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One Signal, Two Behaviors: Odor Discrimination in Unmated versus Mated Female Green Bottle Flies, Lucilia sericata

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Honors Thesis

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Abstract

The green bottle fly, *Lucilia sericata*, is of critical importance in the field of forensic entomology since it is one of the first insects to arrive at a freshly deceased carcass. These flies use a highly tuned and selective olfactory system to identify and locate the carcass; response to the decomposition odor usually occurs within minutes. Male flies use the carcass for a small protein meal and for locating females while females use the carcass for feeding (unmated) and egg-laying (mated). This divergence in behavior to a common odor cue is the subject of this thesis proposal: do unmated (feeding) females respond to the same odor cues as mated (egg-laying) females? The presence of two behaviors indicates that there may be an associated up or down regulation of gene expression of odor binding proteins during the two different stages of female sexual development. Olfactory response to selected decomposition odors in unmated versus mated females was determined using the electroantennogram (EAG) which measures the neuronal depolarization in antennae when an odor triggers a response. Fly heads were mounted on the EAG probes and exposed selected volatiles. Response was measured as the resultant change in voltage (a depolarization). Flies were also subjected to an odor choice behavioral assay. The results show a divergence in antennal response to VOC and choice of VOC at day 4 between the mated and unmated female flies. These results indicate that there are underlying molecular, biochemical, and physiological processes associated with fly response to odors.

Acknowledgements

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Introduction:

Lucilia sericata, the green bottle fly, is an organism of interest in the fields of entomology, ecology, and medicine due to its importance in forensic applications and recent medical applications such as wound debridement (removal of necrotic or infected tissue) and the wound healing and cleaning properties of the larval secretions. This organism is of interest in the field of forensic entomology since it is one of the first fly species to colonize a fresh carcass and is therefore used for post-mortem interval (PMI) assessments, or time since death.

Females and males use the carcass for different functions. It has been postulated that males use the carcass for a small protein meal and for finding and mating with females. The females are known to use the carcass for feeding (unmated females) and egg-laying (mated females). The volatile odor associated with a carcass is a complex suite of chemicals. Given the two very different behaviors displayed by females, this research proposal focuses on whether unmated versus mated females are responding to the same odor cues or if unmated (feeding) females respond to a set of odor cues that is different from those that attract the mated (egg-laying) females. The existence of two very different behaviors could likely be the result of up or down regulation of expression of proteins associated with olfactory sensing in these flies.

This thesis explores the olfactory response of unmated versus mated female flies exposed to volatiles associated with decaying carrion by conducting two different experiments. The experiments done were the physiological study using an Electroantennogram (EAG) followed by a behavioral study using a chamber system.

These two studies showed a divergence in behavior at day 4 of development between mated and unmated female *L. sericata.*

Literature Review:

The green bottle fly, *Lucilia sericata*, is a key organism in the ecological cycle of decomposition since it is one of the first insects to arrive on decaying carrion. As carrion undergoes microbial decay, a suite of odors, or volatile organic compounds (VOC), is released into the air and is the stimulus for attracting insects to the carcass. Decomposition can be viewed as a wave of processes ranging from fresh through dried remains decay phases (Rodriguez and Bass 1983). Different organisms use the carcass at different times during the decomposition period primarily as a food resource and for laying eggs. The presence and growth stages of insects on carrion are used for determining the post-mortem interval (PMI) or time since death (Tarone and Foran 2008). This metric is often a critical component in forensic entomology applications and can be introduced in court proceedings as evidence in murder cases. While PMI (determined from insect colonization data) has been used heavily in forensic cases, there is still a significant information gap in the scientific literature regarding how the insects sense and respond to the odors from a decaying carcass. The research conducted in Dr. Hansen's laboratory over the past five years has focused on finding out how the first colonizer, *L. sericata*, responds to VOC from decaying carcasses and how the resource is used by both male and female flies.

A comprehensive approach to understanding how *Lucilia sericata* responds to decomposition odors has been the focus of several research projects, Honors theses, and a dissertation in Dr. Hansen's laboratory since 2009. Standard culture protocols for *L. sericata* have been established in our laboratory (Blystone and Hansen 2014) and the effect of diet, age, and sex on adult gustatory and olfactory sensing have been explored

(Blystone, Ph.D. Dissertation 2015). Other research projects in the laboratory have focused on: the function of the arista, a featherlike appendage in the fly antenna, in sensing wind direction and odor detection (A. Jacob, Honors Thesis, 2014); the response of males to a specific flower scent, indole (E. Filbrandt, Honors Thesis, 2014); and the response of males to female pheromones (C. Kelly, Honors Thesis, 2015). Collectively, these studies have tried to determine how the males and females arrive at the carcass. These studies have led to a very interesting question on how the carcass is used by females. Females are known to use the carcass for two functions – for feeding (a protein meal to finish internal egg development) and to lay eggs (a food resource for developing larvae). These are very different functions and leads to the question: how are feeding females versus egg-laying females responding to the decomposition odor profile? It is a complex VOC signal that stimulates to two very different behaviors at two different life stages of the insect. The research proposed here focuses on that question: does one odor stimulate two different behaviors?

Olfactory sensing in insects is accomplished using the antenna that has many hair-like structures called sensilla on the surface (Jacquin-Joly and Merlin 2004). Sensilla also occur on other areas of the insect body, for example on the leading edge of the wings. These sensilla are the site of VOC 'capture'. Each sensillum has pores that allow access by the odor to the internal sensillum hemolymph fluid. The hemolymph contains odorant binding proteins (OBP) that bind with the odor and transport it to the sensillum neuron. Once the odor is received on the neuron surface by an odorant (OR), a depolarization cascade occurs and the organism responds (Hansson 1995; Golebiowski et

al. 2007; Wang et al. 2012). For females the response is to fly 'upstream' toward the source of the odor, the carcass.

Hypotheses:

H0: unmated females and mated females respond similarly to the carrion volatile organic compounds (VOC)

HA1: unmated females and mated females respond differently to the carrion volatile organic compounds (VOC)

HA2: unmated females and mated females respond selectively to male pheromones

Methods:

Culturing Flies

The flies that were experimented on were raised in two environments: same sex environment and opposite sex environment. These flies were kept in three kinds of bug dorms: 1) Unmated females 2) Unmated males (used for male pheromones in physiological assay) 3) Mated females with males. The separation was necessary because a parameter of the experiment was needed for the unmated females first exposure to male pheromones is during the experiments. To accomplish the separation the stock flies were cultured in tents where they were fed organic bovine liver, honey water, water, and sugar. The mated female flies' liver was taken out of the bug dorm at day three to ensure that they would not lay their eggs. This is important because it is believed once mated female flies have laid their eggs they start the mating process over again, which is not what these experiments were looking to study on. The flies then lay their eggs on the liver. The liver with eggs are then taken from the tent and put in jars with more liver where they develop through the larval stages into pupae over the course of seven days. Once the flies are in the pupae stage they are separated individually into cups where they emerge as flies. The flies were put in a designated bug dorm based on their sex and if they emerged in the same 24 hour period (= same age). The bug dorms held between 10-40 flies depending on the number that emerged in a given time period. The bug dorms were further separated into different incubators, with unmated females in one incubator and the unmated males and mated females in another. Both incubators were maintained at the same environmental conditions 26.6 degree Celsius, 21% humidity. These conditions were applied to control for different environmental effects on the development of the flies to determine the differences between the unmated and mated females in response to male pheromones and decomposition-related VOCs physiologically, behaviorally and morphologically.

Behavioral Assay

The behavioral responses of unmated and mated females were evaluated by a choice-based experiment. A chamber-apparatus was constructed to give the fly a choice between two different VOCs. The apparatus included a holding-chamber with two inputs

of humidified air delivered at opposite ends of the holding-chamber. Adhesive surfaces were put around the sides of the each of the two inputs of the holding-chambers. The inputs into the holding-chamber are connected to intermediate chambers holding a high concentration to a specific VOC, allowing the humidified VOC to perfume the holding-chamber. A solid line was drawn in the center of the floor of the chamber to distinguish preference of the fly. There are two levels of preference of the fly for the VOC: strong or moderate. A strong preference for a VOC is a fly that has become stuck to the adhesive surface near the input of the VOC. A moderate preference for a VOC is when the fly has crossed the black line to a particular side of the chamber but is not stuck on the adhesive surface.

The two variables in this experiment are male pheromones and a full complement of the carrion (liver). To allow the air stream to attain these volatile profiles, fifteen females and five pieces of organic bovine liver were placed in separate intermediate chambers. The liver was set out an hour before the experiment so it would warm up, allowing it to give off a more potent volatile emission. The negative control for this experiment was humidified air that was sent through an intermediate chamber with no volatile. This experiment was done as a general choice test so a more detailed approach may be done at a later time.

The experiments included unmated and mated female flies evaluated at days 1, 2, 3 and 4 post-eclosion. There were three choice tests: humidified air versus organic bovine liver, humidified air versus male pheromones, and organic bovine liver versus male pheromones. Each experiment consisted of three flies, all mated or unmated, placed in the holding-chamber for 15 minutes. The fifteen minute interval was determined to be an efficient amount of time based on research done by Frederickx et. al. (2012) and Kelly (2015). Each choice experiment was done 3 times. The fly responses were then recorded on Excel spreadsheets and made into graphs.

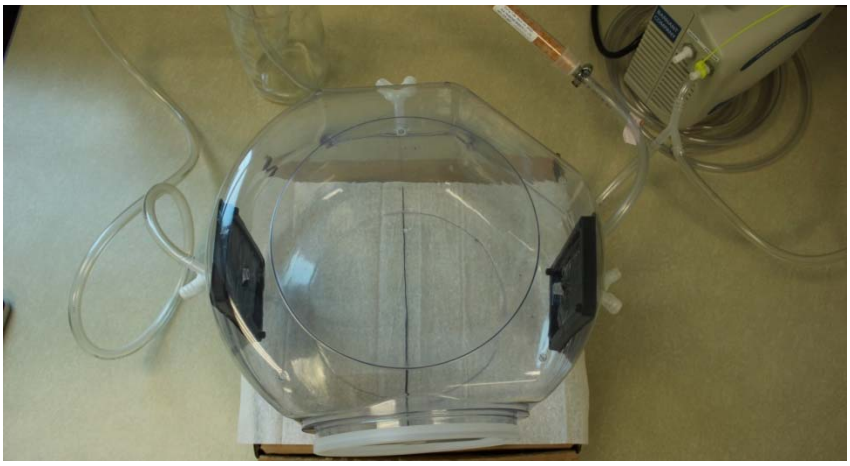

Figure 1. Behavioral test chamber for choice assays. Courtesy of Alexandra Jacob.

Physiological Assay

An Electroantennogram (EAG) was used to detect a neurological response in different VOCs in the unmated and mated females at day 4 post-eclosion. To obtain a neurological response, a strict protocol was followed for each round of experiments. First, each electrode was covered by glass capillary tubes filled with 10% KCl solution. The tips of the capillary tubes were then covered with ultrasound conductive gel which allows the fly head to be attached on one probe and the antennae on the other, completing the circuit and giving an electrical signal. The EAG volatile delivery system consists of cannon apparatus that propels the volatile towards the fly head. The cannon apparatus has a tube attached behind it that gives the cannon a constant supply of humidified air. The cannon apparatus also has a hole at the top where a volatile can be injected to by a hypodermic needle and transmitted to the fly antenna through the stream of humidified air.

The VOCs were created by placing 100µL of a 10% dilution of chemical onto a piece of filter paper which was then placed into a headspace vial. The filter paper was wetted with the specific VOC approximately 15 minutes prior to the testing to allow the air within the headspace vial to assume the chemical composition of the VOC. The hypodermic needle extracted 200µL of air from the headspace vial extracting the VOC which then could be injected into the cannon-apparatus of the EAG.

The VOCs selected for the physiological assay were chosen for being known to give positive response or negative response in EAG literature to consistently giving a

response to both males and females. The positive control for these experiments was dimethyl disulfide (DMDS). Ambient air was used at the negative control for the experiments. Each fly was treated with ambient air at the beginning and the end of each experiment to ensure the flies were not producing an inaccurate depolarization thus producing what was interpreted as a response signal but in reality was not. The experimental VOCs that were selected were cadaverine and unmated male pheromones. Cadaverine was selected to represent decomposition odor because it is an ammonia-rich compound released in the later stages of decomposition during the degradation of proteins (Ashworth 1994; Kasper 2010; Frederickx et al. 2012). If the unmated or mated flies were motivated by a need to consume a protein meal they were expected to exhibit an antenna depolarization upon cadaverine exposure. The other experimental VOC, male pheromones, has not been broken down into the component parts, thus each experiment had 5 live unmated male flies were placed into a headspace vial, and the entire volatile suite was used. The unmated male fly age was three to four days post-eclosion, the same parameters with the behavioral study.

While the VOCs were made and allowed to equilibrate for fifteen minutes, the flies being tested were captured in scintillation vial and placed in an ice bath for 2 minutes to anesthitize the fly. For the physiological assays there was one age group test, which was day four. Day four was chosen for the physiological study because it was the first day at which the difference in behavior between the unmated and mated females was noted. Once the fly was clearly anesthitized it was removed from the ice bath and decapitated. The fly head was then mounted on the ultrasound gel that covered the electrode that was directly connected to the EAG amplifier. Once the fly head was mounted on the electrode the antennae were fluffed up and connected to the opposing electrode. This completes the electrical circuit the EAG utilizes the measure the depolarization signal generated by the fly.

The *Lucilia sericata* nervous system is similar to the human nervous system in that signal transmission is accomplished by depolarization events that originate on the dendritic-like sensillary structures of the fly's antennae. This signal is transmitted through the neuron in the sensillum to the basal ganglia and a response occurs. The EAG enhances the electrical signal produced by the fly and eliminating surrounding electrical

noise (improved signal to noise ratio). The depolarization is measured in millivolts per second.

The EAG was connected to a computer running EAG Pro software. EAG Pro was used to record the data collected by the amplifier. When each recording was completed the images were saved. The images were then imported into Image J (National Institute of Health free software program) and the height of the peak was measured. The height of the peak was the parameter selected to evaluate the degree of male of fly response. These measurements were recorded in millivolts. The testing of the unmated and mated females was done in cohorts of three.

Figure 3. EAG system.

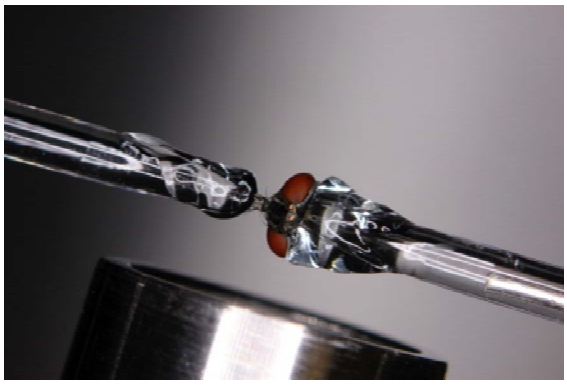

Figure 4. Fly head on EAG system.

(Photos courtesy of Allissa Blystone)

Morphological Assay

The morphological assay was done by capturing unmated and mated flies at ages 1, 2, 3 and 4 post-eclosion individually in a scintillation vial. These flies were then submerged in 95% ethanol, to ensure preservation. These flies were then eventually placed on white paper and a picture was taken of each fly with a Nikon 500 camera. These pictures were taken to document the morphological differences that occur in the development of unmated and mated female flies.

Results:

Behavioral Assay

The behavioral assay choice test for unmated females show a preference for a certain VOC then at day four it changes. In the development of female flies, at first they prefer to have a protein meal. This can be seen in Figure 6 where the unmated female chose liver over ambient air. Also, this preference for a protein meal can be seen in Figure 10 where the first three says post-eclosion the unmated females preferred liver over unmated male pheromones. However, at day four the preference for a protein meal over unmated male pheromones switches so the unmated female is now looking for a male. The mated female flies showed a preference for a VOC throughout their four-day development. From day one post-eclosion to day 4 post-eclosion these flies preferred the liver, which can be seen in Figure 5 where the mated females chose liver over ambient air. Also, they chose the liver over male pheromones which can be seen in Figure 9. These results support the hypothesis that mated and unmated females will respond differently to VOC carrion.

Physiological Assay

Similar to the behavioral assay the physiological assay showed a difference in preference for VOC between the unmated and mated female flies. The unmated female flies had stronger neurological depolarization for male pheromones rather than cadaverine. These results can be seen in Figure 11. The mated female flies respond oppositely to male pheromones and cadaverine. These mated females had a stronger neurological depolarization for cadaverine over male pheromone, which is shown in Figure 11. These results mirror the results in the behavioral assay at day four post-eclosion by showing a difference neurologically for a preference of VOC between the unmated and mated female flies.

Morphological Assay

The morphological study gave further proof a divergence between the mated and unmated female flies. Through the four-day development of these flies both the unmated

and mated females grew larger as a result of their diet. However, in the mated female there starts to be a difference in size in the abdomen at day four where the unmated female is still rather small and the mated female has abdomen has grew due to developing the eggs. These results in can be seen in Figure 12 where pictures show unmated and mated females at days 1, 2, 3 and 4 post-eclosion. These results give further evidence of the divergence in the unmated and mated female flies.

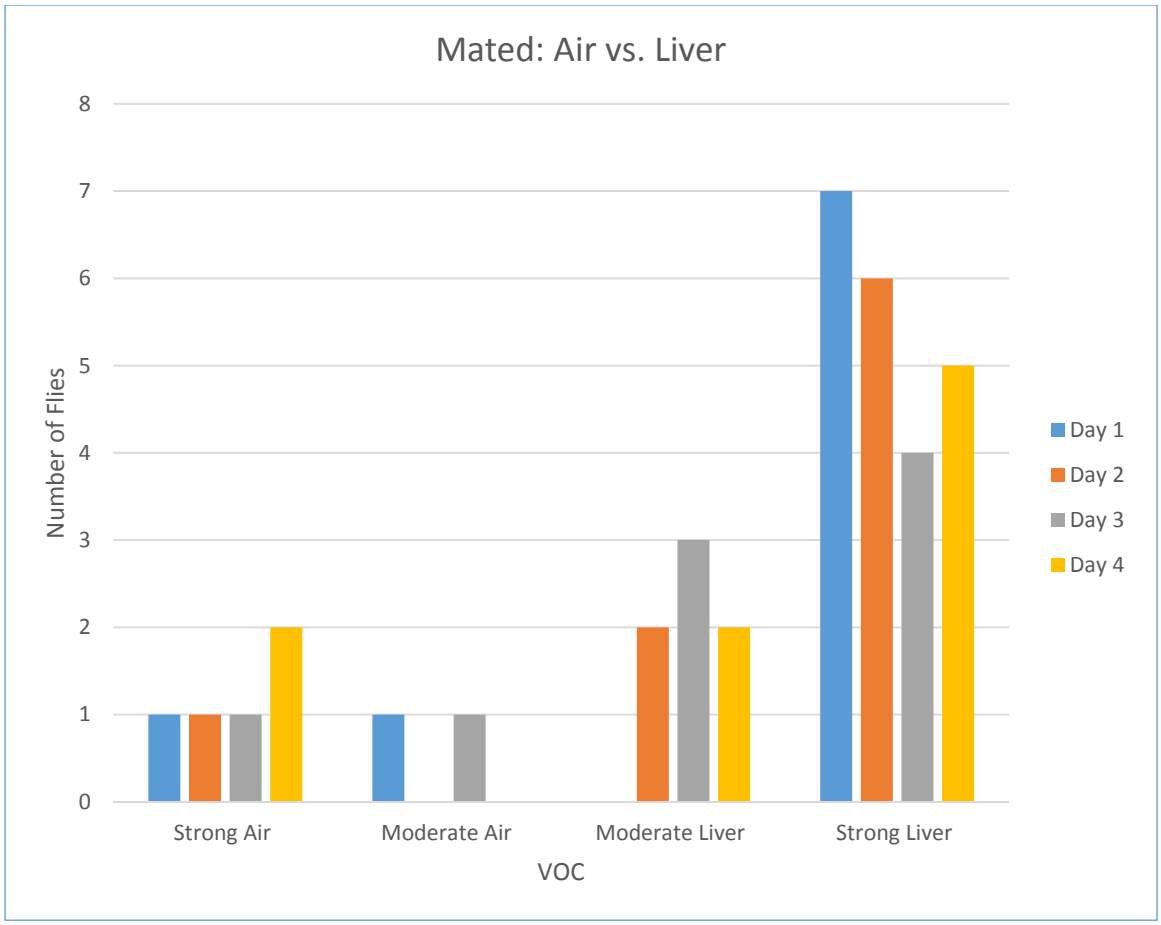

Figure 5. Behavioral Assay Choice – Mated females, Air versus Liver VOC exposure. Bars represent ages of females in days.

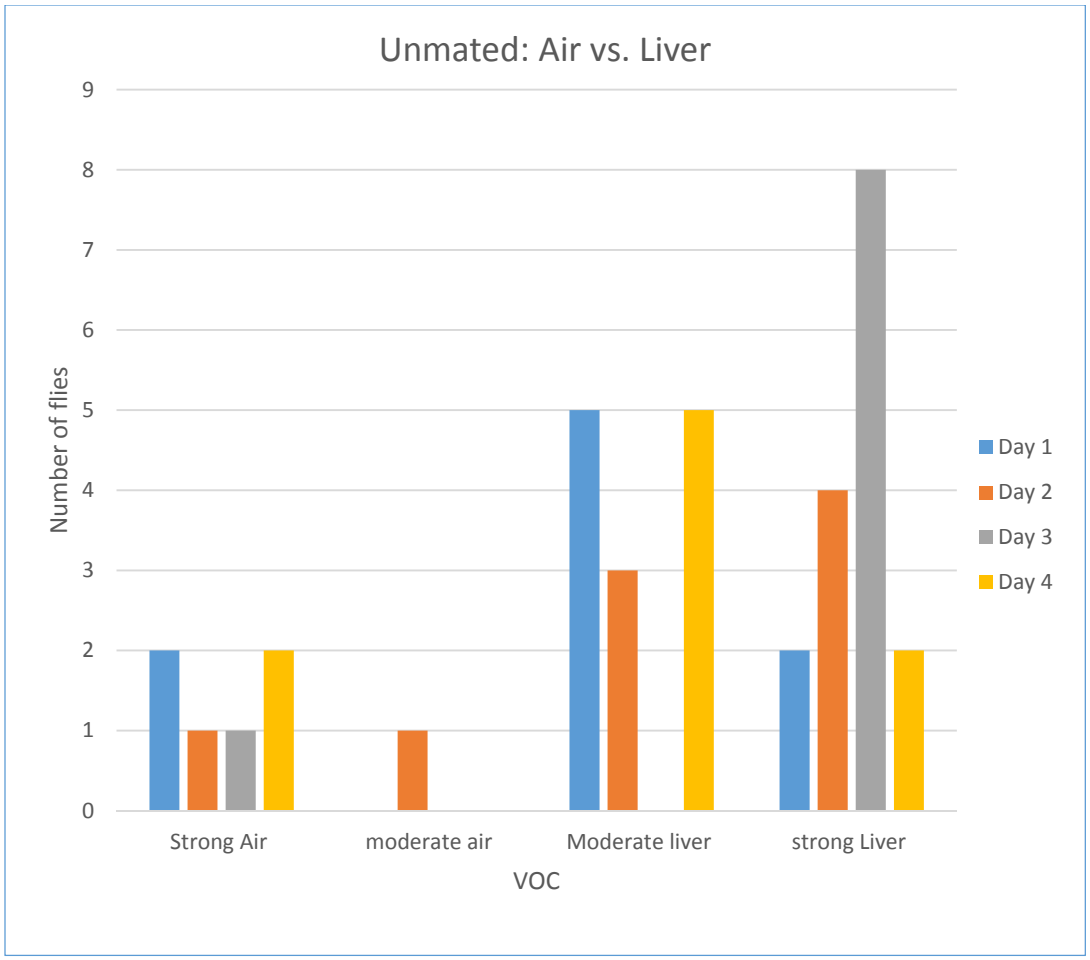

Figure 6. Behavioral Assay Choice – Unmated females, Air versus Liver VOC exposure. Bars represent age of females in days.

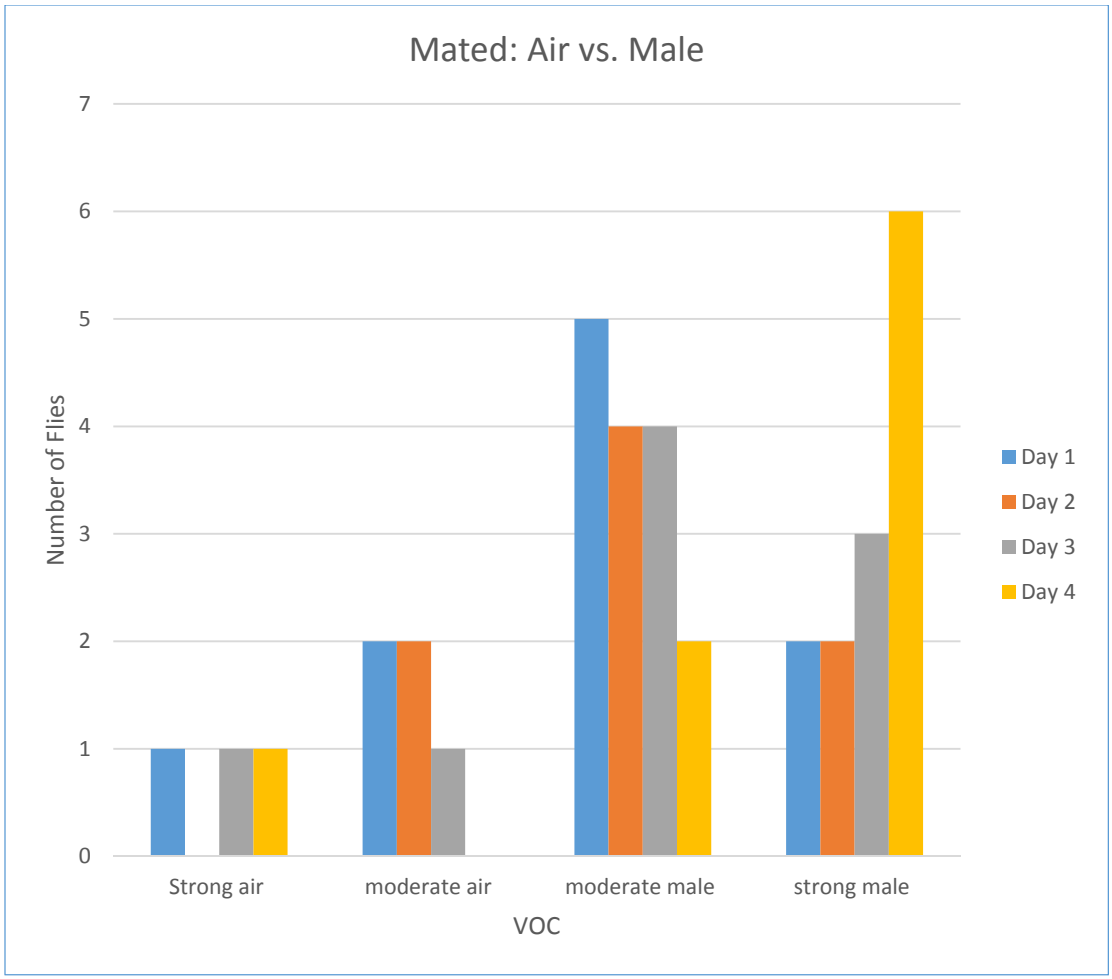

Figure 7. Behavioral Assay Choice – Mated females, Air versus Male VOC exposure. Bars represent age of females in days.

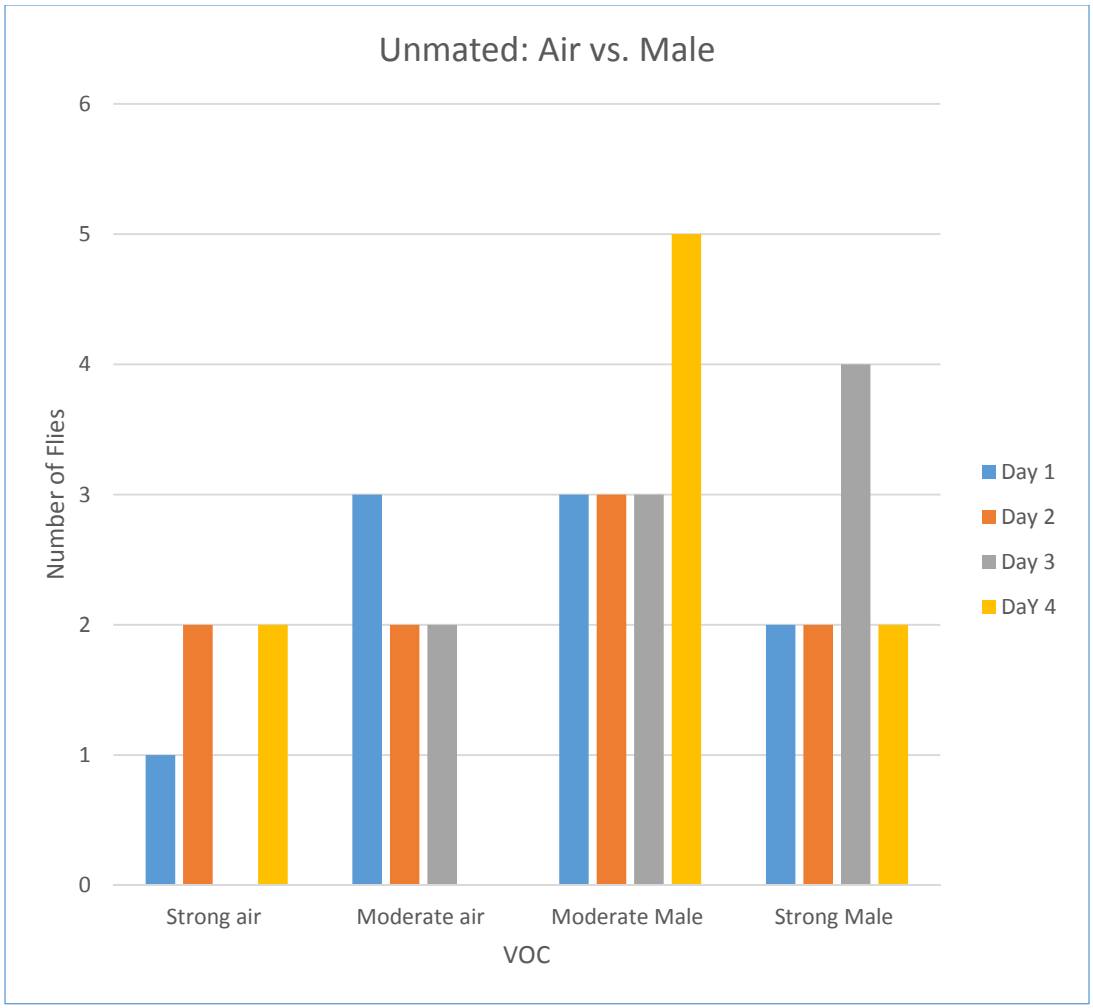

Figure 8. Behavioral Assay Choice – Unmated females, Air versus Male VOC exposure. Bars represent age of females in days.

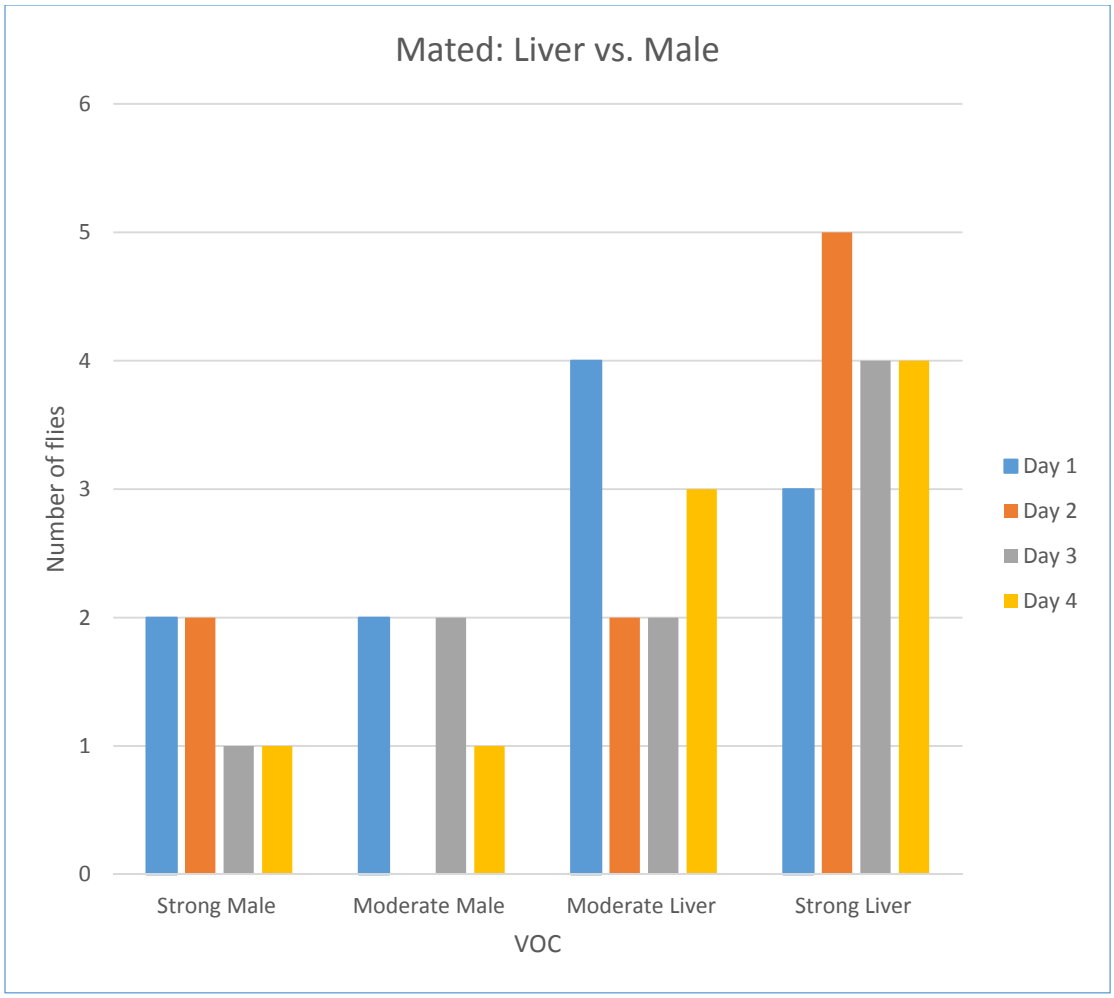

Figure 9. Behavioral Assay Choice – Mated females, liver versus male VOC exposure. Bars represent age of males in days.

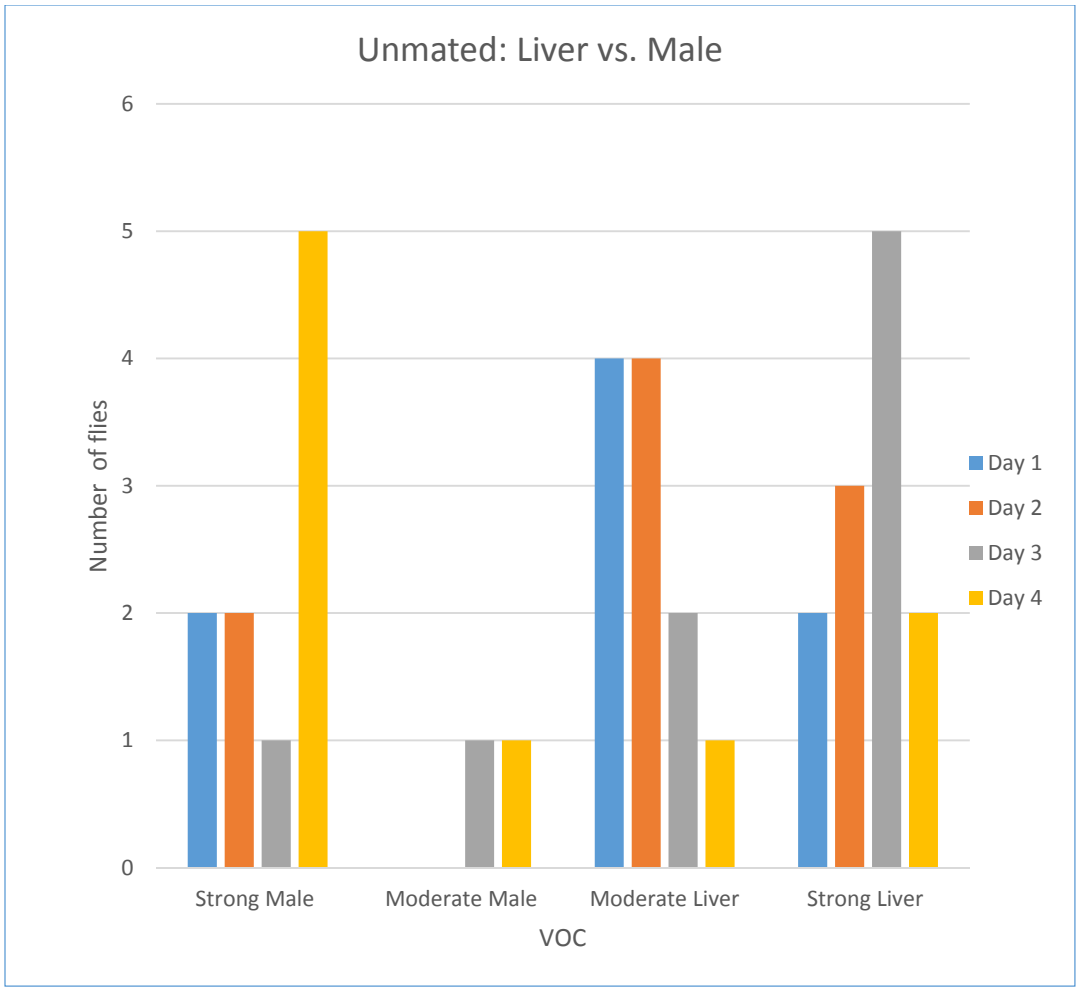

Figure 10. Behavioral Assay Choice – Unmated females, Liver versus Males VOC exposure. Bars represent age of females in days.

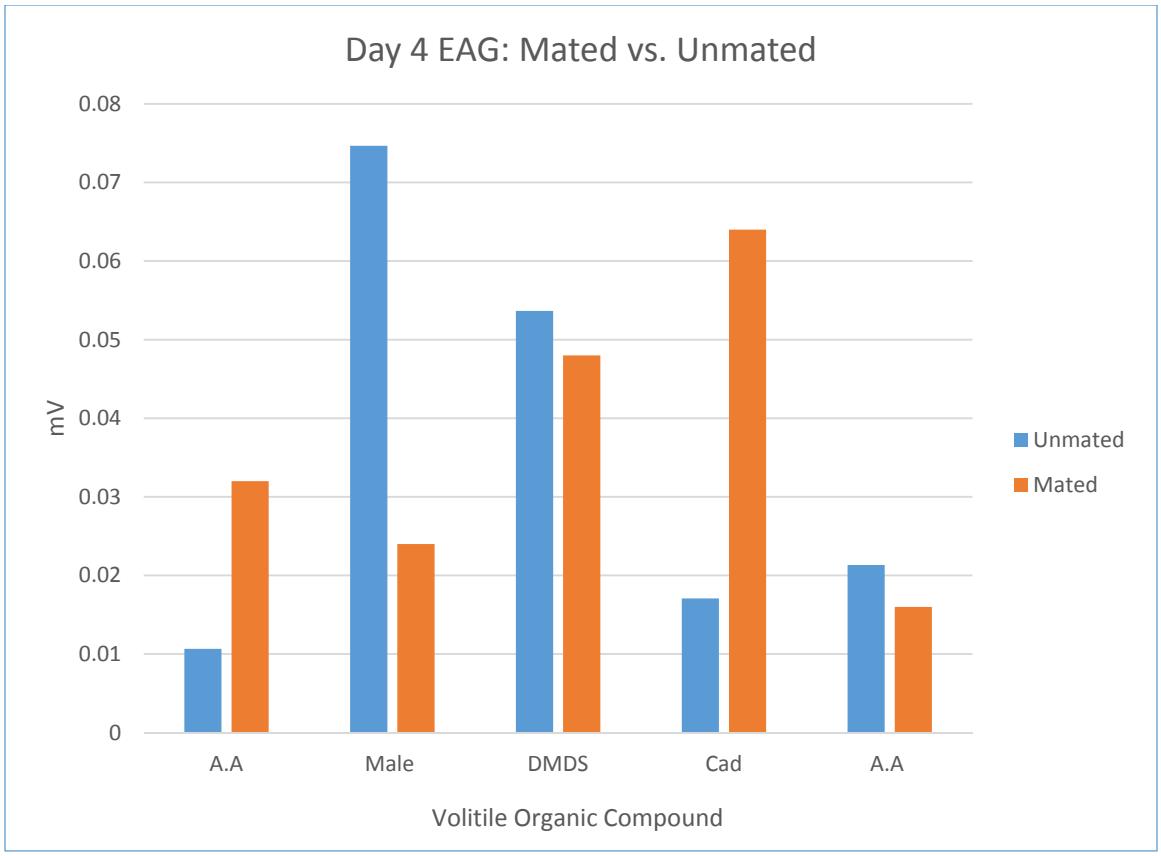

Figure 11. Physiological Assay – Unmated females versus Mated females. The bars present unmated and mated female flies.

Unmated

Day 1 **Day 2** **Day 3** **Day 4**

Mated

Day 1 **Day 2** **Day 3** **Day 4**

Figure 12. Morphological Assay – Unmated females versus Mated females.

Discussion:

Behavioral Assay

The unmated and mated female flies in the behavioral choice assay had a preference for the VOC, both unmated male pheromones and liver, over ambient air. This shows that the negative control worked and that these flies in their development are looking for either a male to mate with or a protein meal to eat. The preference only increased with age since by day 4 there is an even stronger preference for these two VOCs over ambient air except in the unmated females. At day four these unmated females have more of a moderate preference for liver over ambient air (Figure 6). Also, the unmated female shows a stronger preference at day 4 when choosing male pheromones over ambient at day four (Figure 8). These results show that at day four the unmated females are looking for males to mate with rather than look for a protein source to eat.

The third part of the behavioral assay was a choice test for the flies between the male pheromones and liver, which further shows a preference of unmated females for male pheromones and the mated female preference for liver. When given the choice the unmated females chose liver over males for the first three days of their development. Then the unmated females suddenly changed their preference for male pheromones over the liver (Figure 10). Whereas, the mated females continued to show a higher and higher preference for liver as they developed (Figure 9). The male versus liver choice test further gives explicit evidence for a divergence in behavior between the unmated and mated female flies at day 4 post-eclosion. These can due to the difference in physiological needs between the unmated and mated females. The unmated females have had their protein source for the previous three days then at day four are looking to mate because they have not had the chance. Whereas, the mated females have had already mated and are now looking for a protein source not to eat, but to lay their eggs.

Physiological Assay

Since there is a difference in physiological needs at day four post-eclosion between the unmated and mated female flies a physiological study using an

Electroantennogram (EAG) was done to see what was happening at the molecular level. The results showed that there is physiological evidence showing there is a divergence between the unmated females and mated females. These results mirror the behavioral assay conclusion that since there is a physiological difference in needs between unmated and mated female flies that there is a difference in response neurologically between them. Again, the unmated female flies had a larger neurological depolarization for male pheromones because they want to mate, but the mated female flies had a larger depolarization for cadaverine because they have already mated and are now looking for a protein source to lay their eggs (Figure 11).

Morphological Assay

The morphological study mirrors the behavioral and physiological assays but gives a more physical evidence for the divergence at day four post-eclosion. The unmated and mated female flies both grew from day one to day four, however for difference reasons. The unmated female flies developed as a cause of their diet whereas, the mated female flies developed as a result of their diet as well as the eggs in their abdomen developing. We can see this Figure 12, on day four there was a difference in the abdomens of the unmated and mated female flies. These differences show that there is a physical stressor that helps drive the physiological divergence in day four post-eclosion. That is, the abdomen in the mated females is expanding the body, due to the egg development occurring inside it and that it needs to lay the eggs. As for the unmated females they do not have this distension of their abdomen and do not need to look for a protein source but can look for a male to mate.

These three experiments show that there is a divergence in behavior, neurologically and physically between the unmated and mated female flies which corresponds to in a difference in preference for VOCs. The unmated female flies choose male pheromones because they need to mate with males at day 4 post-eclosion. Whereas, the mated female flies choose the protein source because they have already mated and are now looking to lay the eggs.

Future Directions

The data generated through my Honors Thesis does not fully answer the question of if up-down gene regulation is occurring in the female flies and if that regulation triggers the responses to a protein source and male pheromones, especially at day four where we see a divergence between the unmated and mated female flies. Further experiments need to be done at the molecular level at day 4 post-eclosion on unmated and mated female flies to see what differences there are in regulation of odorant binding proteins that contribute to the behavioral differences between the unmated and mated female *Lucilia sericata*.

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