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Assessing Arthropod Responses to Volatile Organic Compounds from the Algal Microbiome of a Sloth using Drosophila Melanogaster

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ASSESSING ARTHROPOD RESPONSES TO VOLATILE ORGANIC COMPOUNDS FROM THE ALGAL MICROBIOME OF A SLOTH USING *DROSOPHILA MELANOGASTER*

by
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ABSTRACT

Sloths contain an unexplored ecosystem of microbial diversity in their fur, including algae, fungi, bacteria, and arthropods. These symbionts are able to communicate through the chemicals they release, including volatile organic compounds (VOCs), which can lead to unique ecological interactions. The VOCs released from the algal polycultures of sloth hairs merit further investigation in their ability to cause a response from other organisms in this microbial community and within a broader ecological context. This research team aimed to assess the response of *Drosophila melanogaster* as test subjects when exposed to VOCs of the algal microbiome of a sloth. Exploratory research collected within the lab indicated that extracted metabolites from algal samples provided attractive responses when cultured between two and four months. Further research of extracted supernatant minus metabolites and other fractions of algal samples will offer more data for analysis, determining an attractive or aversive relationship between these extracted algal compounds and the response of various arthropod test subjects. By examining these interactions, this research highlights the potential of sloths as models for microbial ecology and demonstrates the complex interactions of this intricate microbial community.

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LIST OF ABBREVIATIONS

KSM - kitchen sink medium

mL - milliliter

g - gram

MeOH - methanol

VOCs - volatile organic compounds

SVOCs - semi-volatile compounds

Temperatures: ℃ - degree Celcius, ℉ - degree Fahrenheit

TSI - The Sloth Institute

min - minutes

Sh - shoulder

He- Head

NCNPR- National Center of Natural Products Research

INTRODUCTION

The motivation for this research stemmed from an observation of Azteca ants and sloths at The Sloth Institute in Costa Rica, who both inhabit Cecropia Trees. Sloths, reliant on the leaves for sustenance, prey on these trees, yet the resident ants, which typically attack other herbivores, do not seem to attack sloths that are responsible for destroying their home (Kaup, 2020). Algae, which gives wild sloths a distinctive green hue, serves as a habitat for a variety of arthropods besides ants, including lice, mosquitoes, and ticks. (Marting, 2019). During efforts in the Hom Lab to cultivate algae that grows on the hair of sloths in the wild, our research team observed potent smells produced by the algal cultures, suggesting that these aromas may play a role in mediating sloth-arthropod interactions. Our lab sought to dive deeper into the biological and chemical aspects of the compounds produced by these algal cultures. This thesis summarizes our preliminary efforts to fractionate algal supernatants and to test the responses of fruit flies to these fractions, especially those containing "secondary metabolites," or special metabolites that are not directly involved in "primary" functions of normal growth, development, or reproduction (Sanchez & Demain, 2011). We used the fruit fly, *Drosophila melanogaster*, as a general arthropod model because it is relatively easy to work with in the laboratory and because it is a well-established system to study behavioral responses to smells (Scherer et al., 2003).

1.1 Sloth Microbiome Composition and Ecological Interactions

The extraordinary biological diversity present in tropical forests fosters an abundance of chemical compounds that could be potential sources of bioactivity for pharmaceutical or agrochemical products (Kaup, 2020 and Higginbothom, 2014). The complex tropical forest ecosystem harbors intricate symbioses of microbial biomes that remain to be analyzed, including

those associated with mammals (Paracer, 2000). Arboreal sloths, *Choloepus spp.* and *Bradypus spp.* carry a diverse microbial community on their coarse outer hair (Aiello, 1985; Higginbotham, 2014; Kaup, 2020), including many species of algae that have yet to be characterized (Kaup, 2020). The external cortex, where the algae resides, appears on the thick outer coat of hair; the unique grooves of the *Bradypus* sloth hair provides an environment that encourages colonization of algal communities when soaked with water (Kaup, 2020). Algal species release volatile organic compounds, meriting further investigation of how their excretion may mediate interactions with other organisms in the ecosystem. Arthropod interactions are of particular interest for this study because of their apparent association with the arboreal sloths (Kaup, 2020).

The slow-moving lifestyle of sloths is reflected in the complex ecosystems that inhabit their fur. Moving less than thirty five meters per day, the languid, sluggish pace denotes a slow metabolism and little defecation, less than once per week (Gilmore et al., 2001). Pauli (2014) hypothesized that rather than remain hidden in the safety of the canopy, these arboreal creatures traverse down to the forest floor to relieve themselves, sacrificing their safety from predators. They observed mature moths residing in sloth fur laying eggs in sloth dung After hatching, the larvae feed on the nutrients of the sloth's feces until maturity and presumably fly upward through the canopy back to the sloth (Pauli et al., 2008). Higher moth abundances within the fur of these mammals correlated with higher concentration of nitrogen, which continuously fertilizes the algal community. These three-way interactions of sloths, moths, and algae demonstrate both insect interactions and the importance of algal compounds in microbiome of a sloth (Pauli et al., 2014).

Various bioactive compounds produced by the sloth fur microbiome have recently been investigated for their pharmaceutical potential. A 2014 study isolated eighty-four fungal compounds from the surface of hair that was collected from living brown-throated three-fingered sloths (*Bradypus variegatus*) in the Republic of Panama (Higginbotham, 2014). Metabarcode sequencing of the ITS1 locus revealed the taxonomic composition and bioactivity of fungal species found associated with the hair of sloths. Twenty of the isolated compounds extracted from the fungal strains were active against at least one bacterial strain, and one had an unusual pattern of bioactivity against Gram-negative bacteria that suggests a potentially new mode of action (Higginbotham, 2014). Interestingly, the compound's bioactivity against the breast cancer cell line MCF-17 reduced cancer cell growth by fifty percent or more and some fungal extracts also inhibitory activity towards parasites responsible for malaria (*Plasmodium falciparum*) and Chagas disease (*Trypanosoma cruzi*) (Higginbotham, 2014). Determining the bioactivity of microbiome and specific microbial extracts could provide further insight to the incredible potential this novel microbiome could play in increasing sloth resistance against pathogens, as well as development for human purposes.

1.2 Algal Volatile Organic Compound Bioactivity

Volatile and semi-volatile organic compounds (also referred to as VOCs and SVOCs) serve as a critical group of infochemicals within both aquatic and terrestrial chemical ecology (EPA, 2014). VOCs are low-molecular compounds with low to moderate hydrophilicity, allowing for dissolution in water and dissipation into the gas phase at air-water interfaces (Fink, 2007). SVOCs, while similar, evaporate at a higher temperature, releasing no aroma when emitted (EPA, 2014). Infochemicals are often employed by small organisms to provide critical

information about the surrounding environment changing conditions (Klaschka, 2008). VOCs are a concern in water processing facilities, since aquatic primary producers are the reason for taste and odor problems in drinking water (Fink, 2007). In terrestrial ecosystems, VOCs influence ecological interactions including those between plants and insects, communicating information such as nutrient availability or the presence of water. More specifically, volatile signaling molecules have been well studied as guiding cues for predatory insects in tritrophic interaction (Kergunteuil et al., 2020). Plants can also respond to herbivore attack by releasing volatile infochemicals that attract predators that attack the herbivores, thereby reducing the plants' tissue loss by herbivory (Fink, 2007). Herbivore-induced plant volatiles can act as priming agents that prepare systemic tissues of the same plant, and neighboring plants, for incoming attacks (Mbaluto et al., 2020).

Volatiles released by algae in ecosystems include ketones, aldehydes, esters, alkenes, alkanes, furans, terpenoids, and sufo compounds (Mbaluto et al., 2020). The release of VOCs from plants is influenced by a number of ecological factors such as sunlight, water availability, and nutrient deposition. These abiotic factors alter critical ecological processes and thereby have significant consequences on multitrophic plant-mediated interactions, changing direct defenses such as primary and secondary metabolites (Mbaluto et al., 2020). In terrestrial ecosystems, more than 30,000 VOCs are released from higher order plant leaves, flowers, and underground parts through secondary metabolism pathways (Zuo, 2019). Many of these compounds signal particular responses for the organism such as communication, pathogen defense, and inhibition of seedling germination and growth (Kong et al., 2019). The potency of primary volatile release is affected by varying degrees of sun exposure. For example, isoprene is released by microalgal samples in increasing intensities based on the amount of sunlight available (Achyuthan et al.,

2017). Cyanobacteria, diatoms, and green algae release isoprene through direct synthesis based on the availability of energetic cofactors (Zuo, 2019). Algal VOCs serve important functions in enhancing algal resistance to predators, communicating with homogenous algae, and playing allelopathic roles in heterogeneous algae and aquatic macrophytes (Zuo, 2019). Therefore, fractionating algal samples and isolating compounds from algal samples could provide context to the particular VOCs responsible for these critical interactions in algal communities.

Allelopathy, chemical inhibition of another organism due to chemical release, is noted to result in reduction, and often disappearance, of other algal compounds in both aquatic and terrestrial ecosystems (Holland & Kinnear, 2013). Similar to the algal toxins, cyanobacteria use allelopathy and emit VOCs, reducing the abundance of competitors within the ecosystem (Fink, 2007). Cyanobacteria are capable of rapid growth (algal blooms) when exposed to nitrogen and phosphorus, and VOC production offers them a competitive advantage against other organisms in similar respective ecosystems. When *Chlorella vulgaris* was exposed to the VOCs from the cyanobacterium *Aphanizomenon flos-aquae* under N-depleted conditions, cell growth, photosynthetic pigment content, and photosynthetic abilities drastically decreased (Zuo, 2019). VOCs also serve as information transfer messengers, warning nearby cells of upcoming stress and allowing for necessary preparation through cell signalling.

One final mechanism that promotes propagation and resilience of an algal population is the ability of VOCs to deter predation. For example, when damaged, diatom cells release polyunsaturated eicosapentaenoic acid which is toxic to crustacean herbivores and polyunsaturated aldehydes to repel herbivorous zooplankton (Zuo, 2019). Inhibitory effects deter other organisms from establishing in an environment and often impact their development of other compounds, critical to protection against predators.

VOC communication remains an active field of research in microbiology. Emission acceptors identify and recognize the signalling molecules released in order to make appropriate response decisions. It is important to determine both identification of complex volatile organic compounds released and further analysis of information agents in order to determine these ecological relationships. By analyzing factors that influence VOC emission from algal compounds and its continuous modification, we can better understand how other species within the ecological community respond to these chemical signals. In particular, understanding how arthropods respond to the volatile organic compounds released from algal samples in terrestrial environments such as the Cecropia Tree can provide insight to a specific ecological interaction occurring in Costa Rica.

1.3 Chemical Communication in Arthropods

Arthropods, such as mosquitoes, sandflies, triatomine bugs, lice, ants, and ticks, are commonly found living on sloth hair, communicating with their environment through the use of their olfactory system (Kaup, 2020). Chemical messaging plays a role in arthropod survival, used to avoid predators and pathogens, to locate food, and to find potential mates for reproduction (Mbaluto et al., 2020). The use of chemical senses is one of the earliest mechanisms organisms developed for survival. Arthropods must distinguish between thousands of chemical signals in the environment in order to produce the right behavioral response (Renou, 2014). Although single compounds may trigger behavior, VOCs are often used by arthropods as a complex mixture that may include many odorants, and insects generally respond better to blends (Renou, 2014).

Extensive research regarding arthropod communication has been performed on both ants and fruit flies. Both insects possess antennae that allow them to detect chemical messages. This process also requires the use of an insect's chemosensilla lymph, which aids in the capture of the volatiles (He et al., 2019). The lymph is highly concentrated in soluble proteins that bind to the volatiles. There are two classes of these proteins that are found in the insect olfactory system, with the first class classified as Odorant-Binding Proteins (OBPs). OBPs project from the antennae of insects where they pick up the volatiles and carry them to chemoreceptors that are found on the dendritic membrane of olfactory neurons. Once received, the volatiles are turned into electrical signals that cause a response in the insect. The OBPs have been shown to aid in the discrimination between different chemical signals (Pelosi et al., 2014).

Insects, a class of the phylum Arthropoda, use their chemical communication differently depending if they are eusocial or not. Eusocial insects utilize a caste system, functioning on different levels of organization within their colony. Eusocial insects use communication to effectively delegate resources, divide labor and defend their colony. Pheromones, chemicals released from one individual to influence another, guide these social interactions, and social insects such as ants, bees, and termites create chemical language that guides an array of behaviors essential for the overall function of the colony (Sengupta & Smith, 2014). Non-social insects use chemical communication primarily for mating (Richard & Hunt, 2013). Azteca ants (Marting, 2019) and ants in general are an example of social insects. The ability of ants to communicate with their biotic environment has been critical to their success, communicating with members of their colony, other insect species, fungi and microbes (Chomicki & Renner, 2017). One crucial type of chemical signaling in ants is queen signaling, which alerts the colony that the queen is fertile and instigates brood caretaking (Chomicki & Renner, 2017). Ants also

signal through food, leaving behind trails of pheromones or chemical footprints that the other members of their colony can detect. Thus, ants expend less energy searching for fruitful sources of nutrition, allowing them to refocus their energy on taking care of the colony. In large colonies, ants often rely on each other to make decisions, rather than take unique, separate actions. Eusociality is important because entire colonies within a species can be prompted by one individual to guide interactions and responses within the environment.

Conversely, fruit flies (*D. melanogaster*) are a non-social insect, making decisions surrounding food availability and larvae protection independently. They do not depend on communication between individuals within the colony to search for nutrient availability or protection, and thus, make unique decisions dependent on their own attraction or aversion response to a chemical signal. They instead communicate through chemical signals when mating and when interacting with their environment (Richard & Hunt, 2013). Thus, *D. melanogaster* remains critical to testing responses to volatile organic compounds, as collected data represents the desire of each individual in a species, rather than the desire of the group as a whole responding to social chemical signalling (in the case of Azteca ants). For this reason, *D. melanogaster* constitutes the arthropods utilized in this study to respond to various volatile organic compounds.

As a model organism with a rich genetic and experimental toolkit, the *D. melanogaster* olfactory system and behavioral responses have been extensively researched. *D. melanogaster* has a sophisticated olfactory system with the ability to discriminate between numerous chemicals in the environment (Richard & Hunt, 2013). They have two main types of receptors for olfaction, which are the odorant receptors (ORs) and ionotropic receptors (IRs). Both ORs and IRs are located in the antennae of *D. melanogaster*, in which they are expressed on the olfactory sensory

neurons (OSNs) (Gomez-Diaz et al., 2018). OSNs are located in sensilla, which have pores that allow odorants to dissolve into the sensilla lymph. Volatiles and other odorants attach themselves to the OSNs causing the OSNs to signal the axons to the brain, processing the necessary signal. Certain odorants that attach to the olfactory receptors appear to have a specific biological significance for the flies, such as ethanol (Giang et al., 2017). Defined as key odorants, these compounds elicit a difference in behavioral response when two similar complex odor blends are offered (Giang et al., 2017). At the food source, they prefer to feed to ethanol-enriched food possibly due to its caloric value (Pohl et al., 2012) and/or intoxicating effect (Devineni and Heberlein, 2013). The attraction of flies to ethanol-containing food odors raises the question of what other volatile organic compounds may serve as a key odorant in attracting *D. melanogaster* to a particular source. The *D. melanogaster* olfactory system is important to this research as they reflect independent decision making as non social insects, and numerous fruit flies can be used in the same test without influencing the responses of others in the environment.

1.4 T-MAZE ASSAY DESIGN

The T-maze assay (Fig. 1**)** is an apparatus used in olfactory conditioning, allowing the *Drosophila* to choose between the two odorants placed in the arms of the collection tubes.

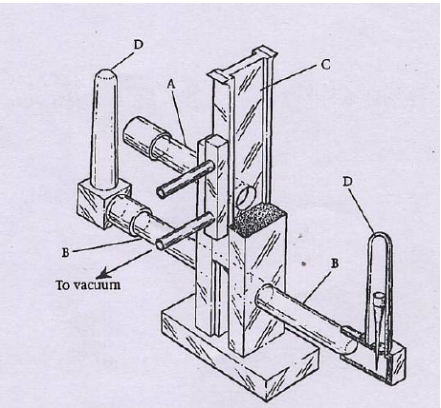

Figure 1. Diagram of simple T-maze apparatus (Tully & Quinn, 1985)

Flies are first placed in the introduction tube (Fig. 1-A) and shaken into the central compartment of the sliding elevator section (Fig. 1-C) where they can adapt to the initial conditions. A section of the right side piece has been cut away in the illustration to show the central compartment. The flies are then lowered to the choice point between the two currents of air. One of the air currents has passed over the algal metabolites samples housed in one of the odor block units (Fig. 1-D), while the other ar current passes over a control sample of KSM. The flies distribute themselves between the arms of the T-maze (Fig. 1-B). After two minutes, the elevator section is partially raised, trapping the flies in the arms of the T-maze. The arms can then be detached, and the flies in each arm can be counted (adapted from Tully & Quinn, 1985).

The assays conducted in this research analyze the effects of volatile emission on arthropod responses, utilizing algal fractions extracted from the hair of sloths from Costa Rica to determine aversion or attraction of *D. melanogaster*, allowing the research team to assess exploratory movement toward the compound or control.

2.1 Methods

The goal of this experiment was to observe the attraction or aversion response from *D. melanogaster* flies to various algal compounds taken from algal cultures derived from hairs on the head and shoulder of wild sloths in Costa Rica (Kaup, 2020). The protocol for conducting this research was divided into two parts: secondary metabolite extraction from liquid algal samples and a subsequent *D. melanogaster* assay using these extracted secondary metabolites. *D. melanogaster* chosen instead of Azteca ants for practical reasons: first and foremost, Azteca ants

have not been studied in a laboratory setting and efforts to do field experiments were not possible due to COVID-19 (Erik Hom, personal communication). In contrast, *D. melanogaster* is a model laboratory organism and there is extensive knowledge regarding olfactory behavioral responses (Pandey & Nichols, 2011). Additionally, they are not social arthropods and will make decisions independently from the rest of the *D. melanogaster* test subjects within each trial. We hypothesized that arthropods in general, and *D. melanogaster* in particular, would exhibit behavioral responses to isolated secondary metabolites from these algal cultures and we set out to test this.

Secondary Metabolite Extraction from Liquid Algal Samples

Secondary metabolites from sloth hair derived algal polycultures were extracted in 5 phases. In Phase 1 (see below) a reverse phase chromatographic resin was prepared to "soak" up these metabolites from algal supernatant, following the centrifugation of the algal polycultures (Phases 2-3). Resin beads were removed, leading to a "supernatant minus extracted" fraction (Fig. 2).

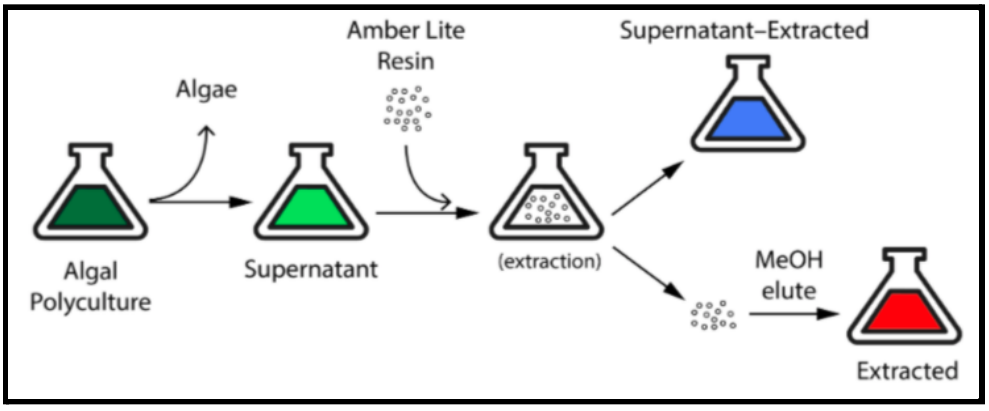

Figure 2. Steps of fractionation for the algal culture sample used. The algae is removed from the polyculture, leaving the remaining supernatant in the flask. Amberlite resin beads are added, extracting the secondary metabolites from the supernatant. Then, the beads, containing the metabolites of interest, are submerged in methanol. The metabolites are suspended in the methanol, and the beads are removed from the flask. Thus, three fractions are prepared for testing: the supernatant, the supernatant minus extracted metabolites, and the metabolite sample.

Extracted metabolites were eluted from the resin beads with methanol (MeOH) and dried down into a powder and weighed (Phases 4-5).

Phase 1: Preparation of Resin

Phase 1 in secondary metabolite extraction prepared Amberlite Resin XAD16N, 20-60 mesh from Sigma Aldrich (Product Number: 104219-63-8) for testing, utilizing a protocol developed in the Hom lab (Quach & Smith, 2021). Amberlite resin contains an antimicrobial and preservative substance that needs to be removed before use. The resin was prepared just before use (at 10 g of dry mass per L of culture supernatant), with the option of preparing it in batch quantities and allowing it to sit in water until further use. Deionized water was added to dry resin (1% w/v) in a large flask or beaker and swirled by hand to suspend and hydrate the resin. After letting the resin beads sit in the water for 30 min, water was decanted. Next, the same volume of MeOH as decanted water was added to the resin beads, and allowed to sit for 30 min after gently shaking to mix, and shaking every 5-10 mins. MeOH was decanted and resin beads were washed with MeOH in the same fashion. After washing, resin beads were stored in Nanopure water (50% w/v) to the container of beads, letting it soak overnight. The Amberlite should have had a wet mass (postprep) to dry mass (pre prep) ratio of 1.54 to 1. We removed the resin from the water the following day, placing them into holding boats and covering them with foil.

Phase 2: Processing of the Algal Cultures

Sloth algae cultures were chosen based on the subjective potency of their smell. After the most potent algal samples were selected by the lab team, preparation of the media began with extraction of algae from the liquid sample using serological pipets. No filtering was done to transfer the big clumps of algae to a new container of media, placing them back into the original

cultures of algae to continue growing. To clarify the media, we used a serological pipet to transfer the rest of the media to a coffee filter placed in a funnel, collecting the supernatant (liquid) in the beaker. We filled the container only ¾ full, so overflow would not occur when resin was added. Once filtration of the media was complete, we scraped the small clumps of algae that are caught in the filter into the container of new media with the big clumps.

Phase 3: Binding of Secondary Metabolites to the Resin

We added the Amberlite to the clarified media (10g resin/1L media), letting it sit overnight.. We stirred the mixture slowly (about one hundred to one hundred twenty rpm) and shook it gently (less than two hundred rpm) for thirty minutes. This resin has been shown to bind secondary metabolites <3000 Da in size in previous studies extracting natural products from microbial cultures (Amberlite, 2021).

Phase 4: MeOH Elution of Secondary Metabolites from Resin

We removed the resin from the supernatant, using a funnel and qualitative filter paper. Most large-pore filter papers are fine as only the Amberlite beads need to be kept. Then, we placed the resin from the filter into a new flask and added MeOH at the same volume as supernatant in Phase 3 to elute off the secondary metabolites into the MeOH fraction. After letting the resin sit overnight, we swirled the flask gently to mix.

Phase 5: Evaporation to a Dry Extract

We removed the resin from the MeOH through gravity filtration. The MeOH now contained the extracted secondary metabolites. We evaporated the MeOH fraction using a Rotavapor, setting the water bath of the Rotavapor to 40°C. We washed the round bottom flask with acetone first, then ethanol, and used the Rotavapor to condense the sample to a volume of

20 mL. Then, we transferred the 20 mL to a 4-dram glass vial. We washed the round bottom flask with ethanol to capture any sample left in the flask and added it to the vial. After further evaporation of the solvent in the 4-dram vial under vacuum with a Speed-Vac until the volume was small enough to transfer into the pre-weighed 1-dram vial. After transferring, we continued to evaporate until completely dry. Finally, we weighed the vial and recorded the new weight to determine the weight of extract.

Drosophila melanogaster Assay Using Extracted Secondary Metabolites

Phase 1: Preparation of Metabolite Vials for Trials

The aim of the *D. melanogaster* T-maze assays using the extracted secondary metabolites was to collect data on the flies' preference or deterrence from the volatiles in the extracted secondary metabolite samples. The first step in this process was to prepare the metabolites for testing, by filling 2 vials with 0.25 mg of dry extract per mL of KSM for each sample. To prepare these vials, the metabolites were resuspended in MeOH to make a 1:1 (w/v) ratio of metabolites to MeOH. Then, we pipetted 2.5 mL of the metabolites and MeOH solution into the testing vials. Next, we pipetted 10 mL of KSM into the same testing vials, thus creating the 0.25 mg metabolite per mL KSM vials needed for fly testing. Control vials were prepared as well by pipetting 10 mL of just KSM into the similar vials used for the extracts. Two were prepared for each algae volatile sample.

Phase 2: Preparation of *D. melanogaster* T-maze assay

We first transferred approximately 25 *Camillus "Buck" Sydney* flies into each of 25 fruit fly bottles containing fly food and allowed approximately 5 days to pass to produce the subsequent generation eggs. After 5 days, we discarded the parent generation, leaving only the eggs of the new generation. The new generation needed approximately 4 more days to mature. After this next generation of flies emerged, they were transferred into smaller food vials for testing. This was done by anesthetizing the flies in the food bottles for easy transfer into the food vials. We collected between 60-80 flies per food vial, using one vial for each T-maze apparatus and two per T-maze trial (see below).

Phase 3: Execution of T-maze Assays

T-maze assays were run using two sets of T-maze apparatuses (T-mazes #1 and #2) at the same time to control for any orientation differences (Fig. 3). For T-maze #1, we loaded an algal extract vial on the left "bubbler" and a control (KSM only) vial on the right. For T-maze #2, we loaded a control vial on the left bubbler and an extract vial on the right bubbler. We transferred *Drosophila* from the food vials to the top compartment of each T-maze. We tapped the left side of the T-maze onto the counter space to make the flies fall into the top compartment. We sealed this compartment by lowering the "elevator." We allowed the flies to rest for one minute so they could adjust to the new environment. Filtered air was "bubbled" into the sample at each end of the T-maze to aerosolized contents of the sample.

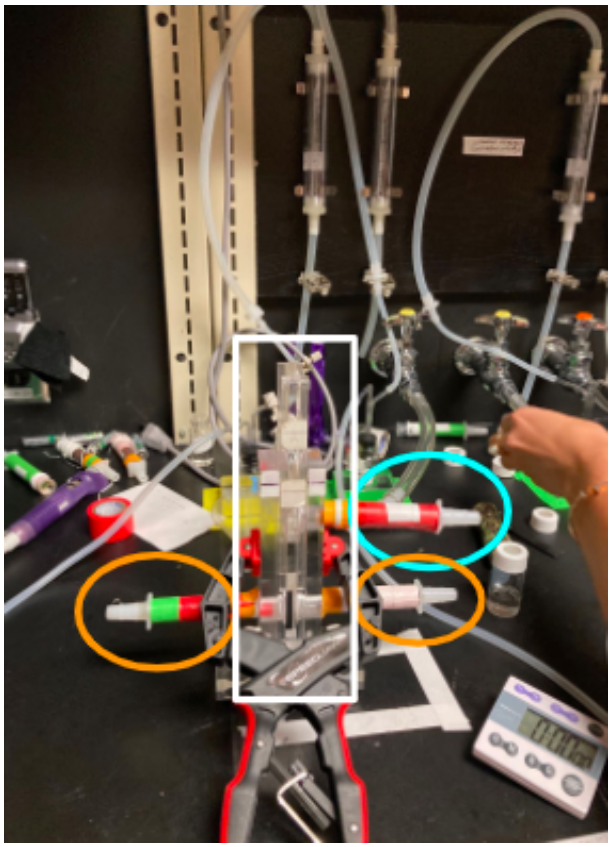

Figure 3. Photo of Simple T-maze apparatus used (real image). The white box highlights the central compartment of the sliding elevator, which is lowered to the orange circles, the arms of the elevator which contain the odor block units of algal metabolites and control samples. The blue circle shows the introduction tube, where the flies enter before being shaken into the central compartment.

We lowered the elevator down to the T-arms connected to the vials with the control and algal extracts to expose the arms to the bubbled sample aerosols. We kept the elevator there for two minutes to give the flies time to choose the preferred arm of the T-maze. After the two minutes, we raised the elevator to the middle line. We collected the flies in each arm of the T-maze, making sure to put them in different tubes based on which side they picked. Any flies that did not make a choice after 2 minutes were discarded. We repeated these steps six times for each

algae fraction. When finished, we placed the used collection tubes into a freezer and counted at the end of the trial.

Experimental Trials

The results of this thesis research is a compilation of three separate testing trials conducted at different times. These trials show the development of the experimental process, as we refined our testing methods and our samples in each trial. The first trial was performed in February 2020, the second trial was performed in November 2020, and the third and final trail was performed in March 2021.

In the first trial, two algal cultures derived from sloth hairs from "Mrs. Potts-shoulder" and "Shuri-shoulder" were selected, as they were two of the most potent smelling cultures that the lab team identified after collectively smell-testing approximately 360 different algal culture samples. In the second trial, we explored a larger group of 9 algal cultures but with a reduced number of replicates (6 instead of 12) to get a qualitative sense of how similar the responses of *D. melanogaster* would be among different potently smelling algal cultures; we focused specifically on the extracted (Amberlite resin) metabolite fraction. For this trial, we made sure to standardize the concentration of extract tested for each sample to 0.25 mg of extract per mL of KSM. COVID-19 delayed these trials by many months so that the age of the algal cultures tested were ~1 year old instead of 4 months old as originally planned. This set of algal cultures also included "Mrs. Potts-shoulder" and "Shuri-shoulder" extracted metabolites to see if age might lead to any change in *Drosophila* response. A third trial was performed in March of 2021 that tested a standardized 0.25 mg/mL concentration of extracted metabolite fractions from just 4 cultures with a greater degree of replication (12 replicates per sample); this included extracts from "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures that were grown for 4 months. This

was performed to confirm the responses we observed in phase 1 for extracted metabolites using a standardized extract concentration, and to examine 2 additional algal culture extracts, "Esperanza-shoulder 40" and "Merlin-head 44".

Analysis of T-maze Data

Based on the counted fly data for each replicate, the "response" of *D. melanogaster* to the sample presented to them was calculated according to the following equation:

$$\text{Response} = \frac{\#\ flies\ choosing\ sample - \#\ flies\ choosing\ control\ (blank\ medium)}{total\ \#\ flies} \quad \text{Eq. 1}$$

For trials 1 and 3, twelve replicate groups of flies (of 40-80 flies) were tested. For trial 2, only six replicate groups of flies were tested, so that we could test more algal culture extracts. Replicate data for each trial was plotted and analyzed in R (v4.0.0) using custom R code (Erik Hom and Damien Barrett, personal communication). Kruskal-Wallis rank sum tests were performed in R to test for differences in the response between sloth samples within a trial.

2.2 RESULTS

Twelve trials were conducted for this first phase of testing with results shown in Fig. 4. A response value with an absolute value ≥0.2 is considered to be a strong olfactory behavioral response (Maria Pena, personal communication). *D. melanogaster* were strongly attracted to the Amberlite extracted metabolites (red boxes) . Extracted metabolites from the "Shuri-shoulder" culture resulted in a stronger attractive response (0.51) than those from the "Mrs. Potts-shoulder" culture (0.23). The supernatant minus extracted metabolites fractions for both "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures were very strongly aversive to *D. melanogaster*

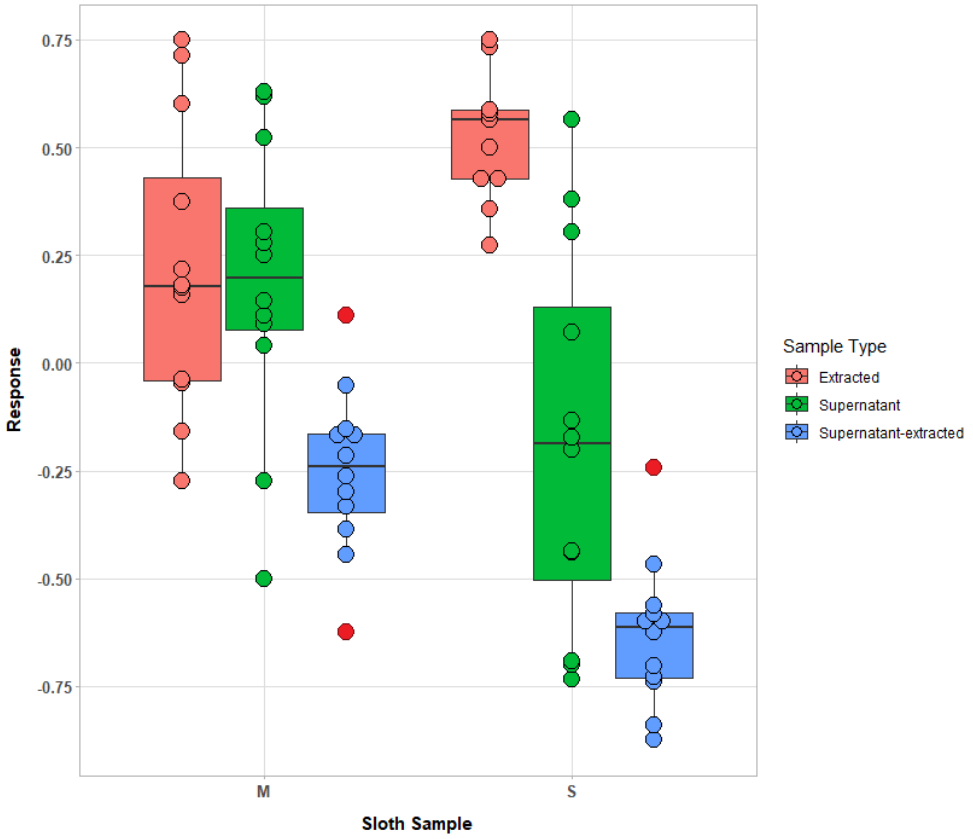

Figure 4. Different fractions of algal cultures show different avoidance/attraction *D. melanogaster* responses. Trial 1 results from *D. melanogaster* responses to fractions (y-axis) from two different algal cultures derived from sloth hairs from "Mrs. Potts-shoulder" (M) and "Shuri-shoulder" (S). Original culture supernatant is shown in green (supernatant), extracted metabolites using Amberlite resin in red (extracted), and the remaining supernatant without extracted metabolites in blue (supernatant-extracted). See Eq. 1 and description in the main text for how "response" was calculated from the results of the *Drosophila* T-maze experiments. Each dot represents a replica (vial of 60-80 flies) in the T-maze assay. Dots located side-by-side recorded similar avoidance values, and coding of the graph did not allow for overlapping dots. A positive response value indicates attraction to the tested fraction and a negative value indicates aversion to the tested fraction. The box spans the interquartile range with the box boundaries representing the upper and lower quartiles.. The horizontal line in the middle of the box denotes the median of the data replicates. The vertical "whisker" lines extend to the highest and lowest response values for each trial that were not outliers; outliers values are highlighted in bright red.

with response values of -0.25 and -0.56, respectively. The supernatants were on average, mildly attractive or aversive for "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures, respectively, although replicate data points span both positive and negative response values. Roughly

speaking, the responses observed for supernatants looked to be the sum of the responses to extracted metabolites and the supernatant minus extracted metabolite fraction.

Six replicates were conducted for the second trial using Amberlite extracted metabolites from 9 one-year old cultures (Fig. 5). For these data, values of the *D. melanogaster* responses were all aversive to the extracted metabolites, even the samples from "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures. Due to the wide range of negative avoidance response values, further statistical analysis was performed to determine a statistical significance for the values.

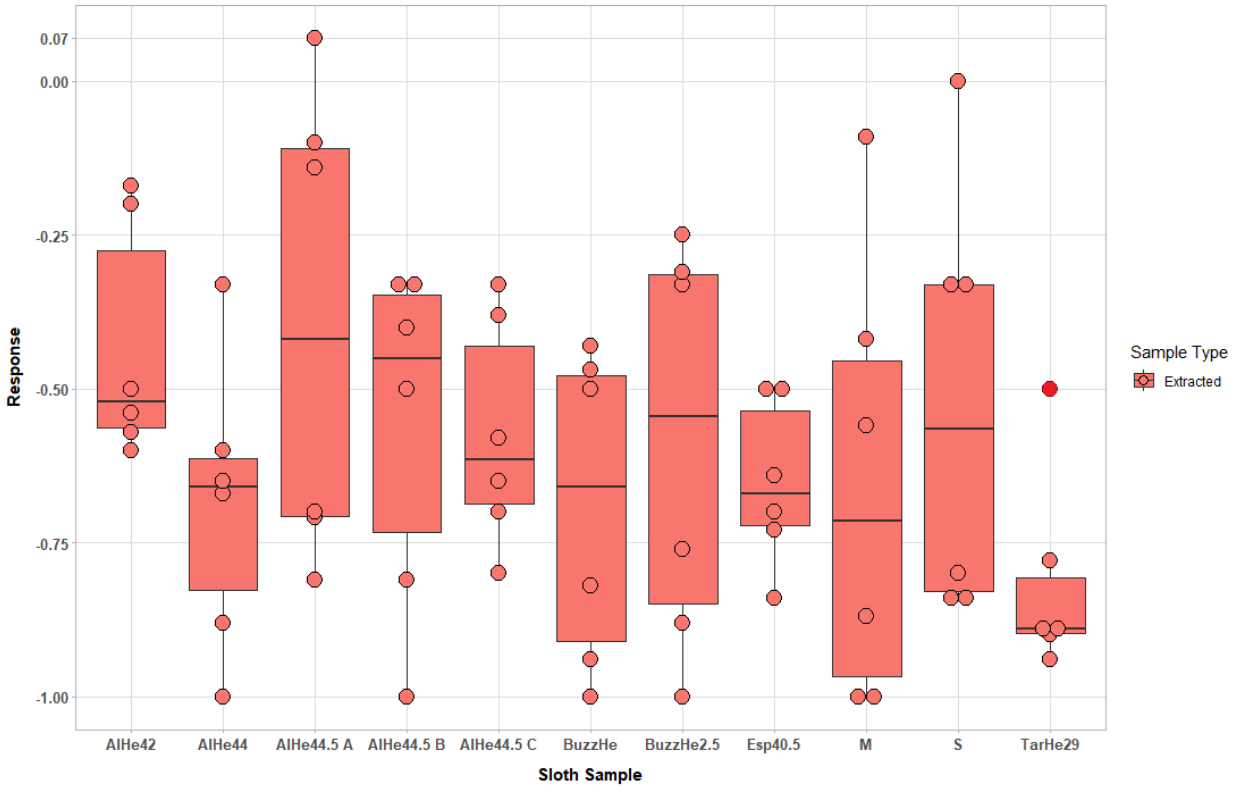

Figure 5. Extracted metabolites from 9 different 1 year old algal cultures are aversive to *D. melanogaster*. Trial 2 results from *D. melanogaster* responses to metabolite fractions (y-axis) with 6 different replicates per sample. One year old cultures of "Mrs. Potts-shoulder" (M) and "Shuri-shoulder" (S) cultures are also tested (cf. Fig. 2). Each dot represents a replica (vial of 40-60 flies) in the T-maze assay. Boxes represent interquartile ranges with median horizontal lines, and vertical "whiskers" indicating the high and low values of the data not considered outliers.

No significant differences in response (Chi square = 10.99), p = 0.36, df = 10) were found among the eleven sloth samples tested.

Twelve replicates were conducted for the third trial for extracted metabolites from 4 four-month old algal cultures (Fig. 6). These included extracts from "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures. *D. melanogaster* was strongly attracted to the extracted metabolites

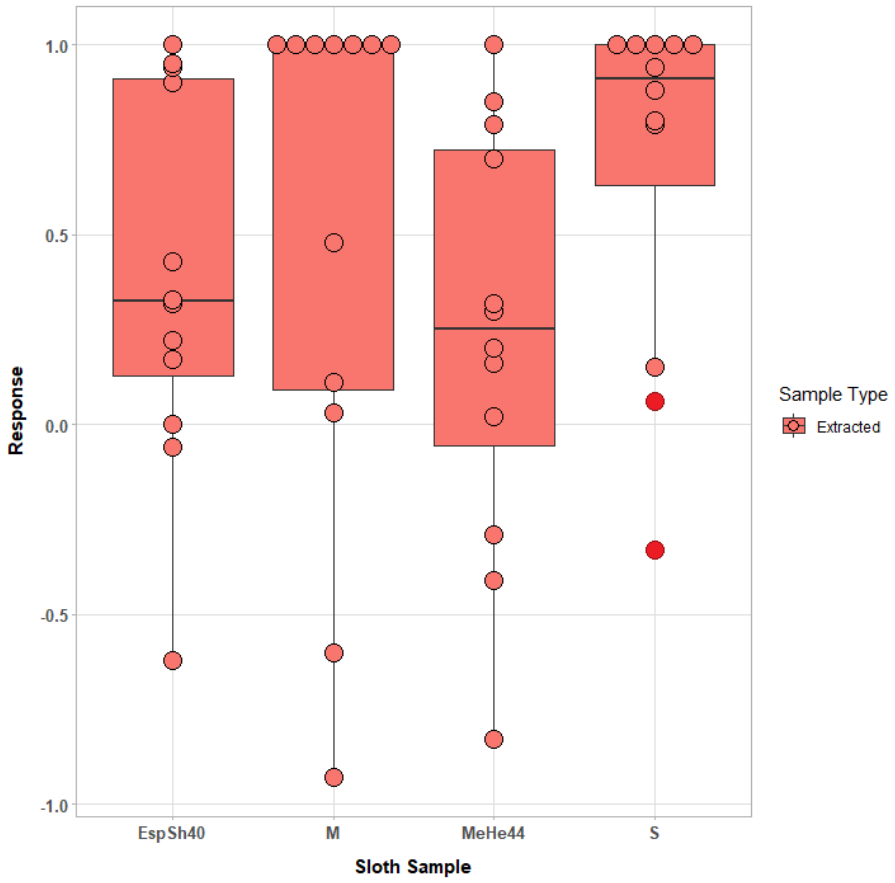

Figure 6. Extracted metabolites from 4 different 4 month algal cultures show different avoidance/attraction D. melanogaster responses. Trial 3 results from *D. melanogaster* responses to metabolite fractions (y-axis) with 6 different replicates per sample. Each dot represents a replica (vial of 60-80 flies) in the T-maze assay. Dots located side-by-side recorded similar avoidance values, and coding of the graph did not allow for overlapping dots. At a standardized concentration of 0.25, trial 3 showsSome of the outliers may be some systematic failure in experiment execution for these particular replicates, and we would like to repeat this experiment. Boxes represent interquartile ranges with median horizontal lines, and vertical "whiskers" indicating the high and low values of the data not considered outliers.

as each of the four sample's median avoidance value was ≥0.2. "Mrs. Potts-shoulder" had the strongest median attractive response (1?), followed by "Shuri-shoulder" (0.69). The two other cultures, "Esperanza-shoulder 40" and "Merlin-head 44", had extracted metabolites that were also attractive, although median responses (0.32 and 0.24, respectively) were not as strong as for "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures. However, due to the wide range of avoidance response values, further statistical analysis was performed to determine a statistical significance for the values. No significant differences in response (Chi square = 5.87, p = 0.12, df = 3) were found among the four sloth samples tested.

2.3 Discussion

The purpose of these assays was to determine whether *D. melanogaster* positively or negatively responses to the presentation of aerosolized material from fractionated sloth algal culture supernatants Because *D. melanogaster* are not a eusocial insect, many flies per replicate vial could be used for each individual T-maze assay without them influencing one another, unlike Azteca ants, which would by leaving a scent trail that would influence other ants that follow. Our extensive knowledge regarding the *D. melanogaster* olfactory system and their use as a test model made them useful for our experiments.

The extracted metabolite fraction was chosen as a particular focus following trial 1 results showing an attractive response by *D. melanogaster* (Fig. 4). Trial 3 experiments confirmed this attractive response in 4-month old algal cultures derived from the hairs of "Mrs. Potts-shoulder" and "Shuri-shoulder" at a standardized concentration of 0.25 mg/mL. Trial 1 was an initial exploratory experiment, and was performed without standardizing the concentration of extract used in the T-maze assay, thus making it hard to compare the response potency of "Mrs.

Potts-shoulder" and "Shuri-shoulder" extracts. However, from trial 3 results with standardized concentrations (Fig. 6), both "Mrs. Potts-shoulder" and "Shuri-shoulder" cultures seem to produce extracted metabolites that are comparable in attraction to *D. melanogaster* as the other sloth algal samples tested. The "Mrs. Potts-shoulder" results were more variable than "Shuri-shoulder", which may point to some problems with some replicates, and we would like to repeat this experiment.

The *D. melanogaster* response values for extracted metabolites in the second trial performed in November of 2020, however, were all negative, meaning that flies were repelled from the aerosolization of these compounds. Since the concentrations were standardized to be the same in the second and third trials,, the age of the polyculture appears to be a key factor. A longer growth period (1 year vs. 4-months) may lead to a transformation of metabolites (e.g., decomposition) into forms that are aversive. Alternatively, the overall composition of the metabolites produced in older cultures may be different due to the accumulation of more aversive compounds than attractive compounds relative to a shorter 4-month cultivation period. It is possible that the extracted pool of secondary metabolites contains a cocktail of compounds each of which would elicit an aversive or attractive response by *Drosophila*; shifting the proportions of these may lead to an overall attractive (e.g., in 4-month old cultures) or aversive (e.g., in 1-year old cultures) responses.

Due to limitations in available materials, the number of replicates used in trial 2 was reduced from 12 to 6 to allow for a greater number of algal samples to be tested (Fig. 5). The restrictions of COVID-19 during these trials also lengthened the growth period for trial 2 algal samples from 4 months to 1 year, which potentially led to a change in chemical makeup of the extracted metabolites as discussed above.

Improvements to this research include pursuing higher replication of data on all three algal fractions: extracted metabolites, supernatant, and supernatant-extracted. We chose to conduct second and third trials solely on the Amberlite extracted metabolites due to the strong attractive response observed in trial 1 testing, our labs general interest in genetically encoded secondary metabolites, and practical limitations due to COVID-19 caused by the pandemic. Given these initial results, the Hom Lab plans to follow up these results by further chemical fractionation of the extracted metabolites and using liquid (for SVOCs) or gas (for VOCs) chromatography coupled with tandem mass spectrometry to identify specific chemical compounds as is pursued in natural product discovery efforts (Hoffmann et al., 2014; Wang et al., 2016; Demarque et al., 2020). More finely fractionated extracts (or even isolated compounds) could then be tested again using the *Drosophila* T-maze assay to determine bioactivity with respect to fruit fly response. Testing fractions or isolated compounds in the T-maze assay using different concentrations other than 0.25 mg/mL may also allow us to determine the dose response of attraction/aversion. We believe the extracted secondary metabolites in our algal cultures comprise a wide variety of VOCs and SVOCs, so identification is critical for further understanding of arthropod response to these samples. Valerie Quach and Paige Smith (2021) used antiSMASH (Medema, 2011), a tool predicting secondary metabolite gene clusters in bacterial genomes, on metagenomic sequencing data the Hom Lab has pursued on the sloth hair microbiome as a complementary approach to determine extracted metabolite composition and potentially the genetic basis for how they are made. Preliminary comparisons between algal cultures derived from sloths obtained in "wet" (summer) or "dry" (winter) seasons show season-dependent differences in the composition biosynthetic gene cluster types (Quach & Smith, 2021). We are currently awaiting results from UM's National Center of Natural Products

Research (NCNPR) analysis of the antibacterial, antifungal, and anticancer activity of our extracted metabolites.

Future studies could use Azteca ants in the field, one of the original motivations for this research, to provide more natural data on the Costan Rican *Bradypus variegatus* sloth and the interactions of arthropods with the microbial community residing in sloth fur. Since Azteca ants are eusocial insects, however, either a single ant needs to be tested in a T-maze assay, or an entire colony would be treated as a single clonal response to the samples/fractions tested.. Future studies could also use other arthropod models including ants (Dupuy et al., 2006; Josens et al., 2009), beetles (Payne et al., 1976; Gruber et al., 2009), mites (Skelton et al., 2007; Zhang et al., 2009), or roaches (Nalyanya & Schal, 2001; Koohbdel et al., 2020).

2.4 Conclusion

By acquiring hair samples from sloth species in Costa Rica, the lab was able to create a collection of sloth-associated algal/cyanobacterial polycultures. Extracted metabolites from algal samples were used as the primary source of testing the aversive or attractive responses of *Drosophila* fruit flies to these metabolites, which we believe are the bases for the various strong and pungent smells we have observed coming from the algal polycultures. In conducting the three trials described, we refined the procedural techniques and best practices to assay *D. melanogaster* responses. Results from the first and third trials of this experiment showed that algal cultures grown for ~4 months provided attractive responses from *D. melanogaster*. More testing with the supernatant-metabolites and other fractions of algal samples will be underwayTogether with metagenomic sequence data and further chromatography-tandem mass spectrometry analysis of fractions, we hope to identify the VOCs and SVOCs that mediate

arthropod interactions with the sloth fur microbiome and of the sloths themselves. Similar research should one day utilize Azteca ants as test subjects, providing a better understanding of the native ecology of the sloth fur ecosystem.

APPENDIX 1

Kitchen Sink Medium (KSM) Composition:

Material	**Amount**	**Material**	**Amount**
$NaNO_3$	17.7 mM	KBr	0.1 nM
K_2HPO_4	0.18 mM	KI	0.05 nM
KH_2PO_4	0.27 mM	Na_2SeO_3	0.01 nM
$MgSO_4$*7 H_2O	0.3 mM	RbCl	0.58 nM
$CaCl_2$*2H_2O	0.24 mM	Na_2SiO_3	0.1 mM
$NaHCO_3$	0.19 mM	$SrCl_2$*6H_2O	0.56 nM
NaCl	0.43 mM	LiCl	7.31 nM
H_3BO_3	46.3 nM	biotin	0.008 nM
$MnCl_2$*4H_2O	9.15 nM	calcium pantothenate	0.84 nM
$ZnSO_4$*7H_2O	0.77 nM	vitamin B12	0.0015 nM
Na_2MoO_4	1.89 nM	folic acid	0.0045 nM
$Co(NO_3)2$*H_2O	0.17 nM	myo-inositol	22.2 nM
$CuCl_2$*2H_2O	0.46 nM	niacin	3.24 nM
Na_2VO_4	0.008 nM	p-aminobenzoic acid	2.9 nM
$AlK(SO_4)_2$*12H_2O	0.1 nM	pyridoxine HCl	1.94 nM
$NiSO_4$*6H_2O	0.05 nM	thiamine	1.33 nM
$CdCl_2$	0.05 nM	riboflavin	0.53 nM
$CrCl_2$*6H_2O	0.01 nM	$FeCl_3$*6H_2O	9.14 nM
Na_2WO_4*2H_2O	0.01 nM	disodium EDTA	9.95 nM

APPENDIX 2

Drosophila and Algae Testing - Standard Operating Procedure

a. **Aim/Rationale of Protocol**
   i. The aim of this protocol is to outline running drosophila T-maze assays using three different fractions of sloth algae, collecting data on the flies' preference or deterrence from certain algae volatiles of the sloth microbiome

b. **Introduction and Workflow**
   i. Preparing Fractions for Trials
   ii. Preparing Drosophila Fly Generations
   iii. T-maze Assay Execution

c. **Required Materials**

Materials	Qty.
Fly Assay Vials	12
Serological Pipette, 25 mL	8
Drosophila Food Bottles	25
CS Drosophila	625
Drosophila Food Vials	36
T-maze	2

d. Preparing Fractions for Trials

i. Fraction A) Supernatant

1. Pipette 2 mL of supernatant into fly assay vial making sure there is no algae in the sample

2. Pipette 8 mL of KSM into the same fly assay vial so that there is now 10 mL of total volume

3. The concentration of of the vials should be 0.25 mL supernatant/ mL KSM
4. Repeat steps to into another fly assay vial
5. Make 2 control fly assay vials by filling them each with 10 mL of KSM

e. Fraction B) Supernatant- Metabolites

- **i.** Pipette 2 mL of supernatant-metabolites into fly assay vial making sure there is no algae in the sample
- **ii.** Pipette 8 mL of KSM into the same fly assay vial so that there is now 10 mL of total volume
- **iii.** The concentration of of the vials should be 0.25 mL supernatant-metabolites/ mL KSM
- **iv.** Repeat steps to into another fly assay vial
- **v.** Make 2 control fly assay vials by filling them each with 10 mL of KSM

f. Fraction C) Metabolites

- **i.** Resuspend metabolites in X mL of MeOH, so that the concentration is equal to 1.00 mg metabolites/ mL MeOH
- **ii.** Pipette 3 mL of metabolites with MeOH into fly assay vial
- iii. Pipette 12 mL of KSM into the same fly assay vial, making 15 mL total
- iv. Let the 3mL MeOH evaporate completely so that the total volume of the fly assay vial is 12 mL
- v. The overall concentration of the vials should be 0.25 mg metabolites / mL KSM
- vi. Repeat these steps into another fly assay vial
- vii. Make 2 control fly assay vials by filling each with 12 mL of KSM

g. How to Prepare Drosophila Fly Generations

- **i.** Transfer approximately 25 CS flies into 25 food bottles
- **ii.** Allow approximately 5 days to produce subsequent generation
- **iii.** Empty parent generation into fly morgue, leaving only the eggs of new generation
- **iv.** Allow 4 days for new generation to mature
- **v.** Using CO2, anesthetize flies to allow for clean transfer to food vials
- **vi.** Collect approximately 50-60 flies per food vial, using one vial for each T-maze assay

h. T-maze Assay Execution

- **i.** Load the volatile and control vials in into the bubbler of the T-maze
- **ii.** For T-maze 1, load the algae volatile on the left bubbler and the control on the right bubbler
- **iii.** For T-maze 2, load the algae volatile on the right bubbler and the control on the left bubbler

a. Transfer Drosophila from food vials to the top compartment of plexiglass T-maze
b. Seal compartment by lowering the elevator
c. Allow flies one minute to adjust to new environment
d. Lower elevator down to goal arms containing control and algae fraction volatile
e. Allow flies two minutes to choose preferred side of the T-maze
f. Seal the two sides of the goal post by raising the elevator
g. Collect resulting flies in each goal post tube
h. Empty the remaining undecided flies into a fly morgue
i. Repeat six times for each algae fraction
j. Place the tubes used in a freezer to prepare them for counting
k. Count and record the number of flies in each tube

*Note: each trial (1-6) will be performed in two different T-mazes, with the control and the volatile occupying opposite sides of the T-mazes, ensuring no extrapolating factors such as side preference

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