholesterol, survival to pupation was similar to diets containing only cholesterol and in some instances better than cholesterol as observed on the 25C:75E diet. As cholesterol increased from 1C:99E to 25C:75E, survival increased from 50% to nearly 80% (Fig. 3.5a) suggesting the use of a sparing mechanism operates above a particular threshold, as has been seen in grasshoppers (Behmer and Elias 1999, 2000) and caterpillars (Nes et al. 1997, Jing et al. 2014). This threshold appears, however, to be species specific. Total sterols were added to the diet in the amount of 1g/L. This amount was determined to support good growth and development on cholesterol only diets (Chapter II) indicating the structural and metabolic sterol need had

been met. Since this concentration was sufficient to support growth and development, the poor performance on diets with ratios low in cholesterol is likely due to a metabolic deficiency – there is not enough cholesterol available for the production of molting hormone. When cholesterol is limited, non-cholesterol sterols can be incorporated into cell membranes and cholesterol is spared for a metabolic role (Clayton 1964). Similarly, Carvalho et al. (2010) reported an 84% increase in survival when ergosterol was supplemented with minimal amounts of cholesterol.

Survival to pupation on 100C was within the range expected from previous studies with varying concentrations of cholesterol (Chapter II). However, it is interesting to note that when only cholesterol was present in the diet (100C), survival to pupation was significantly reduced compared to the 25C:75E diet (by about 10%). This result suggests multiple sterols are beneficial for *Drosophila* and supports the concept of sparing. When both cholesterol and ergosterol are present in the diet individuals can theoretically incorporate ergosterol into membranes and save cholesterol for metabolic purposes; whereas when only cholesterol is present in the diet, it has to be incorporated for both structural and metabolic uses. Survival to pupation on Piper was reduced from 25C:75E but not significantly different from 25C:75E or 100C. The addition of phytosterols in Piper allows for multiple sterols, not just ergosterol to be used in the cellular membranes. Developmental time was shortest on Piper and was significantly delayed in the absence of cholesterol, but there was no difference between 100C and the five C:E ratios. Growth rate was fastest on Piper, but not significant differences were observed in pupal mass.

Based on the number of individuals to hatch, survival to eclosion was equally high on Piper and 25C:75E at 65%. Interestingly, when only cholesterol was present in the diet (100C), survival to eclosion was reduced from Piper and 25C:75E but within the expected range of survival based on a previous study (Chapter II). As the amount of cholesterol decreased, so did the number of individuals eclosing. When cholesterol was absent from the diet, eclosion success was minimal with only 10% of individuals on 100E eclosing. Cholesterol is the precursor to ecdysone and 20-OH ecdysone (Nation 2008) which are important for larval molting and metamorphosis in *Drosophila* (Warren et al. 2002, Spindler et al. 2009). Simply increasing the ratio of cholesterol in the diet from 0 to 0.1 and 0.5 increased survival by 20%. When cholesterol is deficient, normal hormone pulses that regulate molting can be interrupted (Gilbert et al. 2002). Increasing cholesterol by a factor of 10 from 0.1 and 0.5 increased survival to eclosion by an additional 10%. Since larvae can arrest development prior to pupation, eclosion success was also calculated from the percent of individuals that pupated. The pupal stage is highly active metabolically, and no additional feeding occurs during this stage. An ecdysone pulse at the end of the third larval instar initiates pupation (Riddiford 1993) and a series of ecdysone pulses continue to drive development through to eclosion. Eclosion success was highest on Piper, 100C and 25C:75E with more than 80% of pupa eclosing. Within the range of 5C:95E to 0.1C:99.9C, 70-75% survival was observed. Since eclosion is such a metabolically active period, if there were a strong metabolic component involved here we would expect to see a decline in eclosion success as

cholesterol decreased in the diet. It was only when cholesterol was absent from the diet that a significant drop in eclosion success was observed.

This experiment demonstrated 1) the ability of the Piper diet support larval and pupal development and 2) the sparing capabilities of *Drosophila* when reared on a holidic medium. The Piper diet, although reduced from Bloomington, was still able to support upwards of 85% survival to eclosion from pupation. Cholesterol is a suitable sterol for *Drosophila,* as shown by the results of individuals on the 100C diet. Individual performance, in particular pupation success, decreased as the ratio of suitable sterol increased. No major biological differences were observed in pupal mass or developmental time to pupation and eclosion. Sterols are important in physiological processes including membrane function and steroid hormone production. This experiment confirms *Drosophila* can complete development on diets when the majority of sterol is ergosterol, as long as a small amount of cholesterol is present.

CHAPTER IV

CONCLUSIONS

Drosophila are reared in the laboratory on diets containing varying amounts of cholesterol, sitosterol, stigmasterol, and ergosterol. When these sterols are incorporated as individual sterols at different concentrations into a holidic diet, performance and overall survival are significantly affected. The dietary need for sterols, although previous demonstrated (Clayton 1964, Svoboda and Thompson, 1985, Carvalho et al. 2014), was again proven when only one individual (of 270) pupated but did not eclose on the no sterol diet. Individuals reared on cholesterol only diets exhibited the fastest developmental times to pupation and eclosion from pupation; additionally, overall survival to pupation and eclosion was significantly increased compared to sitosterol, stigmasterol, and ergosterol. Ergosterol comprises 75% of the traditional stock diet, but as an individual sterol it minimally supported adult survival at low concentrations and at high concentrations it repeatedly failed to support adult development (Chapter II and Chapter 3, Experiment 2).

When ergosterol was supplemented with cholesterol in different ratios, survival was dramatically improved and in some instances exceeded that of only cholesterol. No major biological differences were observed in pupal mass or in developmental time to pupation and eclosion; however, survival to pupation was significantly reduced as the ratio of cholesterol increased. Sterols are important for multiple physiological processes including membrane function and production of steroid hormones. Taken as a whole,

these experiments demonstrate the need for "good" sterols in the diet to signal the production of steroid hormones. When cholesterol is limited in the diet, survival is compromised. Cholesterol is the known precursor to ecdysteroids and has been shown to support good growth and development in Orthoptera, Coleoptera, Dictyoptera, Diptera, Siphonaptera, Lepidoptera, and Hymenoptera (Behmer and Nes, 2003). Given that *Drosophila* larvae are traditionally mass reared in groups favors prediction for an allee effect, or a positive relationship between individual fitness and population size. In these experiments, larvae were individually reared thus removing any advantage of group rearing and chance of sterol acquisition through the ingestion of excreted cholesterol and also cannibalism or active scavenging.

The use of transcriptional analysis of *Drosophila* sterol use would provide greater mechanistic insights to these variations in sterol structure and concentration, and improve our understanding where physiological differences could not. Identifying genes expressed when individuals are reared on the various sterols could provide insight into which enzymes are involved in sterol metabolism, homeostasis and steroid-based hormone production. Finally, because insects do not produce sterols, incorporating Ezetimibe, a cholesterol uptake inhibitor, into the diet would provide insight into the uptake of sterols.

Experiments with mutants such as NPC1a or NPC1b and also laboratory-selected *D. melanogaster* lines such as lines selected for large cells, thermal tolerance or stress resistance would provide us with a greater understanding of the effects of sterols incorporated into membranes and also sterols spared for hormone production. Different

species within the *Drosophila* genus would be predicted to have different sterol profiles and potentially different dealkylation abilities.

The sterol profiles of *Drosophila* pupae provided a foundation to begin asking questions about the extent to which *Drosophila* are able to metabolize different sterols. Cholesterol was recovered in the sterol profile of all pupae even when it was absent from the diet. With a few exceptions, total body sterols were relatively constant even across high concentrations indicating sterols are maintained by a form cholesterol homeostasis. Subsequent studies looking at the second generation would remove sterols allocated from the parental generation that was reared on the traditional fly diet and provide a greater understanding of sterol metabolism particularly in the lower concentrations. Studies have been conducted on radiolabeled campesterol and sitosterol (Svoboda et al. 1989). Additional studies using the holidic diet with radiolabeled sitosterol, stigmasterol, and ergosterol would provide insight to the metabolic fate of these dietary sterols. Furthermore, sterol analyses of different larval stages and also of larvae that arrested development at known time during development could also provide greater insight to sterol metabolism.

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APPENDIX A

A listing of ingredients used for making the Piper diet and modifications for the current study (major differences are highlighted using bold text). Ingredients are measured by volume (mL) unless otherwise noted. The amounts presented are to prepare 100mL of diet with 1g/L sterol

Step 1	
Agar (g)	2.000
Sucrose (g)	1.712
Sterol (g)	0.1
Chloroform	10
Step 2	
L-isoleucine (g)	0.116
L-leucine (g)	0.164
L-tyrosine (g)	0.084
Step 3	
Solution 1: $CaCl_2$	0.1
Solution 2: $MgSO_4$	0.1
Solution 3: $CuSO_4$	0.1
Solution 4: $FeSO_4$	0.1
Solution 5: $MnCl_2$	0.1
Solution 6: $ZnSO_4$	0.1
Step 4	
10x Buffer Solution	10.0
125x Nucleic acids/Lipid-related	0.80
Essential amino acid solution	6.051
Non-essential amino acid solution	6.051
Glutamate solution	1.821
Cysteine solution	0.528
71.5x vitamin solution	**5.6**
1000x folic acid solution	0.1
Step 5	
Propionic acid	0.6
Nipagin	1.5

APPENDIX B

A listing of ingredients used for making the standard fly diet (Bloomington). Ingredients are measured by mass (grams) unless otherwise noted. The amounts presented are to prepare 100mL of diet.

Ingredient	Amount
Water (mL)	100
Cornmeal	9.6
Light Corn Syrup (mL)	6.8
Malt Extract	6.7
Yeast	2.7
Soy Flour	2.3
Agar	1.1
Antimicrobial Agents (mL)	1.6