JOURNAL OF
EMERGING INVESTIGATORS

Article

Examining effects of *E. muscae* on olfactory function in *D. melanogaster*

Halley Friedman, Brian Dempsey
Acton-Boxborough Regional High School, Acton, Massachusetts

SUMMARY

Many parasites manipulate the behavior of their hosts to induce specific behavior for the benefit of the parasite (i.e., to increase its reproduction and transmission). The complex ability to take over a host's central nervous system has been studied extensively in ants (*Camponotus castaneus*) infected by the fungus *Ophiocordyceps unilateralis s.l.*, but not as much in fruit flies (*Drosophila melanogaster*) infected by the fungus *Entomophthora muscae*. One behavioral change seen in ants infected by *O. unilateralis s.l.* is a reduced response to olfactory stimuli; however, this has yet to be tested in infected fruit flies. In this study, I tested olfactory response in flies infected with *E. muscae*, healthy flies (positive control), and flies without odorant receptors (negative control) via a preference experiment using a saturated sucrose solution and distilled water. The results supported the hypothesis that fruit flies do have a reduced perception of olfactory stimuli when exposed to *E. muscae*, as they perform more like mutant flies without odorant receptors than healthy flies with a functioning olfactory system ($p < 0.05$). Although, it is not fully understood how *E. muscae* enters and takes control of the fruit flies' nervous system, knowing that *E. muscae* reduces their olfaction gives us more insight into the functionality of this parasite overall. Further research that allows us to understand how a nervous system is overtaken could potentially provide insight into the vulnerable parts of the nervous system and thus deepen our understanding of neurology as a whole.

INTRODUCTION

Entomophthora muscae is a fungal parasite that infects Drosophila melanogaster (the common fruit fly). However, this is not an average parasite: *E. muscae* has the ability to overtake and control the entire central nervous system of D. melanogaster before killing it to increase its own transmission, thus coining the name "Zombie Flies" (1). In response to infection from *E. muscae*, *D. melanogaster* experiences drastic behavioral changes. The most infamous response occurs hours before its death, when *E. muscae* induces negative gravitaxis, an instinct to climb upward, in the fly to make it climb to the highest point in its environment, such as a blade of grass. The fungus then prompts the fly to produce a glue-like substance from its mouth to stick the fly's body in place to the elevated surface. *E. muscae* projects the fly's wings upward to ensure the fungal spores emerging out of the fly's abdomen don't get caught in its wings (1). Finally, the fungus kills the fly and uses the nutrients from the fly's body to sporulate from its abdomen, showering spores onto the rest of the fly population.

The results of this experiment support the following hypothesis: If *D. melanogaster* exposed to *E. muscae* are introduced to both a saturated sucrose solution and distilled water 72 hours after exposure, they will not show significant preference between the two, because *E. muscae* will have reduced the flies' responses to olfactory stimuli.

Dr. Carolyn Elya, Harvard University Postdoctoral Researcher, is currently pioneering much of this work at the cellular and molecular levels, specifically concentrating on how *E. muscae* is able to infiltrate and control the complex nervous system of *Drosophila*. The observed abnormal behaviors *D. melanogaster* experience after infection (e.g., the sequence of behaviors before death as explained above) are all that has been deeply studied about the specific behaviors *E. muscae* can induce in its host (1). But how those behaviors occur exactly is unknown; we do not yet know biologically how the fungal spores can enter and eventually take over the nervous system of *D. melanogaster*. This phenomenon, however, has been studied extensively in ants (*Camponotus castaneus)* infected by the fungus *Ophiocordyceps unilateralis s.l.* One study showed multiple behavior changes in the infected ants including a reduced response to stimuli, specifically olfactory stimuli (2). In particular, genes involved with odorant and gustatory perception were down-regulated in ants infected by *O. unilateralis s.l.* (3).

Although *E. muscae* and *O. unilateralis s.l.* are convergently related and thus work differently (for example, *O. unilateralis s.l.* infects ants from outside the body while *E. muscae* infects internally), there are certain behaviors that are universal for all behavior-altering fungi (2, 4). I intended to explore whether *D. melanogaster* infected by *E. muscae* also experienced reduced response to olfactory stimuli, similar to the behavior changes found in infected *C. castaneus*. If this behavior change is induced by both fungi, that will indicate that reducing responses to olfactory stimuli is a necessary action for overtaking an insect host's nervous system in general, since both fungi developed this capability independently as they convergently evolved. This research is important as it will bring better understanding to the full scope of behaviors *E. muscae* induces and the types of traits universal to all behavior manipulating fungi. These findings may prove to broaden the understanding of neurology in general as well.

RESULTS

In order to test the scientific question, "Does *E. muscae* reduce response to olfactory stimuli in *Drosophila*?" I

performed a preference experiment between sucrose and water. Flies infected with *E. muscae* were used as the treatment, which I compared to a negative control (i.e., mutant flies with no olfactory function, and thus cannot detect nutrients in their environment, since drosophila mainly seek food through olfaction) and a positive control (i.e., healthy flies with fully functioning olfactory systems, who can detect their nutrients). The type of fly served as the independent variable and the resulting preference ratios were the dependent variable. Lighting, humidity, time of day, and hours after exposure to fungus were all controlled as outlined in the Methods section.

The data shows that flies infected with *E. muscae* behave more like mutant flies that have no olfactory function than healthy flies with fully functioning olfactory organs. The preference ratios of the infected flies and mutant flies were both close to 50% (n = 357), consistent with the hypothesis that there would be no significant preference for either solution shown by the infected flies (**Figure 1**). There is data for both mutant flies and infected flies that indicates a slight preference for water (about 5% for each group), though this could be due to sampling variability. More experimentation and data are needed to fully suggest water preference. The most significant finding overall is among the preference ratios of infected versus mutant flies, which were observed as more similar (i.e., the average percent preference of both water and sucrose only differs by 0.2% for the two strains). Opposingly, the percent preference of infected flies to sucrose is more than 20% less than healthy flies' percent preference to sucrose (**Figure 1**). These findings suggest *E. muscae* may fully inhibit olfactory perception in *D. melanogaster*.

The difference in solution preference between the infected and healthy flies was also shown to be statistically significant ($p < 0.05$) in the totals laid out in **Table 1**. Using a t-interval and chi-square analysis, I found that the preference ratios in healthy flies are statistically significant at the $p < 0.05$ level of the infected flies' preference ($x^2 = 13.4772$, $p = 0.0002$). This is evident in the sucrose preference percentages of the mutant and infected flies as well, which are each not statistically different ($x^2 = 0.0027$, $p = 0.9589$), as both groups exhibit similar data since they both do not show distinct preference to either sucrose or water. These two samples (infected and mutant) are roughly divided evenly between the sucrose and water sides, indicating that both groups of flies cannot sense a difference between the stimulants.

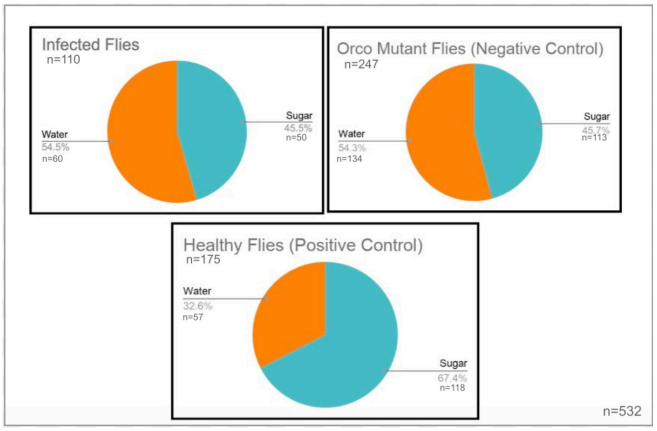

Figure 1: Average Preference Ratio of *D. melanogaster* to Sugar Stimulant. Average preference for both the treatment (i.e., infected flies) and the controls (i.e., mutated flies and healthy flies).

Table 1: Summary Comparison of Treatment vs. Control Preference Ratios. With the sums from each observation, these tables mathematically compare the ratios of preference for the treatment (infected flies) to each control (mutant and healthy). I used both the t-interval and chi-square analysis to analyze the data. There is statistical evidence of difference in the preference ratios of the infected and healthy flies ($p < 0.05$), that is the healthy flies distinctly preferred sucrose, but the infected flies did not. However, the preference ratios of infected and mutant flies were not found to be significantly different ($p > 0.05$).

	Sucrose	**Water**	Total
Infected	50	60	110
Mutant	113	134	247
Total	163	194	357
	Sucrose	**Water**	Total
Infected	50	60	110
Healthy	118	57	175
Total	168	117	285

This idea is further illustrated by the average percentage data present in **Figure 2**. The data differs slightly from the ratios of the raw counts from each trial in **Figure 1**, as this is instead an average of the percent preference from each trial. Overall, again the same result occurred: infected flies acted like mutant flies and differed from healthy flies. The confidence interval ($p < 0.05$) error bars for the infected and healthy flies do not overlap, meaning there is 95% confidence they are statistically different from each other (**Figure 2**). However, the infected flies' percent sucrose preference confidence interval does overlap with the mutant flies' sucrose preference interval, showing the two ratios are not statistically significant in that both do not distinctly prefer sucrose. The results of the experiment reveal that *E. muscae* reduces and may even eliminate olfactory stimuli perception in its Drosophila host, as predicted by the hypothesis.

DISCUSSION

The findings of this study support the hypothesis that *E. muscae* reduces olfactory stimuli response in its *D. melanogaster* host, similar to the effect *Ophiocordyceps*

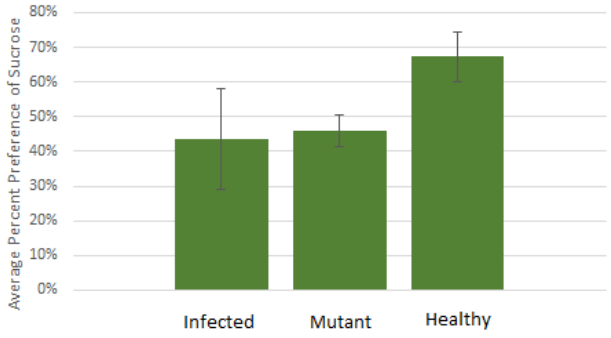

Figure 2: Average Percent Preference of Sucrose. Average of percent sucrose preference from each trial for each group, isolating the percent preference of sucrose from the treatment and controls and expressing variance through 95% confidence intervals ($p < 0.05$). Error bars depict the standard error of the mean.

unilateralis s.l. has on *Camponotus castaneus*. Because I observed infected flies to behave almost exactly like mutant flies with no olfactory function, and healthy flies distinctly prefer sucrose, it can be concluded that healthy flies lose this preference after infection by *E. muscae*. In other words, data provides evidence that *E. muscae* reduces olfaction so much that their sucrose preference is almost equivalent to flies without olfactory function.

Interestingly, I expected infected flies' preference to be somewhere in the middle of flies with a fully functioning olfactory system and a non-functioning one, with their olfactory function simply having been reduced. However, infected flies exhibited behavior distinctly resembling a non-functioning olfactory system, suggesting *E. muscae* fully inhibits olfactory sensory perception in its host instead of only partially inhibiting it. This conclusion, that *E. muscae* could possibly inhibit olfactory perception altogether, is new and involves a more significant behavioral change than predicted in the hypothesis. Isolating the connection between *E. muscae* and the *Drosophila* olfactory system (as done in this study) is imperative to understanding the full scope of behaviors induced by *E. muscae* and its overall complexity, while providing a basis for other *E. muscae* research.

For example, whether *E. muscae* reduces olfactory perception through inhibiting the olfactory receptors themselves or changing preference in the brain cannot be concluded through this preference experiment. What is clear is that the infected flies have inhibited perception to the sugar stimulant, which itself could be more thoroughly investigated in future experiments with specific attention given to the olfactory system and neurochemistry of olfactory perception in the brain. Future studies could test whether *D. melanogaster* solely loses perception to sucrose or if other stimulants yield the same result.

The ability to reduce olfactory perception in a host following infection evolved convergently in both *O. unilateralis s.l.* and *E. muscae*. However, it is not clear how reducing olfaction is advantageous for either parasitic fungi. It is possible that these fungal molecules enter the host's nervous system through the olfactory organs, causing the reduced response to olfactory stimuli as a side effect. It is also possible that these fungi reduce olfaction in their host intentionally, weakening the host by removing its ability to find nutrients, thus making it more susceptible to infection. Future research could uncover whether reduced olfactory perception actually is meant to create an advantage for these fungi or whether it is a side effect of the fungi's permeation of the nervous system.

There were other ideas that emerged during the course of this experiment that could provide next steps to advance this research. For example studying what specific receptors are manipulated in the olfactory system, if not all, and how that affects overall olfactory function. Examiniting exactly when in the 5- to 7-day cycle of *E.muscae* (before day 3) olfactory function is noticeably altered. As well as investigating if *E. muscae* can manipulate its host externally, before it enters the host's body, like *O. unilateralis s.l.* or if instead *E. muscae* only affects olfaction once inside the brain. Executing a preference experiment at different lengths of time after exposure (beyond this study's 72-hour mark), for example only a few hours after exposure versus at 96 hours, could shed light on the stages of infection. Another option to study the exact function and method of infection is simply investigating the specific neural activity in the fly. This could lead to understanding at what point in the 5- to 7-day cycle *E. muscae* actually take hold of its host's nervous system. Studying these ideas will help researchers further their understanding of the *E. muscae* infection and potential relevance to other systems, as well as help researchers gain new insight into unknown parts of this system and the field of behavior manipulation.

However, even before these questions are addressed, it would be ideal if the experiment were replicated in a more controlled setting for the purpose of increased data collection, as this was a preliminary experiment. Although the environment was kept as highly controlled as possible, having to execute experimentation at home inevitably comes with the risk of confounding variables (such as possible contamination) and other problems such as a lack of certain resources, which make it hard to grow large fly populations. Conducting more trials using the same experimental design in a lab would aid greatly in generating stronger conclusions. Specifically, having access to a proper fly lab with sufficient fly populations, resources, and sterile equipment and surfaces is imperative to collecting reliable data.

With access to a full lab, I would repeat these exact trials and also examine different lengths of time after infection, which would help indicate when exactly *E. muscae* reduces olfaction perception in the 5- to 7-day cycle from exposure to the fly's death. Another important factor to consider is testing different olfactory stimuli. While sucrose is a good indicator of overall olfactory perception function, a more diverse set of scents is needed to more strongly conclude that *E. muscae* reduces all olfactory perception, not just sucrose perception in its host.

Overall, continuing this field of research could benefit evolutionary biologists and immunologists interested in this specific parasitic relationship, as well as those seeking to understand more about neuroscience. The similarities and differences between these parasitic fungi could be a possible area for future ecological research. These two fungi have naturally evolved an ability no human has been able to execute, i.e., control over another organism's nervous system, yet how these parasites fully execute this is still unclear (1). One challenge is that there are a multitude of different behavior-altering parasites all infecting their hosts in different ways. Most have evolved convergently, so they all exhibit unique features (2). For example, the ant-infecting fungus *O. unilateralis s.l.* does not have to enter the host's nervous system to overtake it, it infects from outside the body (5, 6). Investigating similarities and differences between the abilities of convergently evolved behavior-altering fungi as they infect a host could shed light on whether there is a universality to their methods.

E. muscae differs from *O. unilateralis s.l.* in that it does enter its host's brain (1, 5–6). However, *E. muscae*'s activity in the brain has not been well-studied, especially how the fungus gets past the blood-brain barrier. Activity in the brains of cockroaches and killifish that have been infected by similar parasites has been well-researched (7–8). Those studies reveal unusual neurotransmitters and hormone concentration in certain parts of the host brain that relate to behavioral function, and suggest that the fungal-produced molecules trigger multiple specific signaling systems to induce behavior (9). What we can take away from this information is that behavior is solely chemical, and that behavior change can be

induced by the introduction of certain specific molecules.

Continuing this line of study to isolate which pathways, chemicals, and combinations of the two affect host behavior using model organisms such as *E. muscae* could have significant implications in the field of neuroscience. For example, studying whether the chemical combinations that affect behavior are universal, or specific to insects, could help us understand if mammalian brains evolved protection against parasitic infestation of the nervous system or if we are unconsciously susceptible to it. Some parasites are already known to affect human behavior, such as *Toxoplasma gondii* (10). Exploring the concentrations of neurotransmitters that occur post-infection could perhaps lead to the ability to artificially induce behavior in model organisms. The nervous system is so incredibly complex and difficult to research, but investigating these behavior-inducing parasites extensively could possibly produce advancements in neurological research.

MATERIALS AND METHODS

This investigation was intended to be done in the de Bivort Lab at Harvard University on a summer internship but due to lab closures caused by the COVID-19 pandemic, it was instead carried out in a private residence. Study mentor Dr. Carolyn Elya of Harvard University also moved her research to her residence and provided general guidance, as well as the proper at-home protocols and procedures. Experimentation was implemented in the following optimal conditions: a sanitary environment at approximately 25°C with 75% humidity and evenly distributed lighting.

Population Growth and Maintenance

A month before beginning the experiment, control strains were cultured: Canton-S (positive control) and Orco mutant strain (negative control) were cultured using the low-yeast brown *Drosophila* media (11). Canton-S are a healthy control strain of *D. melanogaste*r. The mutant strain group is made up of flies without functioning odorant receptors. Specifically, these are flies with the Or83b gene unexpressed—a gene normally expressed in all odorant receptors—effectively making these flies lose all perception of olfactory stimuli (12). Flies were kept inside of capped plastic vials with their food, in an enclosed but well-ventilated space under a 12:12 light:dark cycle. In order to ensure the proper growth of these control populations, flies were switched into new food vials every 3 days.

Once there was a sufficient Canton-S population (about 2 weeks later), *E. muscae*, acquired from spoalating cadavers from Dr. Elya's lab, was introduced into a vial of flies from the Canton-S population in order to start growing the infected fly population. The latter population acted as the treatment in the experiment. The infection was maintained using a detailed protocol provided by Dr. Elya (13). The protocol includes using sporulating cadavers in an enclosed space that was compressed by a plug, in order to expose the healthy flies to the maximum number of spores and increase the possibility of infection (13). The exposed flies die of the infection about 5-7 days later.

The preference experiment took place using flies that were on Day 3 of infection, or 72 hours after their exposure to *E. muscae*. Flies are considered infected about 48 hours after exposure, but their whole nervous system will not be fully compromised until approximately 96 hours have passed (1). At the 72-hour mark the fungus will be in the process of entering and altering the nervous system, thus will have had sufficient time to affect the flies' behavior.

Because of the short lifespan of infected flies, in order to maintain a sufficient number of infected flies to use as a treatment at 72 hours after exposure, the process was repeated using new sets of healthy flies not yet exposed to the fungus. This cycle continued throughout experimentation to ensure a proper population of the infected flies for the experiment, i.e., one vial of approximately 40 viable flies per trial.

Experimental Design/Protocol

The experiment began once sufficient control and treatment populations were achieved and isolated. On a flat surface, in a room with an ambient temperature of ~25°C and with evenly distributed lighting, a sterile 24'' polycarbonate tube was placed on its side. The tube was sealed with a plug at each end. One side of the tube was then opened and the anesthetized flies were gently released from one vial into the center of the tube. The type of fly population was randomized for each trial by randomizing the order by which all three (mutant, healthy, and infected) populations were tested each trial. Cold shocking was used as a form of anesthesia, since there was no access to CO_2 outside the lab. This meant, putting the fly vials in an environment below freezing (i.e., a freezer or outside depending on the time of year), because they go into a natural coma-like state when cold-shocked. Once they're returned to the warmer environment, they wake up. The whole process is a natural form of anesthesia since the fly induces its own coma-state. Then, plugs were removed from the tube and replaced with one cotton ball soaked in a saturated sucrose solution (~2000 g/L at 25°C) on one end of the tube, and one cotton ball soaked in distilled water on the other. The side removed alternated between trials to reduce confounding variables. Three minutes after being placed in the tube, the location of the flies was observed (**Figure 3**). The results, which consisted of the number of flies on the left versus the right side of the tube, were counted and recorded. The amounts indicated the flies' preference for sucrose versus distilled water. This same procedure was then repeated and the data recorded for the remaining two strands. The whole experiment was repeated 3 times, to collect more data and gain evidence of repetition.

Statistical Analysis

Statistical significance was determined in this experiment after aggregating totals from the results of all trials. By

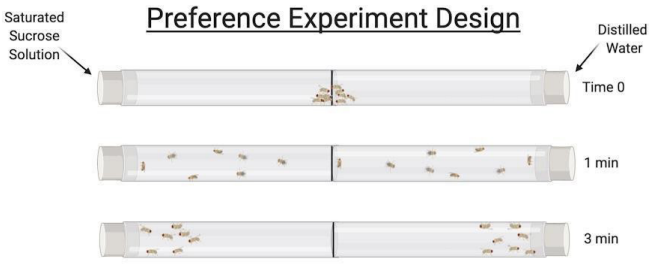

Figure 3: Preference Experiment Design. Visual representation of the preference experiment. Source: H. Friedman 2020.

comparing the preference totals of the treatment (infected flies) to each control (healthy flies and mutant flies), then using chi-square analysis and t-interval to gain evidence for statistical significance ($p < 0.05$), the question of whether the infected flies act more like the healthy or mutant flies 72 hours after exposure to *E. muscae* was answered. Statistical evidence (at $p < 0.05$) for these predicted results was found in the experiment and is analyzed further in the results section.

Risk and Safety

There are no high-risk or life-threatening aspects of this experiment. However, there are some sanitation risks if experimentation is carried out in a private residence (as this experiment was). Therefore, a lab area designated solely for this experiment was created and as such, the cleaning of surfaces, hands, and all materials occurred frequently. Flies were discarded by anesthetizing them, and then placing them in a morgue made of a small sealable container containing ~100 mL of 70% ethanol. They were not released uninhibited.

ACKNOWLEDGEMENTS

I would like to thank my family for their constant support and Mr. Brian Dempsey for his advice, feedback, and help. Also, I thank Dr. Carolyn Elya and Dr. Neal Silverman for their guidance, willingness to share their insight and expertise on *Drosophila*, and aid in getting me the proper resources needed for my at-home experimentation.

Received: December 8, 2020
Accepted: March 25, 2021
Published: July 8, 2021

REFERENCES

1. Elya, Carolyn, *et al*. "Robust Manipulation of the Behavior of D. melanogaster Melanogaster by a Fungal Pathogen in the Laboratory." ELife Sciences Publications, Ltd., 26 July 2018, doi:10.7554/eLife.34414.
2. Hughes, David, *et al*. "From So Simple a Beginning: The Evolution of Behavioral Manipulation by Fungi." Advances in Genetics, vol. 94, February 2016, pp. 437-469. U.S. National Library of Medicine, doi:10.1016/bs.adgen.2016.01.004.
3. de Bekker, Charissa, *et al*. "Gene Expression during Zombie Ant Biting Behavior Reflects the Complexity Underlying Fungal Parasitic Behavioral Manipulation." BMC Genomics, vol. 16, no. 1, 12 August 2015, p. 620. U.S. National Library of Medicine, doi:10.1186/s12864-015-1812-x.
4. Spatafora *et al*. "A Phylum-Level Phylogenetic Classification of Zygomycete Fungi Based on Genome-Scale Data." Mycologia, January 2017, pp. 1028-1046. U.S. National Library of Medicine, doi: 10.3852/16-042.
5. Hughes, David. "Neuroparasitology of Parasite–Insect Associations." Annual Reviews, vol. 63, January 2018, pp. 471-487. https://doi.org/10.1146/annurev-ento-020117-043234.
6. Fredericksen, Maridel A., *et al*. "Three-Dimensional Visualization and a Deep-Learning Model Reveal Complex Fungal Parasite Networks in Behaviorally Manipulated Ants." National Academy of Sciences, vol. 114, no.47, 21 Nov. 2017, pp. 12590-12595. PNAS, https://doi.org/10.1073/pnas.1711673114.
7. Helland-Riise, Siri H., *et al*. "Regional Distribution of a Brain-Encysting Parasite Provides Insight on Parasite-Induced Host Behavioral Manipulation." Journal of Parasitology, vol. 106, no.1, Jan. 2020, pp. 188-197. BioOne, https://doi.org/10.1645/19-86.
8. Arvidson, Ryan, *et al*. "Parasitoid Jewel Wasp Mounts Multipronged Neurochemical Attack to Hijack a Host Brain."American Society for Biochemistry and Molecular Biology, vol. 18, no. 1, 1 Jan. 2019, pp. 99-114. https://doi.org/10.1074/mcp.RA118.000908.
9. Herbison, Ryan. "Lessons in Mind Control: Trends in Research on the Molecular Mechanisms behind Parasite-Host Behavior Manipulation." Frontiers in Ecology and Evolution, 11 September 2017, https://doi.org/10.3389/fevo.2017.00102.
10. Flegr, Jaroslav. "Effects of Toxoplasma on Human Behavior." Schizophrenia Bulletin Oxford University Press, vol. 33, no. 3, May 2007, pp. 757-760. U.S. National Library of Medicine, doi: 10.1093/schbul/sbl074.
11. "Media Recipes." University of Massachusetts Medical School, 21 June 2019, www.umassmed.edu/research/cores/drosophila-resource-facility/media-recipes/.
12. FlyBase Gene Report, Dmel\Orco, flybase.org/reports/FBgn0037324.
13. Elya, Carolyn. "Protocols.", carolynelya.com/protocols/.