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Tobacco Hornworm as an Insect Model System for Cannabinoid Pre-clinical Studies

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TITLE:

Tobacco Hornworm as an Insect Model System for Cannabinoid Pre-clinical Studies

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Cannabis, *Cannabis sativa*, Cannabinoids, Tobacco hornworms *Manduca sexta*

SUMMARY:

The present protocol provides instructional information for using tobacco hornworm *Manduca sexta* in cannabinoid research. The method described here includes all necessary supplies and protocols to monitor physiological and behavioral changes of the insect model in response to cannabidiol (CBD) treatment.

ABSTRACT:

With increased attention on cannabinoids in medicine, several mammalian model organisms have been used to elucidate their unknown pharmaceutical functions. However, many difficulties remain in mammalian research, which necessitates the development of non-mammalian model organisms for cannabinoid research. The authors suggest the tobacco hornworm *Manduca sexta* as a novel insect model system. This protocol provides information on preparing the artificial diet with varying amounts of Cannabidiol (CBD), setting up a cultivation environment, and monitoring their physiological and behavioral changes in response to CBD treatment. Briefly, upon receiving hornworm eggs, the eggs were allowed 1-3 days at 25 °C on a 12:12 light-dark cycle to hatch before being randomly distributed into control (wheat germ-based artificial diet; AD), vehicle (AD + 0.1% medium-chain triglyceride oil; MCT oil) and treatment groups (AD + 0.1% MCT + 1 mM or 2 mM of CBD). Once the media was prepared, 1st instar larvae were individually placed in a 50

mL test tube with a wooden skewer stick, and then the test tube was covered with a cheesecloth. Measurements were taken in 2-day intervals for physiological and behavioral responses to the CBD administration. This simple cultivation procedure allows researchers to test large specimens in a given experiment. Additionally, the relatively short life cycles enable researchers to study the impact of cannabinoid treatments over multiple generations of a homogenous population, allowing for data to support an experimental design in higher mammalian model organisms.

INTRODUCTION:

Over the past years, public attention has been centered on cannabinoids due to their therapeutic potential, including the treatment of epilepsy[1], Parkinson's disease[2], multiple sclerosis[3], and various forms of cancer[4-6] with Cannabidiol (CBD). Since *Cannabis* is legalized as an agricultural commodity in the Agricultural Improvement Act of 2018, Public Law 115-334 (the 2018 Farm Bill), *Cannabis* and its cannabinoid derivatives in the food, cosmetic, and pharmaceutical industries have exponentially increased. Additionally, clinical-grade isolates of single cannabinoids and cannabinoid mixtures have been successfully tested in human subjects[7], cell lines[5,8], and diverse animal model systems[9,10].

A clinical trial would be ideal for validating the efficacy and adverse effects of cannabinoids on a specific disease. However, there are numerous challenges in clinical trials, including ethical/IRB approval, recruitment, and retention of the subjects[11]. To overcome these hurdles, various human cell lines were used because human-derived cell lines are cost-effective, easy to handle, can bypass the ethical issues, and provide consistent and reproducible results as the cell lines are a 'pure population of cells that have no cross-contamination of other cells and chemicals'[12].

Alves et al. (2021)[13] tested CBD in a dose-dependent manner in the placental trophoblasts, which are specialized cells of the placenta that play an essential role in embryo implantation and interaction with the decidualized maternal uterus[14]. Their results showed that CBD caused cell viability loss, cell cycle progression disruption, and apoptosis induction. These observations demonstrate the potential negative impacts of *Cannabis* use by pregnant women[13]. Likewise, a series of cell lines were also used to examine the pharmacological effects of CBD in human diseases, in particular, various forms of cancer. The *in vitro* studies successfully demonstrated anti-cancer effects in pancreatic[15], breast[8], and colorectal cancer cells[16]. However, while being widely available and easy to handle, specific cell lines such as HeLa, HEK293 are prone to genetic and phenotypic changes due to alterations in their growth conditions or handling[17].

In *Cannabis* research, various animal model systems, ranging from small animals such as mouse[18], guinea-pig[19], and rabbit[19] to large animals such as canine[20], piglet[21], monkey[22], horse[23], have been used to explore unknown therapeutic effects. Mice have been the most preferred animal model system for cannabinoid research due to their anatomical, physiological, and genetic similarity to humans[24]. Most significantly, mice have CB1/2 receptors in their nervous system, which are present in humans. They also have a shorter life cycle than human subjects, with easier maintenance and abundant genetic resources, thus making it much easier to monitor the effects of cannabinoids throughout an entire life cycle. The mammalian system is widely used and has successfully demonstrated that CBD relieves seizure disorders[1], post-traumatic stress disorder[9],

oral ulcers[25], and dementia-like symptoms[10]. The mouse model has also enabled a social interaction study of individuals within a community which is extremely difficult in large animals and humans[26].

Despite all the advantages of the animal model system, it is still costly and requires intensive care during drug administration and data collection. Additionally, there is scrutiny of using mice in research because of irreproducibility and poor recapitulation of human conditions due to limitations in experimental design and rigor[27].

With the increasing demand for medical/preclinical studies of cannabinoids, a non-mammalian model system is needed. Invertebrate models traditionally conferred distinctive benefits over vertebrate models. The significant benefits include the ease and low cost of rearing many specimens and enabling researchers to monitor multiple generations of genetically homogeneous populations[28]. A recent study proved the fruit fly, *Drosophila melanogaster,* to be an effective insect model system to investigate pharmacological functions of cannabinoids in modulating feeding behaviors[29]. Among the insect model systems, the authors focused on the tobacco hornworm, *Manduca sexta*, also known as Carolina sphinx moth or hawk moth, as a novel insect model system for cannabinoid research.

Manduca sexta belongs to the family of Sphingidae. The insect is the most common plant pest in the southern United States, where they feed on solanaceous plants. The insect model has a long history in research in insect physiology, biochemistry, neurobiology, and drug interaction studies. *Manduca sexta*'s research portfolio includes a draft genome sequence, allowing for a molecular-level understanding of essential cellular processes[30]. Another crucial benefit of this model system is its large size, reaching more than 100 mm in length and 10 g in weight in the 18-25 days of larval development. The large size enables researchers to easily monitor morphological and behavioral changes in real-time in response to the CBD treatment. Also, due to the size, electrophysiological responses were examined with the abdominal nervous system, including ganglia dissected from the larvae without high-resolution microscope settings. The unique feature allows researchers to readily investigate acute and long-term responses to the administered cannabinoid(s).

Despite such versatility, *M. sexta* has only recently been explored for its suitability as an experimental model for *Cannabis* and cannabinoid studies. In 2019, the authors used the insect model system for the first time to address the hypothesis that *Cannabis* has evolved to produce Cannabidiol to protect itself from insect herbivores[30,31]. The result clearly showed that the plants exploited CBD as a feeding deterrent and inhibited the growth of the pest insect *M. sexta* caterpillar, as well as causing increased mortality[31]. The study also demonstrated the rescuing effects of CBD to intoxicated ethanol larvae, identifying the potential vehicle effect of ethanol as a carrier of the CBD. As shown, the insect model system effectively investigated the therapeutic effects of cannabinoids within 3-4 weeks with less labor and costs than other animal systems. Although the insect model lacks cannabinoid receptors (*i.e*., no CB1/2 receptors), the model system provides a valuable tool to understand the pharmacological roles of cannabinoids through a cannabinoid receptor-independent manner.

The authors of this study have previously worked with the tobacco hornworm as a model system for cannabinoid research[31]. After careful consideration of the benefits and risks of using *M. sexta*, we have provided a method involving the proper care and preparation of diet for preclinical trials that allow for opportunities for future preclinical laboratory use.

PROTOCOL:

1. Hornworm preparation and cannabidiol treatment

1.1. Obtain 150-200 viable *M. sexta* eggs and wheat germ-based artificial diets (see **Table of Materials**).

1.2. Place the hornworm eggs in a polystyrene Petri dish with a wheat germ-based artificial diet (AD) layer and transfer the eggs to an insect rearing chamber (see **Table of Materials**) maintained at 25 °C with 40%-60% relative humidity.

1.3. Allow tobacco hornworm eggs for 1-3 days to hatch inside the insect rearing chamber maintained at 25 °C with 40%-60% relative humidity.

1.4. Prepare cannabidiol (CBD) stock solution (200 mM) by adding 1.26 g of >98% purity CBD isolate in 20 mL of EtOH (200 proof) or 100% medium chain triglycerides (MCT) oil (see **Table of Materials**).

NOTE: CBD isolate is light-sensitive, so handle at dark.

1.5. Add 5 mL and 10 mL of the 200 mM CBD stock solution to the 1,000 g of AD to bring the final concentrations of the diets 1 mM and 2 mM of CBD, respectively.

NOTE: Ensure the diet and CBD stock solution are well-blended until a completely homogeneous mixture is formed. Blend the AD containing stock of CBD in a plastic bag for at least 45 min by hand.

CAUTION: Coffee mixer or any other metal grinder appeared to be ineffective.

1.6. Dispense 20 g of the three media, control (AD), vehicle (AD + 0.1% of EtOH or MCT oil), and CBD containing media (AD + 0.1% of EtOH or MCT oil + 1 mM/2 mM of CBD) to the bottom of the 50 mL tube.

1.7. Randomly distribute 1st instar larvae (~2 mm long) individually in a 50 mL test tube and cover with a perforated lid or cheesecloth (see **Table of Materials**).

NOTE: Place the tube upside down and grow insects at an insect rearing chamber maintained 25 °C with 40%-60% relative humidity.

1.8. Grow them inside an insect rearing chamber (see **Table of Materials**) maintained at 25 °C with a 12 h light/dark cycle.

2. *M. sexta* larval growth, diet consumption, and mortality measurements

2.1. Measure the larval growth (i.e., size and weight) with an analytical balance and mortality at 2-day intervals after being transferred to individual containers until pupation is recognized as the dark brown coloration of a hardened exocuticle layer.

2.1.1. Record the initial mass (in grams) of each group of larvae before introducing the larvae to their respective diets and subtract the mass of the larvae at each measurement from the initial mass to determine mass gains between larvae developmental stages until the larvae complete the pupation stage.

2.1.2. Record the number of days between the instar developmental stages to understand differences in the developmental timeframe between stages of larvae growth until pupation on each diet.

NOTE: Scrape off the fecal matter from the container to avoid any mold contamination. Collect the matter for future testing dependent on experiment purposes (e.g., CBD accumulation rate calculation, microbial profiling). It is important to carefully handle the insect during the fragile periods of apolysis or ecdysis. When taking out of the larvae from a container, gently grab the main body of the insect with a flat-tip and wide forceps and do not force to remove the outer layer of skin when an insect is in the process of shedding.

2.2. Measure the diet consumption[31] by weighing the diet loss of the container between 1st instar larvae and pupation. Record the initial grams of diet at the beginning of the experiment and subtract the initial amount from the remaining amount of diet when the larvae entered the complete pupation stage.

NOTE: The fecal matter should be excluded from the diet measurement. The fecal matter and other debris (*i.e.*, skin sheds) can be easily removed from the media by placing the container upside down.

2.3. Allow the subjected insect to acclimate the chamber environment for at least 5 min and track the distances[31] that three groups of 5th instar insects (80-100 mm in length) traveled using an automated, computerized fear conditioning chamber (see **Table of Materials**).

2.4. Analyze the mobility response[31] through video recorded 60 frames/s for 5 min using a motion detection software (see **Table of Materials**) which generates a motion index.

3. Statistical analysis

3.1. Analyze the differences in the larval growth (i.e., size and weight) and the motion index by one-way ANOVA with Tukey's post-test[32].

3.2. Use the log-rank (Mantel-Cox) test[33] for survival curve comparisons.

NOTE: All the statistical analyses were performed using statistical analysis software (see **Table of Materials**).

REPRESENTATIVE RESULTS:

***Manduca sexta* as a model system to examine cannabinoids toxicity**

Figure 1 depicts the key components of the CBD experiment using tobacco hornworm *Manduca sexta*. Large numbers of insects (>20) were individually reared at 25 °C on a 12 h:12 h = light: dark cycle. The insects' size, weight, and mortality were measured at 2-day intervals to monitor for short-and long-term responses after high-dose CBD (2 mM) treatment.

Figure 2 shows the adverse effects of CBD on the insect's growth and development. The insects reared on an artificial diet (AD) showed the best growth performance. The vehicle control that used 0.1% medium-chain triglyceride (MCT) oil as a dissolving agent for CBD isolate also showed normal growth without any detrimental effects. However, a high dose of CBD (2 mM) induced weight loss (**Figure 2C**) and led to a higher mortality rate than those of control and vehicle groups (**Figure 2D**).

On day 24, the average size of the larvae fed on AD was 63.9 mm (n = 20-22). However, the size of larvae reared on AD containing 2 mM of CBD was 50.7 mm, which was ~21% smaller than the larvae grown on AD (red line in **Figure 2C**)[31]. On day 24, the average weight of larva reared on AD was 6.5 g, which was 2.2-fold greater than those of larvae reared on AD with 2 mM of CBD (n = 12-16, $p < 0.00001$)[31]. Notably, the high dose of CBD (2 mM) significantly increased the mortality rate up to 40%, while the control and vehicle groups showed only a 20% mortality rate (**Figure 2D**)[31]. The results indicated that the high dose of CBD (2 mM) in the diet is detrimental to insect development and correlates to increased mortality.

***Manduca sexta* as a model system to explore unknown therapeutic functions of cannabinoids**

Figure 2 showed that the insect model system effectively monitors any detrimental effects of CBD by monitoring their morphological and physiological changes. The preliminary result indicated that >1% ethanol (EtOH) is negatively related to their growth, mobility, diet consumption, and survival rate. To examine whether CBD improves insect's mobility and feeding behavior in the EtOH-intoxicated *M. sexta* larvae, the total amount of diet consumed by insects and the distance they traveled for 10 min were measured from insects grown under three feeding conditions (AD, AD + 1% EtOH, and AD + 1% EtOH + 1 mM CBD). **Figure 3A** shows that *M. sexta* larvae reared on AD containing 1 mM of CBD consumed at least 3.1-times greater diet mass than those reared on EtOH-added diet[31]. However, the diet consumption of the insects reared on 2 mM of CBD-added media was not significantly different than those of larvae reared on EtOH-only diets ($p > 0.05$)[31].

Larval mobility was also tracked to examine if CBD affected their mobility when intoxicated with EtOH. The mobile index is presented as the percentage (%) of freeze. **Figure 3B** compares the mobile index of *M*. *sexta* larvae reared on different conditions. The results show that 1% EtOH-treated larvae did not affect mobility ($p > 0.05$). The 1 mM CBD administration also did not affect mobility ($p > 0.05$)[31]. The 2% EtOH treatments turned out to be lethal to *M. sexta* larvae; therefore, no mobility index was recorded. With the addition of the high dose of CBD (2 mM) into AD containing 2% EtOH, the mobility remained low (80% freeze)[31].

FIGURE LEGENDS:

Figure 1: The summarized process of using tobacco hornworm *Manduca sexta* caterpillars in cannabidiol study. (**A**) Hornworm eggs hatched in a separate large container with a layer of artificial diet. (**B**) A syringe was used to fill the container to prevent any diet from sticking to the sides of the containers. (**C**) A 2nd instar tobacco hornworm in a 50 mL test tube with cheesecloth. (**D**) A 3rd instar tobacco hornworm. (**E**) Hornworm length (mm) and weight (g) were measured on a scale. (**F**) 5th instar tobacco hornworm which undergoes ecdysis and ready for pupation.

Figure 2: Effects of Cannabidiol (CBD) on the growth and mortality of tobacco hornworm *Manduca sexta*. (**A**) Tobacco hornworm caterpillars at 5th, 3rd instar, and early pupation. The size (**B**), weight (**C**), and mortality (**D**) of *M*. *sexta* when fed on artificial diet (AD), AD + 0.1% of medium-chain triglyceride (MCT), and AD + 0.1% of MCT + 2 mM of CBD. For statistical analyses on insect growth and survival rate, a one-way ANOVA with Tukey's multiple comparisons test (n = 20–22, $p < 0.05$) and Mantel-Cox test (n = 20–22, $p < 0.05$) were used, respectively. The figure is adapted from Reference[31].

Figure 3: The effects of Cannabidiol (CBD) on insect feeding behavior and mobility. (**A**) Diet consumption of tobacco hornworm caterpillars reared on artificial diet (AD), AD + 1-2% of ethanol (EtOH), and AD + 1-2% of EtOH + 1-2mM of CBD (one-way ANOVA, Tukey's multiple comparison at $p < 0.05$). (**B**) Insect mobility. The mobility is depicted as freeze %. *** indicates $p < 0.01$. The figure is adapted from Reference[31].

DISCUSSION:

The feeding study demonstrated that high doses of CBD (2 mM) inhibited the insect's growth and increased mortality[31]. The insect model also showed sensitivity to ethanol; however, CBD effectively detoxicated the ethanol toxicity, increasing their survival rate, diet consumption, and food searching behaviors to similar levels to the control group (**Figure 3A,B**)[31]. The described insect model system is composed of three critical steps: (1) ensuring *M. sexta*'s eggs are hatched uniformly in size and timing, (2) preparing the growth media that are homogeneously blended with cannabinoids to a targeted concentration, and (3) maintaining the growth media to be free of fungal contamination while maintaining ideal humidity level at 40%-60%. The insect model system enabled us to address the research question within 25 days, from media preparation to data collection and interpretation. Most importantly, the insect system produced consistent results from large specimens.

To ensure the success of the cultivated *M. sexta* larvae, maintaining the relative humidity at 40%-60% inside the container is essential. If a container fails to hold the high humidity, an artificial diet containing the cannabinoids will be desiccated rapidly, causing early experiment termination due to the insects' death. However, in a closed system, the high humidity provides an ideal condition for the fungal outbreak, which is difficult to eradicate. The authors suggest using a perforated lid or cheesecloth to supply sufficient air circulation while minimizing water loss from the media. In a natural environment, the caterpillars prefer to feed on the abaxial side of a leaf where humidity is higher while presenting fewer trichomes than the leaf's surface area[34]. Thus, placing a container upside down was exceptionally helpful while providing a refuge area or crawling wood stick. This also helps to remove fecal matter from the media area and makes it easy to collect the waste for further assays.

As cannabinoids receptors are absent in invertabrates[35], the tobacco hornworm *M. sexta* might not be suitable for therapeutic studies mediated by the endocannabinoid system. However, with the numerous benefits demonstrated in our pilot study, the insect should be considered a new model system to investigate the pharmacological functions of cannabinoids, particularly studies involving non-CB receptor-mediated pharmacokinetics. The relatively short life cycle of *M. sexta* allows researchers to understand the impacts of a cannabinoid-containing diet over multiple generations, allowing for an experimental design in higher mammal model organisms.

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DISCLOSURES:
The authors have no conflicts of interest.

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Figure 1

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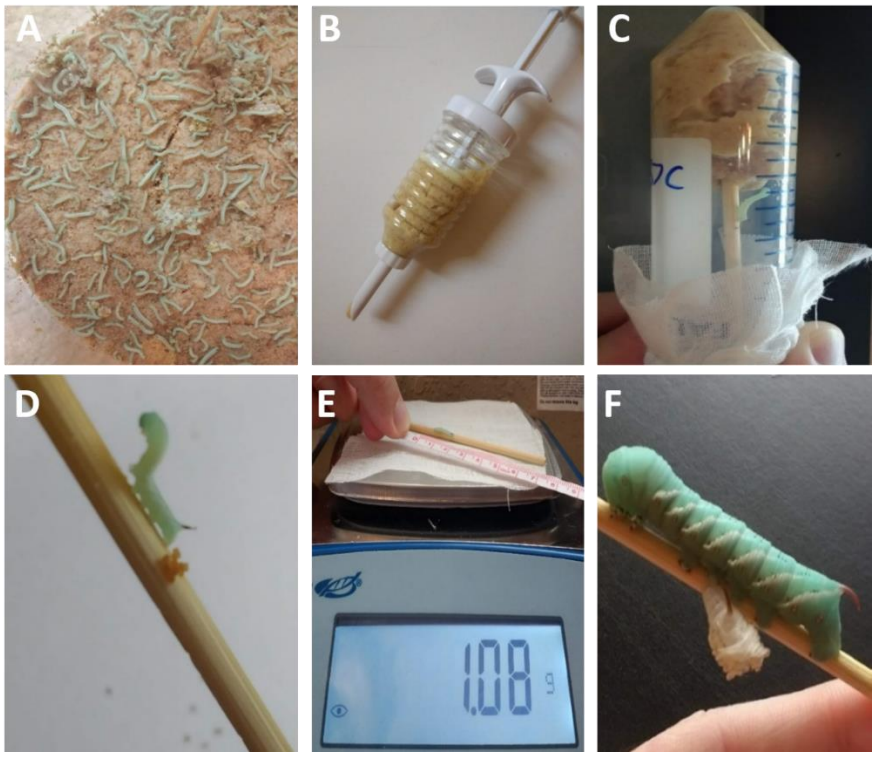

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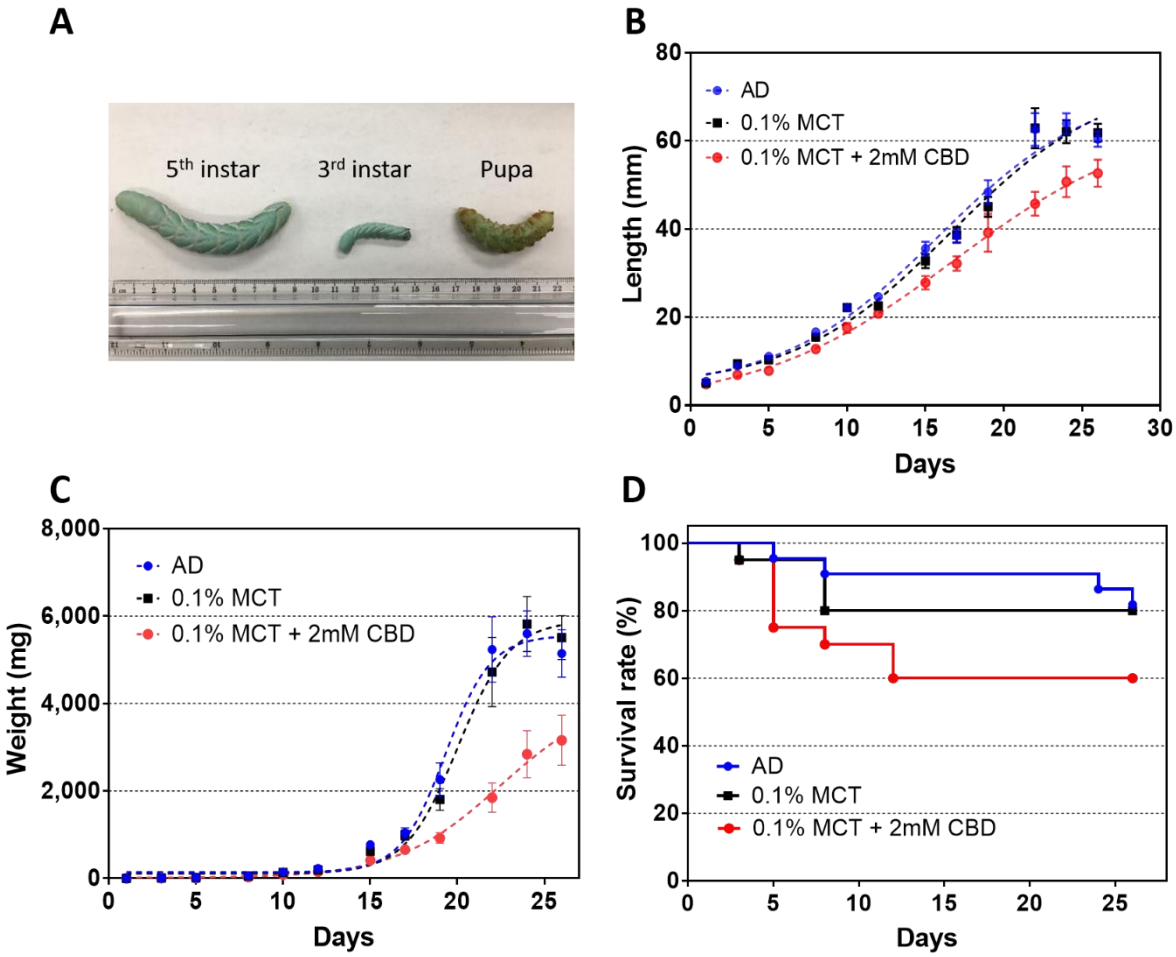
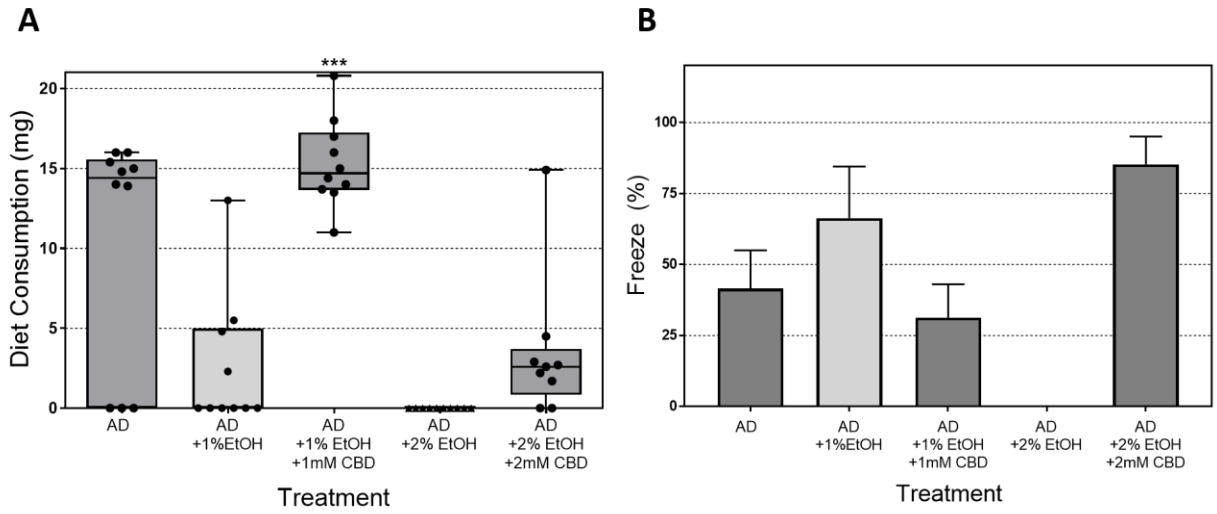


Figure 3



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Dear Dr. Park,

Your manuscript, JoVE63228 "Tobacco Hornworm (Manduca sexta) as an Insect model System for Cannabinoid Pre-clinical Studies," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Nov 22, 2021**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Nilanjana Saha, PhD
Review Editor
JoVE
nilanjana.saha@jove.com
617.674.1888
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Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
The authors carefully reviewed and improve the quality of the manuscript.

2. Please reword the following lines to avoid previously published work: 68-69, 74-75, 151-152, 156-157, 169-173, 188-194, 200-204, 208-209, 211-212.
The authors have revised those stated lines to highlight their importance in relation to our current protocol and avoid previously published work that may distract from main body of the manuscript. Thank you.

3. Please provide email for each author.
All author's email addresses have been provided in the manuscript. Thank you.

4. Please remove the media compositions from the Abstract.
The authors have considered three media compositions – control (wheat germ based artificial diet), vehicle (AD+MCT oil), and AD+MCT oil+CBD). These diets are very important to understand the study, so the information has been kept. However, media composition for preparing stock solution has been removed as suggested. Thank you.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Coulbourn Instruments, etc.
All the commercial brand and company information have been removed from the body of manuscript. The information is referred in the 'Table of Materials'. Thank you.

6. Was any ethical clearance needed to carry out this this? If yes, please include the ethics statement before the numbered steps of the protocol.
The authors have a confirmation from the University that nothing is needed for insect studies.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
The authors revisited the protocol steps and added detail information including reference to ensure viewers to follow the protocols without any issues. Thank you.

8. Please add more details to your protocol steps:
Line 138: Please mention the volume of stock solution added.
The volume of stock solution is added.
Line 143: How to recognize the 1st intar larvae?
1st instar larva is recognized by the size (~2mm long). The information has been added.
Line 156: How is the pupation stage recognized?
Pupation is recognized as 1) dehydration (losing weight and size), 2) immobilization, 3) hardened exocuticle layer, 4) brown coloration, and 5) hibernation. The information has been added.
Line 160: How to recognize the 5th intar larvae?
5th instar insects average 80 mm in length. The information has been added.

Line 164: Is the Actimetrics software freely available?
The program comes with the monitoring chamber.

Line 169: Please include citations for Tukey's post-test. Citation is added.
Line 172: Please include citations for log-rank (Mantel-Cox) test. Citation is added.

9. Please include one line space between the protocol steps and highlight that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
The essential steps of the protocol for the video has been highlighted with yellow.

10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. However, the NOTEs cannot be filmed, so please do not highlight.
The essential steps of the protocol for the video has been highlighted yellow. All the notes were excluded. Thank you.

11. Figure 1 legend: Please include the description of the (E) panel.
The description of the E panel is added. Thank you.

12. Here, the Figures are reused from a previous publication. Hence, please obtain explicit copyright permission to reuse any figure/table from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure/Table must be cited appropriately in the Figure/Table Legend, i.e. "This figure/Table has been modified from [citation]."
The copyright permission letter has been uploaded to the Editorial Manager account. Thank you.

13. As we are a methods journal, please ensure that the Discussion cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) significance with respect to existing methods
e) Any future applications of the technique

The discussion has been extended to include all the suggested items.

14. Please do not abbreviate the journal names in the References.
All the references have been reformatted according to the author's guideline. Thank you.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript focuses on the tobacco hornworm Manduca sexta and its potential use as an important insect model to study cannabinoid functions. The study demonstrated how cannabidiol (CBD) inhibited M. sexta growth and increased mortality of the insect-Manduca. Interestingly, CBD was also shown to increase the diet consumption and mobility of ethanol-intoxicated M. sexta. In this sense, the manuscript functions as an excellent guide in using M. sexta to study cannabinoids. I would strongly support the publication of this manuscript after the following points are well addressed.

Major Concerns:
No major concerns

Minor Concerns:
1. Line 154, "It is important to carefully handle the insect during the fragile periods of apolysis or ecdysis." The authors need to describe how careful handling is conducted or what to avoid.
The detail information has been added as following. "It is important to carefully handle the insect during the fragile periods of apolysis or ecdysis. When taking out of the larvae from a container, gently

grab the main body of the insect with a flat-tip and wide forceps and do not force to remove the outer layer of skin when an insect is in the process of shedding."

2. There is lack of some explanation for Section 2 of the Protocol Text. For diet loss, is there a specific set up for this assay? The detail information to measure diet consumption has been added in the section 2. For the distance tracking assay, are the instars allowed to familiarize with their surroundings before being forced to travel? Please clarify. Thank you for the comment. The authors allowed the insects to acclimate the chamber condition for at least 5 min prior to mobility response monitoring. The information is added.

3. On the Results part:
a) In Figure 2A, it is nice to clearly label the stages of the M. sexta on the figure. The stage for each insect is added.
b) The description of Figure 1(E) is missing in the figure legend. The missing legend is added.
c) In Figure 3B, are there any significant differences between the treated insects and control? No significant difference was shown between control and treated insects. As no statistical significance was shown so the results are re-written to avoid any confusion. Thank you.

d) Line 207, "Figure 3A" should be "Figure 3B". Changed
4. Authors mentioned the important impacts of cannabis on pregnant women in the Introduction part. Have the authors conducted fertility or fecundity assays using M. sexta? This question is for authors to consider but not required for publication of this manuscript. The authors also monitored post-pupation phenomenon (i.e., pupal size, moth transformation rate, fecundity, etc) and gathered preliminary data which is not included in this manuscript. The authors have found some meaningful results and working on publication. Thank you for the comments.

5. The fly model system for cannabinoid studies has been recently published as another insect model, well complementary with the Manduca model. The authors should cite the paper "He J, et la., Sci Rep. 2021".

The authors need to carefully proofread the manuscript before the publication.
Some typos are included but not limited to the following:
Line 32, "The authors suggest tobacco hornworm Manduca sexta as novel insect model system". Need to add "a" in between "as" and "novel".
Corrected.
Line 98, "The authors paid attention tobacco hornworm Manduca sexta". "to" is missing between "attention" and "tobacco".
Corrected.
Line 100, "The insect is most common plant pest". "the" is missing between "is" and "most".
Corrected.
Line 103, "Allowing molecular level understanding essential cellular processes". Please add "of" in between "understanding" and "essential".
Corrected.
Line 246, "To ensure the success cultivation of M. sexta larvae". Please change "success" to "successful".
Corrected.
Line 250, "The authors suggest use a perporated lid". Please change "use" to "using" and "perporated" to "perforated".
Corrected.
Line 252, "Caterpillars prefered to locate at the abaxial side of a leaf". Please correct spelling of "preferred".

Corrected.

Reviewer #2:

Manuscript Summary:

This manuscript alleges to develop a ne insect model for the study of cannabis. However, it in no way develops or characterizes the model, only describes how to mix a diet essentially. Everything else is focused on general insect rearing (which is already known and published) and even that is questionable in that the author's mention a fungal problem, but don't recommend the use of an antifungal like tegosept. Lastly, this paper for some reason looks at the effect of cannabis on insects that have been fed high ethanol diets, but there is no justification for this in the paper or explanation of why they tried to look at the effect of cannabis on intoxicated larva. These descriptions of the methods are unclear as well. Overall, this paper is not suitable for publication.

The simplicity of experimental setup (i.e., diet preparation, environment controlling) with large number of insect species is the biggest strength of this insect model system. Also the data consistency and repeatability over multiple generation gives another advantages to cannabinoid researchers who have limited access to animal model systems. Our research team developed the insect system for the first time for the cannabinoid research. The detail protocol has not been published, although a brief summary was published in our previous article in 2019 at *Scientific Reports*. In order to properly setup the insect experiment, comprehensive understanding of the insect life cycle, physiological and behavioral characters are critical to ensure the success of the experiment. The authors investigated *M. Sexta* for almost 5 years to establish the best growth conditions and to find the best solvent agents for cannabinoid testing. We hope the system is of great help for other cannabinoid research scientists, especially for those who are interested in therapeutic functions of cannabinoids that are mediated by non-CB receptors.

The proposed insect model system efficiently proved the insecticidal effects of CBD when used MCT oil as solvent agent. Also the authors tried to demonstrate that the insect model system was used to prove the therapeutic function of CBD on ethanol intoxicated insects. The model enabled us to investigate non-CB receptor mediated therapeutic reaction of cannabinoids in only 20 days. Currently, other minor cannabinoids are being tested with the model, showing very different effects on each treatment, providing hints for us to design animal experiment. The authors wish to share our know-hows on media preparation, establishment insect rearing environment, contamination management, and data collection etc.

Major Concerns:

The authors do not develop the necessity for this model, nor do they characterize the model. The experimental design is not explained nor justified. The logic behind why cannabis would affect alcohol induced behavior is not present nor clear. The negative effects of cannabis are demonstrated, but the doses are very high and it is unclear how these are relevant to human data at all, which is the point of a "model". Overall, the writing needs to be improved and edited. Overall, this does not appear to be an appropriate paper for a Methods journal.

As this article was initially intended to a protocol-focused manuscript, the experimental background has not provided. Instead, the author referenced our previous paper published in Scientific Reports, 2019. In this JoVE manuscript, the authors focused high CBD dosages (1mM and 2mM) to investigate the defensive role of the plant secondary metabolite and rescuing effects on the ethanol intoxicated larvae. In our previous article, the author investigated different CBD solvent agents ranging from DMSO, MCT coconut oil, EtOH, Tween 20, and methanol, as well as varying concentrations of CBD (0uM, 10uM, 100uM, 1000um to 5mM, 10mM). It appeared that 1mM CBD was the concentration that the insects were able to survive while presenting clear

detrimental effects of CBD. While testing different solvent agents, we also learned that the insects faced lethality at >1% EtOH. However, the high dose CBD administration (>1mM), which was detrimental to growth, showed clear rescuing effects on the EtOH intoxicated larvae. More than >40% of EtOH-stressed insects survived while only >2% EtOH treatment control group showed 100% mortality within a day. From our previous work, we learned that the insect model system was very effective show any beneficial- or detrimental effects of cannabinoids within relatively short time of period compared to other mice model system with less labor and costs.

The authors agree with the reviewer's comments. For better understanding of this study, the brief background/justification of the experiments were added to the main text in the manuscript. Thank you so much.

Minor Concerns:
line 46: What is a "higher mammal"? Compared to insects or other mammals?

It means various animal model systems, ranging from small animals such as a mouse, guinea-pig, or rabbit to large animals such as a canine, piglet, monkey, or horse have been used to explore unknown therapeutic effects. Mice have been the most preferred animal model system for cannabinoid research due to their anatomical, physiological, and genetic similarity to humans. It is indicated in the introduction lines 77-80. Thank you.

line 53: should be "public attention has been centered on" Corrected.
line 56: something can not be "exponentially used in food". Do the author's mean its use in food has exponentially increased? Corrected. Thank you.
line 61: missing "the" Added
line 65: What is a "pure" population of cells? A 'pure' cell line means no cross-contamination of other cells as well as other chemical and metabolite contaminants. The pure cell lines provide a consistent sample and reproducible results. The additional information has been added. Thank you.
line 77: Why does size matter in these comparisons? This question will come up later as well. As all the animals regardless of the size have endocannabinoid system, the size of the animal is important in regard to handling and examining a given treatment group. Particularly, from a maintenance perspective, smaller animals made it much easier to monitor the effects of cannabinoids in large populations, producing robust statistics.

line 81: It is stated that mice are similar to humans. As compared to? In addition to their phylogenetic relatedness, anatomical, and physiological similarity to humans, mice and humans contain about 3.1 billion base pairs and 85% similarity of protein-coding regions between the mouse and human genomes. Also, the genetic regulatory system of two species are largely same and has a similar endocannabinoid system (lines 83-85).

line 98: "focused" instead of "paid attention" also missing a "the". Corrected.
line 104: missing "of" Added.
line 105-107: Why does the large size matter? It is irrelevant as a model for humans. Does it make them easier to manipulate? Compared to other insect model system such as fruit fly, the larger size enables researchers to easily monitor morphological-, behavioral changes in real-time in response to CBD treatment. Also due to the size, electrophysiological responses were also able to be examined with abdominal nervous system including ganglia that were dissected from the larvae without high resolution microscope settings (lines 111-114). Thank you for the comment.

line 112-116: It is unclear whether these animals would naturally feed on Cannabis. Is this truly an adaptive response or random? In hemp fields in Colorado, there are several pest insects

observed which includes different caterpillars. What is the mechanism by which this deters the animals from ingesting it? The authors conducted RNA-Seq and metabolomics to understand the defensive mechanism with the insects that reared on high dose CBD containing artificial diet and found very promising mechanism. The results will be published soon.
line 140; an extra "a" Corrected.
line 141: This seems extraneous and if this is the level of methods to be included it would benefit from more details on the actual rearing chambers. The author wish to emphasize the importance hand blending instead of grinder due to the viscosity of the artificial diet. We agree with the reviewer that this seems irrelevant but practical advice.

line 145 "in an insect rearing" Please describe the chamber.
The information can be found at the Table of Materials. Thank you.

line 152: "Scrape" not scrap Corrected.
line 153: Which purposes are the fecal matter being collected for? The fecal matters can be used to calculate how much CBD is accumulated and/or passed through the system, as well as how CBD affects the microbial community in the system.

line 160: No details provided. More detail information has been added with reference.
Line 163: No details provided. More information has been added with reference.
line 196: This is the most confusing part. It is not at all clear why an ethanol study was carried out or how it relates to anything. The authors appreciate the reviewer's comment. The results 2 and 3 were presented to demonstrate Manduca sexta as a model system for 1) does-dependent CBD toxicity and 2) therapeutic function investigation. Thus, the subheadings have been changed to reflect the purpose of the experiments. In addition, the ethanol study is important to mention in the protocol as the authors have previously identified potential lethality issues of ethanol as a delivery vehicle for the CBD that should be considered in future experiments.

line 238-242: How is this relevant to humans? Doses? Responses? This is unclear.
The reviewer is correct that the results from the proposed insect model system is not directly relevant to humans. However, the insect system would provide clues of the unknown therapeutic function of cannabinoids, allowing for an experimental design in higher mammal model organisms, eventually leading to a human clinical trial. As invertebrates that do not have CB-receptors, any pharmacokinetic actions of cannabinoids shown in the *M. sexta* model system is mediated through non-CB receptors (e.g., TRPV, 5HT). The system can be used to investigate pharmacological functions of cannabinoid(s), particularly studies involving non-CB receptor mediated pharmacokinetics.

line 246: missing "of the" Added.
line 248: "cease" should be "death" Corrected.
line 251: If fungus is an issue, why not consider an anti-fungal. This paragraph argues against this model system as being appropriate and understood by the authors
By establishing the grow environment as suggested, the mold was effectively controlled and also the authors wanted to minimize possible effects of fungicide in the system.

Figure One: What type of scale?
Mettler AE 100S precision digital analytic laboratory scale balance. The information is added in the Table of Materials. Thank you.

Reviewer #3:
Manuscript Summary:
The manuscript is well written. Clear introduction, reasoning and detailed protocol. And data looks solid.

Major Concerns:
None

Minor Concerns:
Yes, my two minor concerns are
1. If the authors are interested in understanding physiological modifications in Manduca that affects its growth and development, they are missing a few major mile stones. This include mass gains (final mass over initial) at different stages. I encourage authors to look at recent manuscripts on plant defenses, secondary metabolites and their impact on Manduca. Second is about pupal mass, pupal volume and time to complete each life stages these are quite important and should be discussed.

The authors appreciate your concern and have addressed your concerns about larval and pupil measurements within the protocol, as data that can be collected as part of this protocol (see steps 2.1, 2.1.1, 2.1.2, and 2.2). The authors agree these measurements and the timeframe of developmental stages are important to include to understand the physiological effects of cannabinoids on the larvae and pupil. These changes have been incorporated into the protocol. Thank you.

2. The rearing method using tubes seems to be more vulnerable to infection- not much aeration, fecal matter build up, condensation from artificial diet. Standard use of plastic containers with mesh and paper towels that would absorb moisture and let fecal matter fall through the mesh is a good alternative.

The authors have tried varying size containers with a perforated lid. One of them we used was a round plastic container (100 mm ×50 mm) that provided more aeration but once mold outbreaks, a lot of media (>30 grams) had to be replaced. It was costly and labor intensive to replace. Also the round container takes up too much space in a rearing chamber as we have at least 50 replications for each group (control, vehicle, and treated). To avoid the condensation with aeration, the authors sealed the 50 mL tubes with cheese cloth and placed the tube upside down to allow aeration but minimize humidity loss. Additionally, by doing so, all the fecal matter and skin sheds were easily separated from the media. Thank you.

Journalpermissions

Tue, Nov 16, 9:35 AM

to me

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