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Evaluating Effects of a Critical Micronutrient (24-Methylenecholesterol) on Honey Bee Physiology

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Abstract

Although poor nutrition is cited as one of the crucial factors in global pollinator decline, the requirements and role of several important nutrients (especially micronutrients) in honey bees are not well understood. Micronutrients, viz. phytosterols, play a physiologically vital role in insects as precursors of important molting hormones and building blocks of cellular membranes. There is a gap in comprehensive understanding of the impacts of dietary sterols on honey bee physiology. In the present study, we investigated the role of 24-methylenecholesterol—a key phytosterol—in honey bee nutritional physiology. Artificial diets with varying concentrations of 24-methylenecholesterol (0%, 0.1%, 0.25%, 0.5%, 0.75%, and 1% dry diet weight) were formulated and fed to honey bees in a laboratory cage experiment. Survival, diet consumption, head protein content, and abdominal lipid contents were significantly higher in dietary sterol-supplemented bees. Our findings provide additional insights regarding the role of this important sterol in honey bee nutritional physiology. The insights gleaned from this study could also advance the understanding of sterol metabolism and regulation in other bee species that are dependent on pollen for sterols, and assist in formulation of a more complete artificial diet for honey bees (*Apis mellifera* Linnaeus, 1758) (Hymenoptera: Apidae).

Key words: honey bee physiology, pollen, honey bee nutrition, 24-methylenecholesterol, phytosterol

Habitat loss, monocultures, and changes in plant flowering phenology are considered the major contributors to poor honey bee nutrition and subsequent honey bee declines (Kremen et al. 2002, Naug 2009, Vanbergen et al. 2013, Otto et al. 2016). Research to date has demonstrated that adequate nutrition is critical for maintaining strong immunity and survival in honey bees. Honey bee colonies that consume sufficient amounts of high-quality pollen are less susceptible to the gut parasite *Nosema ceranae*, have lower pathogen loads, overwinter more successfully, exhibit enhanced immunocompetence, produce drones with better semen quality, and are able to better resist stressors such as parasites, diseases, and pesticides (Alaux et al. 2010, 2011, Brodschneider and Crailsheim 2010, Di Pasquale et al. 2013, Mao et al. 2013, Schmehl et al. 2014, Niño and Jasper 2015, Jack et al. 2016, McMenamin et al. 2016, 2018, Smart et al. 2016). Poor nutrition may alter the physiology of emergent spring workers (Mattila and Otis 2006) and may subsequently influence behavior. In one study, limited nutrition during larval stages produced adults that were inefficient foragers and waggle dancers (Scofield and Mattila 2015). Hence, optimal nutrition may be a colony's first line of defense, enabling it to withstand the effects of both biotic and abiotic stressors.

Pollen and nectar are the two principal sources of macronutrients for honey bees. Carbohydrate-rich nectar supplies bees with

energy, while pollen serves as the primary source of proteins, lipids, vitamins, and vital micronutrients (viz. sterols; Brodschneider and Crailsheim 2010). Micronutrients are critical and yet are understudied in honey bees (Bonoan et al. 2018). Pollen is consumed by nurse bees, whose hypopharyngeal glands biosynthesize the proteinaceous secretions that are progressively provisioned to developing larvae (Knecht and Kaatz 1990, Crailsheim et al. 1992). Hence, understanding the effects of critical micronutrients on nurse bee physiology is important in understanding its long-term implications on colony health. Research on honey bee nutrition is still a poorly developed area (Somerville 2005, Bonoan et al. 2018). Even though bee nutrition has been studied for a significant amount of time, a substantial gap in knowledge exists regarding the physiological impacts of sterols in honey bees.

Sterols play a vital role in insect physiology. They are precursors for molting hormones, act as signaling molecules that influence development, and are critical for cell membrane development and function (Behmer and Nes 2003). All insects are sterol auxotrophs in that they are unable to synthesize sterols, thereby, depending on dietary sources for these important micronutrients (Carvalho et al. 2010). In honey bees, a specific sterol called 24-methylenecholesterol (24MC) has been reported to be the most critical for colony growth

and worker longevity (Herbert et al. 1980), as the researchers observed higher survival of worker bees and greater brood production in 24MC diet treatments. However, Herbert et al. (1980) do not discuss proximate mechanisms resulting in higher survival and brood rearing. Indeed, concentrations of this sterol in pupae were higher than any other sterol (Feldlaufer 1986, Svoboda et al. 1986). Elevated concentrations of this sterol have also been attributed to the formation of ecdysteroids in honey bees (Svoboda et al. 1986). Like all dietary sterols, honey bees obtain it from pollen. Colonies used for crop pollination often face nutritional stress because the quality or quantity of pollen forage available to them in such agricultural landscapes is inadequate (Naug 2009). Hence, it is crucial to understand the nutritional physiology and impacts of this important sterol in honey bees.

In this study, we examined the effects of 24MC on honey bee physiology by measuring a few important physiological parameters. The dietary concentrations of sterols vary among different insect species, but the optimal concentration of sterols appears to be around 0.1% (Clayton 1964, Jing et al. 2013) for better fitness. For species whose sterol requirements are not known, significantly higher concentrations than 0.1% were chosen in some cholesterol-related studies, and diets with higher concentrations of cholesterol, such as 1%, 3%, or even 10%, enhanced insect growth and showed no deleterious effects (Clayton 1964). In addition, plant pollens contain varying concentrations of 24MC (Standifer et al. 1968, Villette et al. 2015, Chakrabarti et al. 2019). In a previous seminal study with honey bees, Herbert et al. (1980) used 0.1% 24MC to examine the effects of this sterol on brood rearing. This study did not provide justification for selecting this specific concentration of 24MC. We speculate that these researchers chose this concentration based on information available in the literature pertaining to sterol needs of other insects. Furthermore, this study only measured effects of 24MC on brood rearing and longevity of bees and did not examine effects on any physiological parameters. Our goal in the present study was to evaluate the effects of 24MC on honey bee physiology and longevity. In our study, artificial diets with varying concentrations of 24MC (ranging from 0 to 1.0%) were formulated and fed to honey bees in a laboratory cage experiment. We chose the concentrations of 24MC to include the concentrations used in

Herbert et al. (1980) and the concentrations deemed to be optimal with respect to longevity, fitness, and growth based on studies with other insects (Clayton 1964, Jing et al. 2013). For each treatment group, we measured the consumption of diet and survival of bees. In addition, we measured abdominal fat content and head protein content as abdominal fat is a crucial indicator of fitness traits in insects, including bees (Amdam et al. 2003, Toth et al. 2005, Arrese and Soulagés 2010) and the head capsule houses major brood food-producing glands in bees (Kucharski and Maleszka 1998). These two physiological parameters are relatively precise indicators and are directly related to the broad and general parameters (i.e., longevity and brood rearing) that were measured by Herbert et al. (1980). Our findings provide new insights regarding sterol nutritional physiology in honey bees.

Materials and Methods

Sample Collection and Experimental Design

The study was conducted in June 2017. Three frames containing ready-to-emerge adult honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae) were collected from each of the 6 sister-queen colonies (ensuring genetic similarities) designated for this study at the Oregon State University apiaries located in Corvallis, OR. These 18 frames were brought to the laboratory and placed in an incubator overnight at 33°C, 55% RH (Percival Intellus I-36VL, Percival Scientific Inc.). All newly emerged bees from all the frames were thoroughly mixed and then 170 of these newly emerged bees were randomly allocated to each of the three experimental replicate cages per treatment group. The mixing was done to negate any bias in the population mix, especially because newly emerged bees were obviously fed some natural sterols via brood food when they were larvae. The treatment groups are described in the next section. The total duration of the study was 3 wk. Each cylindrical cage was custom built with ¼ inch hardware cloth and contained an artificially formulated diet placed on the cage floor, and water and 40% sugar syrup (w/v) were fed from inverted vials above (Fig. 1). Three evaporation control cages—empty cages with only sugar syrup, water, and control diets—were also included in this study to account for the loss in weight of diets due to moisture evaporation and loss in volume of

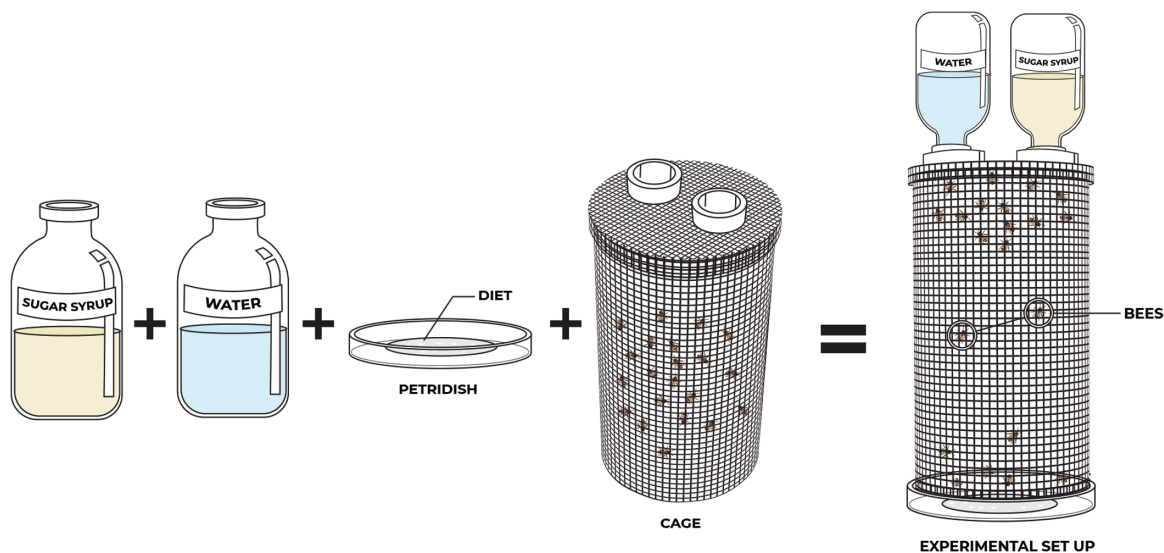


Fig. 1. Line diagram of the experimental cage set-up depicting how the artificial diets, sugar syrup, and water were administered to the honey bees in the laboratory.

water and sugar syrups due to evaporation. To reiterate, there were 170 bees per cage, and three replicate cages were designated for every control and treatment group.

Formulation of Artificial Diets

Artificial diets with varying concentrations of 24MC (ranging from 0.1 to 1%) were formulated based on the diets used in couple of previous studies—a seminal study by Herbert et al. (1980) and another study by Clayton (1964)—that documented optimal concentrations of sterols for insects to enhance insect fitness. To incorporate 24MC (Expert Synthesis Solutions, London, ON, Canada) into treatment diets, it was first dissolved in acetone, a common solvent (Gregorc et al. 2012, Zhu et al. 2014). The 24MC–acetone stock solution was further diluted as per the required sterol concentrations of the treatment groups. An equal volume of acetone solution was next added to the dry diet mixtures (Table 1) to create treatment diets with the following concentrations of 24MC (as percent dry diet weight): 0.1% (treatment group S1), 0.25% (treatment group S2), 0.5% (treatment group S3), 0.75% (treatment group S4), and 1.0% (treatment group S5).

A 2-g patty (dry diet weight) was provided to bees in each cage, and all patties were replaced weekly. Each 2 g of dry diet contained 810 mg of a complete amino acid powder containing all 20 amino acids (Nutricia, Zoetermeer, Netherlands), 1.171 g sucrose (C&H sugar, Crockett, CA), 17 mg Wesson's salt (MP Biochemicals, Irvine, CA), and 2 mg of zinc gluconate (Millipore Sigma, Burlington, MA). These dry ingredients were thoroughly mixed by hand using a sterile glass rod. Equal volumes of sterol–acetone solution were mixed into the dry ingredients thoroughly, and then the acetone was evaporated off the diets under a fume hood for 24 h. Four microliters of a B-vitamin mixture (Durvet) was then added to the diet. Finally, 450 μ l of 40% sucrose syrup was mixed into each diet to form a patty.

To test any potential effects of acetone addition to the diets, we included an additional control group that received acetone. Hence, overall, we had two control groups, C0 (no acetone and no 24MC) and C1 (acetone and no 24MC) along with five 24MC groups. All cages in all experimental groups received 2 g of the appropriate diet, freshly prepared, at the beginning of each week. The dead bees were removed when the diet patties were replaced each week.

Survival

Bee mortality in each cage was recorded at 2-d intervals and total mortality was calculated at the end of each week for each replicate cage in all treatment and control groups. Kaplan–Meier survival analysis was performed based on previous studies (Klein and Moeschberger 2003, Chakrabarti et al. 2015) using MedCalc

Version 18.2.1 software. Survival curves were calculated for a period up to 21 d for the study.

Diet Consumption

Diet consumption in each experimental cage was recorded each week and was calculated as the change in patty weight from the beginning of each week when it was placed in the cage to the end of the week when it was removed from the cage (seven days later). Cages that contained artificial diets, sugar syrup, and water, but no bees, were also placed in the incubator and treated identical to cages with bees. The change in diet weight within these empty cages represented the effects of evaporation. Before calculating diet consumption, the mean weekly weight loss of diet patties in these 'evaporation control' cages was subtracted from the patty weight loss of each experimental cage.

The average diet consumption per bee per week was calculated as follows:

$$\text{Weekly consumption per bee per cage (mg)} = \frac{(I_x - F_x) - x'}{N_y}$$

where I_x = initial weight (mg) of diet placed in cage 'y' at the beginning of week 'x'; F_x = final weight (mg) of diet in cage 'y' at the end of week 'x'; x' = mean change in diet patty weight in evaporation control cages during week 'x'; and N_y = average number of live honey bees in cage 'y' during week 'x'.

Head Protein Content

Live honey bees were collected at the end of the experiment. Head protein content was analyzed using previously published methods (Jack et al. 2016). For each experimental replicate cage, the heads of 10 bees were pooled together and homogenized in 600 μ l of phosphate-buffered saline (Sigma–Aldrich, St. Louis, MO; 10 mM phosphate, 2.7 mM potassium chloride, and 137 mM sodium chloride, pH 7.4) with one 3-mm tungsten carbide bead (Qiagen, Hilden, Germany) in a Tissue Lyser II (Qiagen; two rounds of 1.5 min at 30 oscillations/s). Homogenized samples were then centrifuged at 4°C for 6 min at 20,000 \times g (Eppendorf model 5430R, Eppendorf, Hamburg, Germany) and supernatant collected. The microplate assay protocol of a standard BCA assay (Pierce Biotech BCA Assay Kit, Thermo Scientific, Waltham, MA) was followed, and the absorbance at 562 nm was measured on a BioTek Synergy 2 plate reader (BioTek Instruments, Winooski, VT).

Abdominal Lipid Content

At the end of 3 wk, from each experimental replicate, live honey bees were collected and the abdomens of 10 honey bees were pooled and tested for abdominal fat content following an ether wash protocol

Table 1. Acetone volumes added to the control and treatment groups

Treatment group	Volume of acetone (μ l)	Volume used from stock (μ l)	Final volume (μ l)	Volume per 2 g patty (μ l) used	Final concentration of sterol (% of dry diet weight)
S5 (stock)	1,500	N/A	1,500	500	1
S4	125	375	500	500	0.75
S3	250	250	500	500	0.50
S2	375	125	500	500	0.25
S1	450	50	500	500	0.1
C1 (acetone control)	500	0	500	500	0
C0 (no acetone, control)	0	0	0	0	0

Sixty milligrams of 24-methylenecholesterol was dissolved in 1.5-ml acetone to produce the stock solution.

adapted from previous studies (Wilson-Rich et al. 2008). Each sample of 10 abdomens was dried at 45°C for 72 h in a drying oven (VWR, Radnor, PA) to reach constant dry mass and then weighed (Ohaus Pioneer Analytical, Parsippany, NJ). The guts were not removed from the abdomens. The abdominal lipids were next solubilized by gently shaking each set of 10 abdomens in 3-ml anhydrous ethyl ether (Avantor Performance Materials Inc., Radnor Township, PA) for 24 h on a microplate shaker (VWR). The abdomens were then dried under a fume hood for 72 h. The difference between initial dry weight and final dry weight was used to quantify abdominal lipid content, reported as percent initial dry weight.

Statistical Analyses

Kaplan–Meier survival analysis was performed using MedCalc Version 18.2.1 software and GraphPad Prism Version 7.03. Data were checked for normality using Shapiro–Wilk test. Statistical significance was tested using two-tailed *t*-tests for two groups and one-way analysis of variance (ANOVA) between multiple groups. Tukey's post hoc test was also conducted for multiple comparisons between groups. A logarithmic transformation was performed for data that were not normally distributed. One-way ANOVA and *t*-tests were performed using GraphPad Prism Version 7.03 software and R version 3.3.3. Results are presented as mean values \pm SEM. Data were pooled for the replicates for each control and treatment group when comparing means.

Results

Survival in Experimental Groups

Kaplan–Meier survival analyses indicated that significant difference between the survival curves for controls (C1 and C0) and 24MC supplemented treatment groups ($\chi^2 = 230.5$, $df = 6$, $P < 0.01$; Fig. 2). The highest survival was observed in group S3 (treated with 0.5% dry diet weight of 24MC), followed by groups S5, S4, S2, S1, C1, and C0 (with declining concentration of 24MC in the diet). The proportion of bees surviving at the end of 3 wk for S3, S5, S4, S2, S1, C0, and C1 were 0.383 ± 0.02 , 0.363 ± 0.02 , 0.307 ± 0.03 , 0.275 ± 0.015 , 0.197 ± 0.013 , 0.106 ± 0.009 , and 0.117 ± 0.01 , respectively.

Diet Consumption

Consumption data from all three replicates of a given experimental group (diet treatment) were pooled together for each week. The

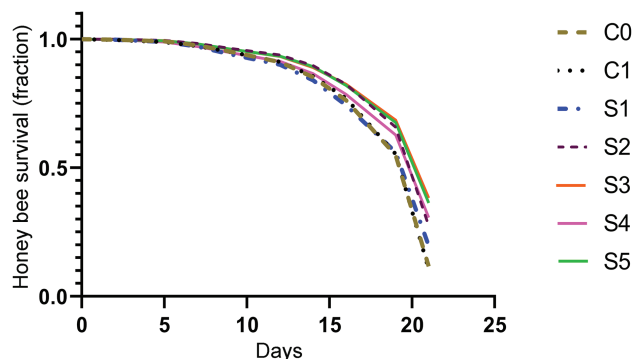


Fig. 2. Kaplan–Meier survival curves for the experimental groups S1–S5 when compared with controls C0 and C1. The experimental duration was 3 wk (21 d). C0 and C1: control groups without and with acetone, respectively; S1, S2, S3, S4, and S5: 0.1, 0.25, 0.5, 0.75, and 1.0% dry diet weights of 24-methylenecholesterol in artificial diets.

results for all Tukey's post hoc tests for every week are provided in Supp Table 1 (online only). There was no significant difference in diet consumption between the two control groups C0 (week 1: 7.66 ± 0.87 mg per bee; week 2: 5.84 ± 0.46 mg per bee; week 3: 9.07 ± 0.86 mg per bee) and C1 (week 1: 8.20 ± 0.58 mg per bee; week 2: 5.67 ± 0.47 mg per bee; week 3: 9.05 ± 1.63 mg per bee) for week 1 ($t = -0.519$, $df = 4$, $P = 0.32$), week 2 ($t = 0.274$, $df = 4$, $P = 0.60$), and week 3 ($t = 0.0065$, $df = 4$, $P = 0.502$).

In week 1, a significant difference in consumption was observed between treatment groups (one-way ANOVA, $F_{6,14} = 19.52$, $P < 0.001$). Tukey's post hoc test revealed no significant differences between groups C0, C1, and S1 and between groups S2, S3, S4, and S5 (Fig. 3; Supp Table 1 [online only]). In week 1, the average consumption of the sterol diets were 8.47 ± 1.01 , 12.81 ± 0.44 , 12.61 ± 0.25 , 13.00 ± 0.44 , and 14.10 ± 0.31 mg per bee for experimental groups S1, S2, S3, S4, and S5, respectively (Fig. 3). Significant differences in consumption were also observed between treatment groups in week 2 (one-way ANOVA, $F_{6,14} = 3.854$, $P < 0.05$; Fig. 3; Supp Table 1 [online only]). The average diet consumptions were 7.02 ± 1.08 , 7.18 ± 0.93 , 7.95 ± 0.49 , 8.97 ± 0.69 , and 9.63 ± 0.93 mg per bee for S1, S2, S3, S4, and S5 groups, respectively (Fig. 3). Likewise, in week 3, significant differences in diet consumption were observed (one-way ANOVA, $F_{6,14} = 3.757$, $P < 0.05$; Fig. 3; Supp Table 1 [online only]). The average diet consumptions per bee during week 3 were 9.18 ± 0.30 , 10.71 ± 0.80 , 11.49 ± 0.052 , 11.94 ± 0.44 , and 13.87 ± 1.35 mg, respectively, in groups S1, S2, S3, S4, and S5 (Fig. 3).

Total Head Protein Content

The total head protein content was significantly different between controls and the five treatment groups (one-way ANOVA, $F_{6,14} = 30.64$, $P < 0.001$). Tukey's post hoc test showed that S5 was significantly higher than the other groups, and groups S2, S3, S4, and S1 had significantly more head protein content than C0, C1, and S1 (Fig. 4). Mean head protein content at the end of the 3-wk study was 276.468 ± 12.75 , 267.88 ± 24.25 , 308.87 ± 1.587 , 352.34 ± 6.24 , 359.23 ± 13.08 , 416.58 ± 31.68 , and 539.26 ± 8.32 μ g per honey bee, respectively, for groups C0, C1, S1, S2, S3, S4, and S5. The results from Tukey's post hoc tests are provided in Supp Table 2 (online only).

Abdominal Lipid Content

The experimental groups S2, S3, S4, and S5 had significantly higher abdominal lipid content than groups C0, C1, and S1 (one-way ANOVA and Tukey's post hoc test, $F_{6,14} = 27.77$, $P < 0.001$, Fig. 5). After 3 wk on the experimental diets, mean lipid content (as percent dry abdominal weight) of bees in groups C0, C1, S1, S2, S3, S4, and S5 were 5.25 ± 0.27 , 5.74 ± 0.39 , 5.90 ± 0.33 , 9.91 ± 0.38 , 10.27 ± 0.60 , 10.41 ± 0.85 , and 10.48 ± 0.45 , respectively. The results from Tukey's post hoc tests are provided in Supp Table 3 (online only).

Discussion

To our knowledge, this is the first comprehensive study to investigate the effects of 24MC (a critical micronutrient) on honey bee physiology and survival. We found that bees consumed higher amounts of the provided artificial diets when supplemented with 24MC, suggesting that bees perceive the presence of sterol in their diet, which in turn may stimulate them to consume greater quantities of the diet. Some other phytophagous insects exhibit similar behavior. For example, silkworm larvae preferentially consumed diet blocks coated in their most physiologically important sterol over those coated in

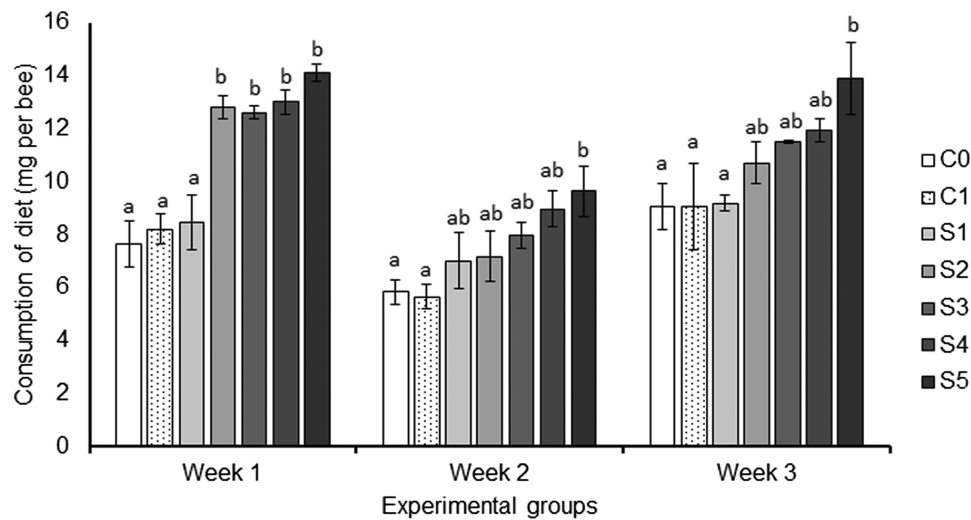


Fig. 3. Mean consumption of artificial diets (mg per honey bee) in different experimental groups for 3 wk. Error bars indicate SEM. For every week, different letters indicate statistically significant differences between experimental groups. C0 and C1: control groups without and with acetone, respectively; S1, S2, S3, S4, and S5: 0.1, 0.25, 0.5, 0.75, and 1.0% dry diet weights of 24-methylenecholesterol in artificial diets.

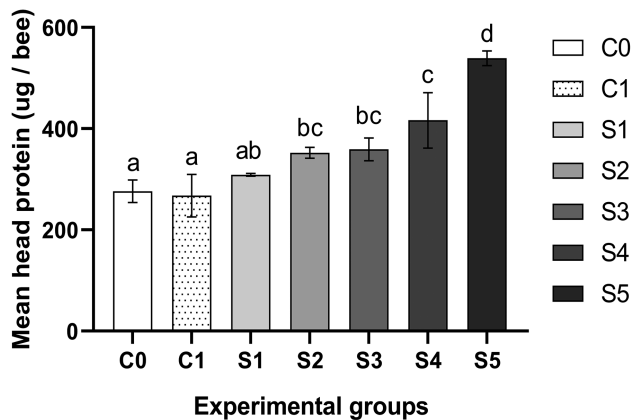


Fig. 4. Mean head protein content in bees that received 24-methylenecholesterol-supplemented diets or control diets C0 (without acetone) and C1 (with acetone). Diets for treatment groups S1, S2, S3, S4, and S5 contained 0.1, 0.25, 0.5, 0.75, and 1.0% 24-methylenecholesterol, respectively. Error bars indicate SEM. Different letters indicate significant differences among groups.

other sterols (Nagata et al. 2006). However, sterol concentrations may not be a predominant factor in diet preference when honey bees are able to choose between multiple diets varying in several nutritional components (Corby-Harris et al. 2018), wherein pollen diversity, protein to lipid ratio, or greater quantities of certain amino acids or fatty acids may serve as the dominant phagostimulant.

In our study, honey bee survival, head protein content, and abdominal lipid content were all significantly higher in bees that were fed diets with higher concentrations of sterols (even though the proportions of carbohydrates and proteins were identical among all diets). A similar effect of dietary sterols on fitness has been demonstrated in other insects. Adult female ambrosia beetles exhibited decreased longevity, locomotion, and reproduction when fed sterol-deficient diets (Norris and Moore 1980). Likewise, nymphs of the generalist grasshopper, *Schistocerca americana*, survived to adulthood in significantly greater proportions in less time when raised on diets containing sitosterol concentrations of 0.05% or greater than when fed diets with 0.025% sitosterol (Behmer and Elias 1999).

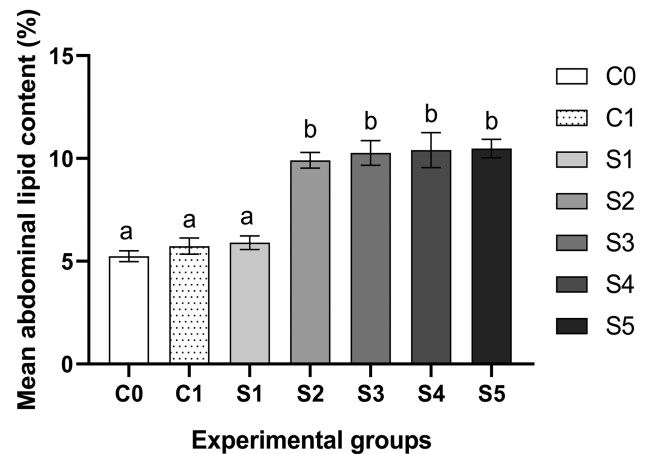


Fig. 5. Mean abdominal lipid content (%) of bees receiving 24-methylenecholesterol-supplemented diets or control diets C0 (without acetone) and C1 (with acetone). Diets for treatment groups S1, S2, S3, S4, and S5 contained 0.1, 0.25, 0.5, 0.75, and 1.0% dry diet weights of 24-methylenecholesterol, respectively. Error bars indicate SEM. Different letters indicate significant differences among groups.

Although consumption of the artificial protein diets was not significantly different between S2, S3, S4, and S5 groups during any week of our experiment, at the end of 3 wk, head protein content was significantly higher in the S5 group than in the others. The protein content of the honey bee in whole body or a specific tissue is crucial to gaining insights on the physiology of bees and all correlated biological processes (Hartfelder et al. 2013). In an earlier study (Svoboda et al. 1986), 24MC was found in high quantities in the hypopharyngeal glands (located in the head capsule) of honey bees that consumed an artificial diet supplemented with 24MC. In addition, the 16-molecule architecture of the major royal jelly protein (MRJP1) oligomer was recently reported to be able to hold eight 24MC molecules (Tian et al. 2018). These findings are further supported by studies that report hypopharyngeal glands as the sites of MRJP1 production (Kucharski and Maleszka 1998). MRJP1 is an important component of the proteinaceous glandular secretions of the nurse bee hypopharyngeal and mandibular glands (both located

in the honey bee head) and plays a role in age polyethism in honey bees (Buttstedt et al. 2014). The evidence of 24MC accumulation in these glands, coupled with our findings of significantly higher head protein content in bees fed greater concentrations of 24MC, suggests the potential role of this sterol (24MC) in protein synthesis in the brood food-producing glands (particularly, the hypopharyngeal glands). Thus, in our study, head proteins may be a good indicator of the state of brood food-producing glands in the experimental groups.

Abdominal fat is a crucial indicator of bee fitness and performance and regulates various aspects of insect physiology, including the synthesis and use of energy reserves (Arrese and Soulages 2010), lipid storage (Olofsson et al. 2009), detoxification (Arrese and Soulages 2010), synthesis of vitellogenin (Amdam et al. 2003, Arrese and Soulages 2010), and initiating foraging tasks in worker bees (Schulz et al. 1998, Toth et al. 2005) etc. Diet has previously been reported to influence gene expressions of the bee abdominal fat body tissues (Ament et al. 2011). In our study, lipid content in abdomens was higher in bees from sterol-rich treatment groups than in bees from the control group. Our lipid assays only quantified lipids in general, rather than distinguishing between fatty acids, triglycerides, sterols, etc. Therefore, we cannot predict what proportion each major lipid class contributed to the overall lipid content that we observed in abdomens. However, we surmise that sterols account for the difference in lipid stores between honey bees from high sterol groups and control groups.

The amount of lipids in insects may vary with life cycle stages, age, and nutritional needs (Beenackers et al. 1985). This is especially true in honey bees, where the newly emerged adult workers (nurse bees) mature into foragers as they age (Crailsheim et al. 1992, Johnson 2010). Bees with access to greater quantities of dietary sterols were probably better able to replace the endogenous sterols they used over time and/or store excess sterols in the lipids of the abdominal fat bodies. It has been reported that nurse bees can selectively transfer significant amounts of this particular sterol from their endogenous pools to the developing brood via brood food (Svoboda et al. 1980), just as they do with amino acids and proteins when rearing brood during pollen shortages (Haydak 1970). It can be presumed that because ample fat body increases fitness in overwintering bees, sufficient consumption of sterols may potentially lead to a healthier overwintering colony in field with adequate abdominal lipid stores.

In honey bees, protein and lipid stores decline as they mature into foragers (Chan et al. 2011). We measured the effects of dietary sterols in bees that were nutritionally manipulated only in our experimental cages, as adults; the physiological parameters were examined only at the end of the experiment. Before these bees emerged from their respective cells and became part of our study, they were reared as brood in naturally foraging colonies. Thus, as larvae, they were fed by nurse bees that had access to pollen. Although we do not know the specific sterol content of this pollen, we can certainly assume that the pollen contained some phytosterols—as all plant pollens do (Villette et al. 2015). Thus, as newly emerged bees, in our experiment, they were presumably equipped with some amount of endogenous sterols. To negate the effects of endogenous sterols as a factor in our study, we thoroughly mixed all newly emerged bees of the same age group and randomly allocated them to the experimental cages. This ensured an unbiased homogenous mix of honey bees.

In our study, honey bees fed diets with a sterol concentration of 0.5% survived the longest and their head protein and abdominal lipid levels were also among the highest. Our findings provide additional insights regarding the role of this important sterol (24MC)

in honey bee nutritional physiology and suggest that 0.5% concentration of 24MC may be an ideal concentration for formulating supplemental protein diets for honey bees. Research in the future should be conducted under realistic field conditions to verify the findings from our laboratory cage study. In addition, future research should also explore the feasibility of providing predetermined, specific amounts of diets to experimental bees instead of ad libitum for nuanced understanding of the effects of sterols, even though that approach appears to have significant limitations. Furthermore, the vast majority of bee species rely on pollen for their protein needs. The results of this study can be used for building an understanding of the impacts of pollen sterols on the physiology of other bee species. With multifactorial stressors contributing to pollinator decline, a fundamental knowledge of all nutritional needs of bees is crucial to improve and sustain pollinator health. Insights gleaned from this study have the potential to help formulate a more complete diet for honey bees in the future and help the beleaguered beekeeping industry.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

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Data Availability

All data generated or analyzed during this study are included in this published article. Any other information is available from the corresponding author on a reasonable request.

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