A Semi-Synthetic Diet and the Potential Important Chemicals for *Mythimna separata* (Lepidoptera: Noctuidae)

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Subject Editor: Muhammad Chaudhury

Received 27 June 2019; Editorial decision 5 October 2019

Abstract

Armyworm feeding in large, destructive groups is hugely difficult to control and the oriental armyworm, *Mythimna separata* (Walk), is one such pest. In this study, we reported a semisynthetic artificial diet for the oriental armyworm. This diet is based on Ritter’s diet, a formula developed for *Heliothis zea*. The survival of *M. separata* was extremely low and only around 2% insects can reach the adult stage on Ritter’s diet. But, it can reach up to 100% if corn leaf powder (CLP) was mixed, and insects grew faster and gained more mass. After testing a set of mixtures of Ritter’s diet and CLP, we found that 14.3% was the optimal proportion of CLP for making the artificial diet. We then used chloroform to extract CLP. Insect performance was still much better on Ch-extracted CLP diets than that on Ritter’s diet, but it was poorer than that on the diets containing unprocessed CLP, suggesting that the essential factor(s) was only partially extracted from corn leaf. We then used methanol and dichloromethane, two solvents differing in their polarity, to process the extractions and analyzed the extracted chemicals using gas chromatography–mass spectrometry (GC-MS). Insects had a better performance on dichloromethane-extracted CLP diet in comparison to methanol-extracted one, indicating that the important factor(s) is more prone to methanol extraction. The reported recipe here is useful for the research on *M. separata* and possibly other grain-crop eating armyworms. The functions of the chemicals extracted from corn leaf tissue can be investigated in the future studies.

Key words: artificial diet, corn leaf, *Mythimna separata*, armyworms, GC-MS

Armyworms refer to the larva of any of the *Mythimna* and *Spodoptera* genera in the order of Lepidoptera. These economically important pests move across fields to available food resource in an army-like fashion and feed in large, destructive groups to take a heavy toll of grain production. They may also threaten other agricultural products when preferred food is not available. Armyworm outbreak is usually episodic, but it is hugely difficult to control. As one of the examples, the oriental armyworm *Mythimna separata* (Walk) is a devastating pest in Asia. The insects are unable to over-winter in high latitudes, but they can migrate into the temperate zone and become the most devastating pest there during summer (Sharma and Davies 1983, Jiang et al. 2011, Lv et al. 2014).

Synthetic or semisynthetic artificial diets, which completely or mainly comprise chemically defined constituents, are critical to standardize experimentation including experimental animal health status and experimental conditions (Siviter et al. 2018). These diets have been developed for a number of economically important insect species, such as *Blattella germanica*, *Musca domestica*, *Schistocerca gregaria*, *Calliphora erythrocephala*, *Xyleborus ferrugineus*, *Drosophila melanogaster*, *Heliothis zea*, *Acrystosiphon pism*, *Aedes aegypti*, etc., and contributed to various research such as insect nutrition, genetics, ecology, and pesticide evaluation (Noland 1954; Monroe 1959; Dadd 1960a,b; Kodicek and Levinson 1960; Chu et al. 1970; Cooke and Sang 1970; Piper et al. 2014; Talyuli et al. 2015). The advantage of using chemically defined constituents includes the ease of manipulating components, evaluating the impact of certain nutrients and, more importantly, reducing confounding effects by unidentified components or inconsistent composition in diets (Deans et al. 2017). However, plant materials are currently the dominant component (25.36–86.72%) in the published diet recipes for
M. separata, and larval survival is relatively low, i.e., 41.8–61.6%., especially on the diets containing low proportions of plant materials (Be 1981, Zhou et al. 2009).

Ritter and Nes reported a semisynthetic diet for Heliothis zea, and all components are chemically defined except for casein. This diet was latter modified and used in research on insect physiology for several important lepidopteran pests (Ritter and Nes 1981; Jing et al. 2012, 2013, 2014; Deans et al. 2015). In a pilot experiment, we observed that M. separata cannot grow on Ritter’s diet unless corn leaf tissue was mixed. In this study, we tested a set of mixtures of Ritter’s diet and corn leaf powder (CLP). The effect of CLP on insect performance was characterized and the optimal proportion of CLP was suggested. We then conducted chemical analyses on corn leaf extractions using GC coupled to a mass spectrometer (GC-MS). The potential important ingredients in corn leaf tissue were screened out for further investigation. The reported recipe here will significantly contribute to the research on M. separata and possibly other grain-crop lepidopteran pests.

Materials and Methods

Experimental Animals

The eggs of M. separata were obtained from Keyun Industry Co., Ltd. (Jiyuan, Henan, China). The hatchlings were used in this study.

Preparation of CLP

Maize cv. SIMON 208 was planted in a greenhouse. All leaves were removed and freeze-dried when the seedlings reached about 30-cm high. The dry leaves were ground into powder by a food processor and then kept in ~20°C for further use. CLP was sent to an analytical service company (Sci-tech Innovation, Qingdao, China) and it contained approximately 21.52% carbohydrate, 18.4% protein, 42.3% cellulose, and 0.5% fat on a dry mass basis. This information was verified by another independent analytical analysis (The Test Center, Northwest A&F University, Yangling, China).

Artificial Diet Preparation

Ritter’s diet was originally developed for Heliothis zea by Ritter and Nes (Ritter and Nes 1981). We modified this diet by adding CLP (Supp Table 1 [online only]). First, the casein, albumin, peptone, and cellulose were combined and mixed. The casein, albumin, and peptone are the major protein resources, and the only other protein resource is from corn leaf. Second, cholesterol was dissolved in chloroform. This solution was added into the above mixture and stirred, so that the chloroform solution was evenly distributed. The mixture was left under a fume hood for 24 h, so that the chloroform could completely evaporate. The third step was to add the other macro-nutrients and antibiotic mixture to the mortar, later thorough grinding them to powder and adding the powder to the above beaker. The fourth step was to mix CLP into each beaker. At step 5, a number of ethanol-soluble components were added to 100% ethanol, whereas at step 6, a number of water-soluble components were added to water. These two solutions, plus one-fourth of the distilled water from step 7, were combined with the sucrose needed. After a thorough mixing, the heated and melted agar solution using the remaining three-fourth of the water in step 7 was made, and mixed with the previously diet ingredients. Around 50 ml of this liquid diet was then poured into each of four Petri dishes and allowed to set. After the diets cooled, caps were placed on each Petri dish of food. All diets were stored at 4°C.

Massive Extraction for CLP

CLP was processed in a 1,000-ml beaker. We weighed out a certain amount of CLP (25 g maximally) and transferred to the beaker. Solvent (chloroform, methanol, or dichloromethane) was then added (10: 1 volume/mass) and the mixture was stirred thoroughly. Two hours later, the mixture was filtered to drain the solvent out as much as possible. This procedure repeated six times. The processed CLP was left under a fume hood for 24 h to dry and then transferred into an oven at 55°C to dry thoroughly for another 48 h.

Artificial Diets

To determine the amount of CLP added to the diet, a set of mixtures were tested. In total, nine diets were prepared and these diets differed by the proportion of CLP added and whether CLP was chloroform-extracted. The same amount of cholesterol was added into each of these diets, respectively (Supp Table 1 [online only]). CLP, either chloroform-extracted or not, accounted for 4.8, 9.5, 14.3, and 19% of the total diet dry mass, respectively. These diets were prepared as above, and protein and carbohydrate contents were adjusted to the same level. We used Ritter’s diet as control. For convenience, the diets containing chloroform-extracted CLP were designated as Ch-extracted CLP diets and those containing normal CLP as unprocessed CLP diets. As a result, we had one control diet, four unprocessed CLP diets, and four Ch-extracted CLP diets.

Each newly emerged neonate was randomly transferred onto a diet with a fine-tipped paintbrush. Each diet had 30 biological replicates. All insect rearing cups (3-cm in diameter and 3.5-cm tall) capped with lids were put in an incubator at 25 ± 1°C and a photoperiod of 14:10 (L:D) h. Lids were drilled a few ventilation holes for oxygen supply. The insects were checked twice a day and the artificial diets were changed every 2 d till the pupation stage. This observation process continued at least 20 d, and larval survival and larval stage were recorded. Pupae were weighed 2 d after pupation and checked daily for eclosion.

Insect Performance Comparison Between Di-extracted and Methanol-Extracted CLP Diets

We supplemented Ritter’s diet with dichloromethane-extracted (Di-extracted) CLP and methanol-extracted (Me-extracted) CLP, respectively, and compared insect performance between these two diets. Rearing procedure was same as above with 30 insects for each diet.

Chemical Analysis

To analyze the chemicals extracted by the solvents, around 0.1-g CLP was extracted in 5-ml dichloromethane or methanol using Stainless Steel Advanced Multi-Tube Vortexer (Troemner, Thorofare, NJ). The mixture was shaken at 2,500 rpm for 5 min and then centrifuged at 4,000 rpm for 5 min. The supernatant solution was filtered (pore size: 0.45 μm). A 2-μl volume of this filtered solution was injected into a gas chromatograph–mass spectrometer (GC-MS; PerkinElmer Clarus 680) maintaining an inlet temperature of 300°C and a transfer line temperature of 260°C. The oven temperature was programmed for three phases: in the first phase, oven was programmed from 50 to 150°C with the initial temperature maintained for 3 min, the final temperature for 3 min, and a ramp rate of 10°C/min; in the second phase, oven temperature increased from 150 to 250°C at a ramp rate of 5°C/min with the final temperature for 3 min; in the last phase, oven
temperature increased from 250 to 300°C at a ramp rate of 5°C/min with the final temperature for 3 min. The column used was a capillary DB-5MS column (30 m) (Agilent) with a film thickness of 0.25 μm. Helium at a flow rate of 1.5 ml/min served as carrier gas. The MS was operated in an electron ionization (EI) mode with mass ranges from 35 to 450 Da. Compounds were matched with the National Institute of Standards and Technology library (NIST 2011, Washington, DC) and Wiley Registry of Mass Spectral Data (8th Edition).

Data Analysis

Developmental time and pupal mass were analyzed by analysis of variance. Larval survival and pupal survival (i.e., pupation and eclosion), which followed binomial distributions, were analyzed by using Likelihood Ratio χ^2 statistics. All statistical analyses were performed in SPSS V.22.0.

Results

Insect Performance on Artificial Diets

Larval Performance

The larvae grew faster on the diets containing CLP (unprocessed CLP diets: F (4,120) = 61.12, P < 0.001; Ch-extracted CLP diets: F (4,120) = 29.639, P < 0.001), but the developmental time was longer on Ch-extracted CLP diets in comparison to the corresponding unprocessed ones, particularly those on 9.5, 14.3, and 19%, respectively (t (54) = 4.63, P < 0.001; t (51) = 5.71, P < 0.001; t (54) = 10.33, P < 0.001; Fig. 1a). Most larvae died on Ritter’s diet. In contrast, pupation rate on the diets containing CLP was significantly higher than that on Ritter’s diet (unprocessed CLP diets: χ^2 = 80.22, P < 0.001; Ch-extracted CLP diets: χ^2 = 45.84, P < 0.001; Fig. 1b). It was 86.7% on 4.8% unprocessed CLP diet and 100% on other unprocessed CLP diets. It was relatively lower on Ch-extracted CLP diets in comparison to the corresponding unprocessed ones, although the significant difference was only found on 4.8 and 9.5% diet, respectively (χ^2 = 4.37, P = 0.037; χ^2 = 6.67, P = 0.01). Larval performance kept increasing along with the elevated amount of CLP and the improvement was not significant when the proportion of CLP was higher than 9.5%.

Pupal Performance

Pupal performance was also correlated with CLP content in the diets (Fig. 2). First, pupae were lighter on Ritter’s diet than those on CLP diets except for 4.8% Ch-extracted CLP diet, and pupae became heavier when the proportion of CLP increased (unprocessed CLP diets: F (4,120) = 24.15, P < 0.001; Ch-extracted CLP diets: F (4,120) = 27.86, P < 0.001; Fig. 2a). Pupae on CH-extracted CLP diets were lighter than those on the corresponding unprocessed ones (4.8%: t (57) = 5.20, P < 0.001; 9.5%: t (54) = 6.66, P < 0.001; 14.3%: t (53) = 5.38, P < 0.001; 19%: t (55) = 2.40, P = 0.021), but the difference kept reducing along with the increase of CLP proportion. Second, pupal developmental time on 19.0% unprocessed CLP diet was shorter in comparison to Ritter’s diet (unprocessed CLP diets: F (4,120) = 5.14, P = 0.001; Ch-extracted CLP diets: F (4,120) = 2.12, P = 0.08; Fig. 2b). Pupal development time was also shorter on 14.3 and 19% Ch-extracted CLP diets than that on the corresponding unprocessed diets, respectively (14.3%: t (52) = 5.08, P < 0.001; 19%: t (56) = 2.94, P = 0.005). Third, only one-fourth of the pupae eclosed on Ritter’s diet. In another word, only around 2% of the experimental larvae can reach the adult stage. In contrast, eclosion rate dramatically increased when CLP was mixed in the diet (unprocessed CLP diets: χ^2 = 53.19, P < 0.001; Ch-extracted CLP diets: χ^2 = 40.82, P < 0.001; Fig. 2c), and it was similar between the pairs of unprocessed and Ch-extracted diets (4.8%: χ^2 = 2.97, P = 0.085; 9.5%: χ^2 = 0.85, P = 0.357; 14.3%: χ^2 = 0.022, P = 0.96; 19%: χ^2 = 1.017, P = 0.313).

Characterization of Important Corn Leaf Factors for M. separata

Insect Performance on Di-Extracted and Methanol-Extracted CLP Diets

To investigate what ingredients in CLP may potentially affect insect performance, we conducted another experiment. We used two solvents, methanol and dichloromethane, to extract CLP. These two solvents have different polarity from chloroform (4.40). Methanol is higher (6.40) and dichloromethane is lower (3.40). We expected that chemicals can be extracted differentially from CLP by these solvents and insect performance differed accordingly. Based on the above results, we used 14.3% CLP diet. We then found that insects had a better performance on Di-extracted CLP diet than that on methanol-extracted one. For example, the insects grew faster (t (53) = 3.75,
The chemicals extracted by these two solvents were analyzed using GC-MS (Table 1, Supp Fig. 1 [online only]). Four chemicals, hydroxyacetone, propylacetate, 2,3-dihydro-3,5-dihydroxy-6-hexadecenoic acid, phytol, and linolenic acid were more preferentially extracted by methanol over dichloromethane. Five chemicals such as neophytadiene, 3,7,11,15-tetramethyl-2-hexadecene, \(\chi^2 = 3.29, P = 0.07; \text{Fig. 3b and d)}. \)

Chemical Analysis in Dichloromethane-Extraction and Methanol-Extraction

The chemicals extracted from these two solvents were analyzed using GC-MS (Table 1, Supp Fig. 1 [online only]). Chemicals, hydroxyacetone, propylacetate, 2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyrans-4-one, and 2,3-dihydrobenzofuran were only detected in methanol extract, whereas 2,4-dimethylethylamine, 2,4-dimethyl-1-heptene, octadecanal, and 1-heptacosanol only in dichloromethane extract. Methyl pyruvate, 6-methoxy-2-benzoazolinone, (E)-3,7,11,15-tetramethyl-2-hexadecene, \(\chi^2 = 0.22, P = 0.64; \chi^2 = 3.29, P = 0.07; \text{Fig. 3b and d)}. \)

Discussion

Here, we reported a semisynthetic diet for an armyworm pest, *M. separata*, an economically important pest in Asia. According to the results, 14.3% was an optimal proportion of CLP for the diet. The proportion can be further reduced to 4.8% with a marginal loss in insect performance. The plant material used was substantially less in comparison to the previously published diet recipes and, more importantly, the survival rate from larvae to adults dramatically increased (Zhou et al. 2009). Among ingredients in this formula, CLP is the only one providing multiple dimensions of nutrients, and the left 85.7% content can give substantial flexibility to adjust each individual nutrient, such as carbohydrates, proteins, lipids, sterols, vitamins, fatty acids, and minerals. This is particularly important for the research requiring a controlled nutritional context. As one example, studies on insect sterol physiology are largely dependent on the availability of a sterol-free diet (Noland 1954; Kaplanis et al. 1960; Jing et al. 2012, 2014). Removing sterols from bulk materials is a costly and time-consuming process, and cumulated sterol residues in extracted materials can sometimes meet insect sterol requirement (Guo et al. 2017). Phytochemicals such as campestere, stigmasterol, and sitosterol were successfully removed by the extractions (Table 1) and *M. separata* larvae were arrested in the first or second instar on Ch-extracted CLP (19%) diet unless cholesterol was added (Supp Fig. 2 [online only]). Moreover, reducing natural materials which may vary from batch to batch can greatly enhance the reproducibility of experiments. Therefore, the reported dietary information is highly useful for studies on *M. separata*. Other grain-crop eating armyworms which have similar host plants (e.g., *Spodoptera frugiperda*) may also accept this recipe.

CLP contained essential ingredients for *M. separata* which are lacking in Ritter’s recipe. In another study, Huynh et al. found that some unknown ingredient(s) in corn root powder was essential for the larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Huynh et al. 2017, 2019). Ritter’s diet cannot support *M. separata* growth, despite its complete nutritional matrix for other caterpillar species and the same composition of macronutrients (i.e., carbohydrates and proteins) as CLP-contained diets (Jing et al. 2014). It was reported that the absence of a nonnutrient phagostimulant can lead to the low rate of food intake and the subsequently insect poor growth (Dadd 1960a,b), so we suspect that the essential factor(s) in corn may serve as an ingestion stimulus. *Mythimna separata* performance on the diets containing Ch-extracted CLP was much better than that on Ritter’s diet but relatively poorer than that on unprocessed CLP diets, indicating that the essential ingredient(s) in CLP was only partially extracted by chloroform. We used two solvents with different polarity, methanol, and dichloromethane, to extract CLP, and correlated the different chemical profiles in these two extractions with the corresponding insect performance. Indeed, we found that insect performance was
Fig. 3. Pupal performance on Me-extracted (Methanol) and Di-extracted (CH₂Cl₂) diets. a) Larval developmental time (means ± SEM) on two diets. b) Pupation percentage on two diets. Percentage was calculated by the number of pupae divided by the total number of larvae for each diet. c) Pupal mass (means ± SEM) on two diets. d) Eclosion percentage on two diets. Percentage was calculated by the number of adults divided by the total number of pupae. ***P < 0.001.

Table 1. Compounds identified from methanol or dichloromethane extracts

| No. | Retention time | CAS no. | Chemical name |
|-----|----------------|---------|---------------|
|     |                |         |               |
|     |                | 116-09-6| Hydroxyacetone|
|     |                | 109-60-4| Propylacetate  |
|     |                | 28564-83-2| 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one |
|     |                | 496-16-2| 2,3-Dihydrobenzofuran |
|     | 4.24           | 2213-23-2| 2,4-Dimethylheptane |
|     | 4.62           | 19549-87-2| 2,4-Dimethyl-1-heptene |
|     | 51.76          | 638-66-4| Octadecanal    |
|     | 52.93          | 2004-39-9| 1-Heptacosanol |
|     |                | 600-22-6| Methyl pyruvate |
|     |                | 532-91-2| 6-Methoxy-2-benzoxazolinone |
|     |                | 14237-73-1| (2E)-3,7,11,15-Tetramethyl-2-hexadecene |
|     |                | 57-10-3| n-Hexadecanoic acid |
|     |                | 150-86-7| Phytol         |
|     |                | 463-40-1| Linolenic acid |
|     | 3.70           | 600-22-6| Methyl pyruvate |
|     | 22.50          | 532-91-2| 6-Methoxy-2-benzoxazolinone |
|     | 24.02          | 14237-73-1| (2E)-3,7,11,15-Tetramethyl-2-hexadecene |
|     | 26.46          | 57-10-3| n-Hexadecanoic acid |
|     | 29.28          | 150-86-7| Phytol         |
|     | 29.91          | 463-40-1| Linolenic acid |
|     | 23.88          | 504-96-1| Neophytadiene  |
|     | 24.78          | 102608-53-7| 3,7,11,15-Tetramethyl-2-hexadecen-1-ol |
|     | 48.08          | 474-62-4| Campesterol    |
|     | 48.47          | 83-48-7| Stigmasterol   |
|     | 49.37          | 83-47-6| Stigmasterol   |

The mass spectra of all detected chemicals were identified against spectra in the NIST and Wiley library using a cut-off value of 80%. The identifications were manually validated to reduce deconvolution errors during automated data-processing and any chemicals with the identity lower than the cut-off value was not included.

*No chemical was more preferentially extracted by dichloromethane over methanol.

*The peak area in methanol extract was more than twofold larger than that in dichloromethane extract.
relatively poorer on methanol-extracted CLP diet, suggesting that the key chemical(s) may be more prone to methanol extraction. Ten chemicals were only detected or enriched (more than twofold) in the methanol extract (Table 1). Linolenic acid was added in the diet, so it should not be the essential factor. Among the nine chemicals left, 6-methoxy-2-benzoxazolinone and 2,3-dihydrobenzofuran can affect insect food searching and feeding behavior (Bjostad and Hibbard 1992, Morris et al. 2000, Drijfhout and Morgan 2010). But these chemicals, plus hydroxyacetone, α-hexadecanoic acid, and phytol, were also detected in dicotyledonous plants which M. separata rarely feed on (Nascimento and Lopes 1999, Katonoguchi and Macias 2008, Mach 2015, Bourguignon 2016, Ravi and Krishnan 2017). The information on propylacetate, 2,3-dihydro-3,5-dihydroxy-6-methylpyran-4-one, methyl pyruvate, and (2E)-3,7,11,15-tetramethyl-2-hexadecene is scarce. The effect of these chemicals on M. separata can be further investigated by mixing the authentic chemicals into Ritter’s diet. Notably, we cannot exclude the potential interactions of these chemicals, which should also be considered in the future studies.

In conclusion, M. separata can only grow well on Ritter’s diet when CLP was mixed. The optimal CLP proportion in the diet was 14.3% and it may be further reduced depending on research purpose. Chloroform extraction can reduce the content of the essential factor(s) in corn leaf which seems more prone to the extraction by methanol than dichloromethane. The reported recipe is useful for the research on M. separata and possibly other grain-crop armyworms. The functions of the chemicals characterized in corn leaf tissue can be investigated in the future studies.

Supplementary Data
Supplementary data are available at Journal of Insect Science online

Acknowledgments
We are sincerely thankful for the help of students at the Key Laboratory of Applied Entomology, Northwest A&F University, Yangling, Shaanxi, China. This research was funded by the General Program of National Natural Science Foundation of China (31672349), and the Introduction of Talent Research Start-up Fund of Northwest A&F University (Z111021503).

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